Welcome

to Stockholm and Advances in Neuroblastoma Research!

Dear all,

We are so happy that you all finally made it here, to ANR 2010, to Scandinavia, and most of all, to our beautiful capital of Sweden, Stockholm!

We are excited to have received more than 400 abstracts which is the basis of our scientific programme. We are therefore confident that this year's programme will satisfy every single participant, being a researcher, a clinician, a nurse, a student or a parent. Numerous sessions are prepared, spanning all aspects of neuroblastoma from bench to bedside.

We are thrilled to have such excellent invited lecturers including four Nobel laureates visiting us, together with several future ones, we are sure!

We are grateful to our sponsors and all others who have contributed to make the meeting possible.

Please enjoy the multitude of oral and poster presentations. Discuss and interact! Enjoy the social programme, explore the light summer in the beautiful city of Stockholm. After the meeting you should go home exhausted with important experiences, new ideas and friends.

On behalf of the local organising committee,

Per Kogner

CONTENTS

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ANRA Incoming President: Michelle Haber

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* as before election spring 2010.
Please see www.anrmeeting.org for updates and contact details.

Acknowledgements and Sponsors

The Advances in Neuroblastoma Research Conference would like to thank the following sponsors for their generous support and contributions

Co-organisers and main contributors

Swedish Childhood Cancer Foundation/Barncancerfonden
Karolinska Institutet
ANRA
Swedish Cancer Society/Cancerfonden
Swedish Institute/Svenska Institutet

Silver sponsorship

Mary Béve Foundation

Bronze Sponsorship

Pierre Fabre Oncology
Visual Sonics

Exhibiting Organisations

The Advances in Neuroblastoma Research Conference would like to thank the following exhibiting organisations for their contribution and participation. Exhibiting organisations are listed in alphabetical order.

ACGT Advancing Clinico Genomic Trials on Cancer
ANR2012
Covidien
Hospira
Pierre Fabre Oncology
Ridgeview Instruments AB
Swedish Childhood Cancer Foundation/Barncancerfonden
Visual Sonics

Additional contributors

Amgen Oncology
Medac
Scandinavian Airlines SAS
The Vasa Museum
Waldemarsudde

Social Programme

The Social Programme is open to all registered participants and accompanying persons, and must be pre-booked. Additional tickets may be booked and/or purchased at the Registration desk, should space be available.

Welcome Programme (Welcome to Stockholm and Welcome to Sweden)

The Welcome Programme will take place at the Stockholm City Conference Centre Monday June 21st at 16:30. The programme includes an introduction to Stockholm, Karolinska Institutet and the Nobel Prize, followed by a lecture by the Swedish Nobel Laureate, Professor Bengt Samuelsson. This will be followed by traditional Swedish music and performances by various artists, and you will get to know Sweden culturally and historically. The Welcome Programme is followed by a Welcome Reception at the Stockholm City Hall. The Welcome Programme is open to all participants and registered accompanying guests.

Welcome Reception at Stockholm City Hall

The Welcome Reception, Monday June 21st, at 19:00, is hosted by the City of Stockholm and the County Council at Stockholm City Hall, the venue for the Nobel Prize dinner. A buffet dinner will be served followed by a guided tour of the building.

Please note: The reception is open to participants and registered accompanying guests, only if marked on the registration form. Please bring your ticket.

Opening Ceremony

All participants and registered accompanying guest are invited to attend the Opening Ceremony on June 22nd at 08:00 in the Stockholm City Conference Centre where ANR 2010 officially will be opened.

Archipelago Boat Tour

The Archipelago Boat Tour, Tuesday June 22nd, will depart from, and return to Nybrokajen, in Stockholm. The duration of the Tour is approximately three and a half hours and you will be given the opportunity to see some of the most beautiful scenery of Stockholm. A light dinner, including a glass of wine or non-alcoholic beverage and coffee, will be served on-board. A cash bar will be open, at own expense, during the Tour.

Please note: The Archipelago Boat Tour is open for participants and registered accompanying guests. The boat will depart at 19:00 sharp. Please bring your ticket. A light jacket and/or scarf is recommended.

Gala Dinner

The Gala Dinner will take place at Solliden, a classic restaurant with beautiful decor and a stunning view of Stockholm and south inlet heights. The Gala Dinner will be held on Wednesday June 23rd, at 19:00 and buses will depart from City Conference Centre/Norra Bantorget from 18:45. Buses will return to Norra Bantorget from 22:45.

Solliden is located at Skansen, Sweden's largest outdoor museum, where there is always something happening. Every season has its own charm. Summer and winter, every day, all year round, there are lots to see and experience. Skansen is known for its wild animals and culture and out-door exhibitions of Sweden's cultural heritage. It is also the stage from where entertainment programmes are often shown live on TV.

Registered Accompanying Guests

The registration fee for accompanying guests includes admission to the Welcome Programme at the City Conference Centre and Welcome Reception at the City Hall on Monday June 21st, as well as the Opening Ceremony on Tuesday June 22nd. Registered accompanying guests are also invited to listen to the lectures "The Road to Stockholm and Beyond" 1 and 2 in conjunction with the above mentioned events. In addition it gives accompanying guests the possibility to attend the Archipelago Boat Tour and the Gala Dinner for an additional fee.

Please note that the fee for accompanying guests does not include admission to the scientific sessions, the commercial- or the poster exhibitions nor does it entitle them to any Conference documentation or meals during breaks.

Exhibitor Registration Fee

The registration fee for exhibitors includes daily tea/coffee and lunch as well as the Welcome Reception at Stockholm City Hall.

Please note that the fee for exhibitors and sponsors does not include admission to the scientific sessions.

General Information

Badges

Each participant will receive a name badge upon registration at the Conference venue. For security reasons all participants are requested to wear their badge during all the Conference activities and social events. The cost for replacing a lost badge is SEK 100.

Banks, Credit Cards and Currency Exchange

Banks are open between 09:30/10:00 and 15:00 on weekdays. Some banks in central Stockholm are open from 09:00 to 17:00. Major credit cards are accepted in hotels, restaurants, taxis and shops. It is advisable to carry an identity card or some form of photo identification.

The official currency is Swedish Krona (SEK). USD 1 = SEK 7.75, EUR 1 = SEK 10.00 (May, 2010). For money exchange, the companies Forex and X-change have offices at the airports and in the city. Opening hours and other information about exchange can be found at www.forex.se and www.x-change.se.

Certificate of Attendance

A certificate of attendance will be given to registered participants upon request.

Climate and Dress

The weather in Stockholm at this time of the year is usually warm and sunny with temperatures of approximately 15–20 degrees Celsius; showers may occur. Informal dress is recommended for the Conference sessions and smart casual is recommended for the Welcome Reception and the Gala Dinner. A light jacket and/or scarf is recommended for the Archipelago Boat Tour.

Commercial Exhibition

A commercial exhibition will be arranged in the Conference Hall Foyer in conjunction with the Conference. Please see the list of exhibitors and further details on the cover.

Disclaimer/Liability

The Organising Committee and Congrex Sweden AB accept no liability for any injuries/losses incurred by participants and/or accompanying persons, nor loss of, or damage to, any luggage and/or personal belongings.

Electricity

Electrical current in Sweden is 220 V/50 Hz. Round, European-style two-pin plugs are used. Appliances designed to operate on 110/120 Volts need a voltage converter and a plug adapter.

European Green Capital

Stockholm is the first city to be awarded European Green Capital, 2010, by the European Commission. The award marks a city's wish and capability to solve environmental problems in order to both improve the quality of life of its citizens and reduce the impact it makes to the global environment as a whole. Everything from water protection plans, to public transport and work to omit fossil fuel emissions was reviewed. In all aspects, Stockholm is considered a front-runner.

Internet

High-speed wireless Internet is available at no charge in the Conference venue.

Language

The official language of the Conference is English. No simultaneous translation will be provided.

Meals

Daily tea/coffee and lunches are included in the registration fee.

Official Airline

SAS is proud to be the Official Airline for the Advances in Neuroblastoma Research meeting – ANR 2010 and welcomes you to Stockholm.

Professional Congress Organiser

CONGREX

Congrex Sweden AB is the official organiser of the Advances in Neuroblastoma Research Congress – ANR 2010. Congrex Sweden AB is a leading international management company offering comprehensive services for meetings, events, conferences, association management, travel and accommodation. Let's meet, visit www.congrex.com

On-site Registration at the City Conference Centre

On-site registration will start Sunday June 20th, at 15:00. The registration desk and Conference secretariat will be open for the duration of the Conference.

Time Zone

The time zone in Stockholm is GMT + 1 hour. Daylight Saving Time is used during the summer.

Tipping

A gratuity is included in the price of hotels and taxis. It is common, however, to leave a tip of around 10%. When visiting restaurants, you can show your appreciation for good service by leaving a little extra.

Tourist Information

The Conference secretariat will be available to give you more information about Stockholm. For additional information please contact the official tourist guide of Stockholm at:

Web site: www.stockholmtown.se
Visiting address: Sverigehuset, Hamngatan 27
E-mail: info@svb.stockholm.se
Phone: +46 8 508 28 508

Scientific Information

Poster Sessions

All posters will be displayed for the duration of the Conference. Special time is set aside for poster viewing only, and presenting authors of the abstracts in focus are asked to stand by their posters at this time.

Time for mounting and dismantling

Mounting: June 22nd, 10:15 – 12:00
Dismantling: June 24th, 13:00

Poster discussion time with presenting author present by their poster board:

June 22nd 16:45 – 17:30 (odd numbered/left aligned posters, see page 29)
June 23rd 16:45 – 17:30 (even numbered/indented posters, see page 29)

Poster map is found on the cover

Selected Posters

In addition to the oral presentation of selected posters, all selected posters will be displayed throughout the Conference. Selected posters will be displayed at the "Mezzanine level" on the day of the lecture, and in the main poster area the other days.

Selected posters 1–24 will be displayed at the "Mezzanine level" on June 22nd and selected posters 25–48 on June 23rd. Therefore, a switch of posters from the "Mezzanine level" to the main poster area needs to be done after the poster session on June 22nd. We ask all authors for their kind assistance to make this switch run smoothly.

Detailed instructions for mounting/dismantling Biology 1, Clinical 1 and Translational 1 (selected posters 1–24):

Mounting: June 22nd, 10:15 – 12:00 on Mezzanine level
Dismantling: June 22nd, 18.30 (posters can be stored at the venue over night)
Mounting: June 23rd, 07:30 – 10:00 in main poster area
Dismantling: June 24th, 13:00

Detailed instructions for mounting/dismantling Biology 2, Biology 3 and Translational 2 (selected posters 25–48):

Mounting: June 22nd, 09:00 – 10:15 in main poster area
Dismantling: June 22nd, 18.30 (posters can be stored at the venue over night)
Mounting: June 23rd, 07:30 – 10:00 on Mezzanine level
Dismantling: June 24th, 13:00

Selected poster discussion time (presenting author present by their poster board):

Authors to selected posters SEL1–24, presented in oral session on June 22nd should be present by their poster
June 22nd 16:45 – 17:30 (Mezzanine level)
Authors to selected posters SEL25–48, presented in oral session on June 23rd should be present by their poster
June 23rd 16:45 – 17:30 (Mezzanine level)

Speakers Preview room and AV information

The Speakers Preview room is located back stage of the Conference Halls and will open one hour before the first session each day and stay open throughout the day.

Speakers are requested to use this facility before their session to ensure that their slides project clearly and are in correct order. Slides should then be handed over to the technical staff no later than three hours before the start of that particular session.

Awards

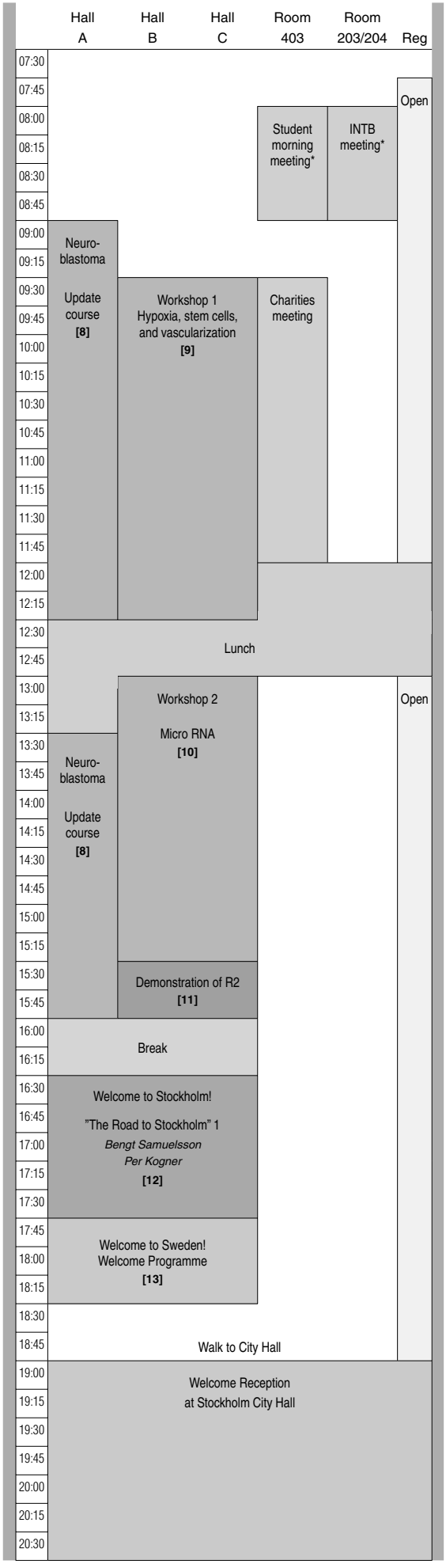
Awards for oral presentations

The Conference Organisers offered abstract presenters to apply for an award. Among these applicants, the abstracts with best rating were selected for a second round of rating by unbiased experts and a final set of candidates were selected. After final assessments of the oral presentations during the conference, awardees will be appointed and honored at the Gala Dinner, June 23rd (Biology and Translational award winners) and the Closing and Award Session June 24th (Clinical award winners).

Poster Prizes

Two kinds of poster prizes will be awarded. One set of awardees will be selected by three different committees (Biology, Translational and Clinical). Another set of awardees will be selected by all participants by using the voting form enclosed in your conference bag. All votes must be returned to the registration desk no later than Thursday lunch, 13:00. All poster prizes will be presented during the Closing and Award Session on Thursday June 24th.

Programme
Monday June 21st



* By invitation only

Monday June 21st

09:00 – 16:00 Monday June 21st
Hall A
Neuroblastoma Update Course
Organisers: Susan L. Cohn and Andrew Pearson

Chairs: Susan L. Cohn and Rani George

Neuroblastoma biology

09:00 **C1 Using genome-wide strategies to discover new gene aberrations** Page 78
Frank Speleman, Belgium

09:25 **C2 Biological and clinical relevance of ALK mutations** Page 78
Rani George and Yael Mosse, United States

Risk classification

10:05 **C3 INRG: Next steps** Page 78
Andy Pearson, United States

10:30 BREAK

Imaging

10:50 **C4 Using PET and MIBG to evaluate disease and response** Page 78
Sue Sharp, United States

Minimally invasive surgery

11:15 **C5 Is there a role for laparoscopic surgery?** Page 78
Jed Nuctern, United States

Low and intermediate-risk disease

11:40 **C6 Therapy and segmental aberrations for treatment stratification** Page 79
Gudrun Schleiermacher, France

12:05 **C7 When should a we use a "wait and see" approach** Page 79
Frank Berthold, Germany

12:30 LUNCH

Chairs: Andrew Pearson and Yaël Mossé

High-risk disease

13:30 **C8 Immunotherapy plus retinoic acid: A new standard of care** Page 79
Alice Yu, United States

MIBG radiotherapy

13:55 **C9 Update on MIBG therapy** Page 79
Kate Matthay, United States

Late Effects

14:20 **C10 Late effects in neuroblastoma** Page 80
Lisa Diller, United States

14:45 BREAK

Relapsed Disease

15:05 **C11 Factors that predict outcome in relapsed disease** Page 80
Wendy London, United States

Novel Treatments

15:30 **C12 Molecularly targeted therapy** Page 80
Louis Chesler, United Kingdom

15:55 **Closing**

Made possible thanks to a special grant from Mary Béve Foundation.

09:30 – 12:30 Monday June 21st

Hall B/C

Workshop 1 – Tumor initiating/stem cells, hypoxia and tumor vascularization

Organisers and chairs: David Kaplan and Sven Påhlman

09:30 **WS1 Tumor initiating/stem cells, hypoxia and vascularization - what are the connections?** Page 81
Sven Påhlman, Sweden

09:50 **WS2 Development of the autonomic nervous system, a molecular view** Page 81
Herman Rohrer, Germany

10:10 **WS3 Tumor initiating cells from bone marrow of high-stage neuroblastoma patients** Page 81
David Kaplan, Canada

10:30 BREAK

10:45 **WS4 Tumor initiating cells from MYCN amplified neuroblastomas** Page 81
Jan Molenaar, Netherlands

10:55 **WS5 The miRNAome of TICs in relation to tumor cells and fetal neuroblasts** Page 81
Katleen de Preter, Belgium

11:05 **WS6 Identification and molecular characterization of human neuroblastoma tumor-initiating cells** Page 81
Aurelie Coulon, Switzerland

11:15 **WS7 Vascular mimicry in human neuroblastom: identification of the progenitors of tumor derived endothelial cells** Page 82
Vito Pistoia, Italy

11:25 **WS8 Identification *in vitro* and *in vivo* of tumoral glial precursor cells in neuroblastic tumors** Page 82
Jaume Mora, Spain

11:35 **WS9 Exploiting the embryonic environment to reprogram cancer stem cells in neuroblastoma** Page 82
Rachel Carter, United Kingdom

11:45 **WS10 Low Dose Metronomic anti-angiogenic (LDM) oral Topotecan and Pazopanib as a potential model for maintenance therapy neuroblastoma** Page 83
Sylvain Baruchel, Canada

11:55 **Discussion**
- Phenotype of NB initiating/stem cells
- Primary vs cell line TICs/SCs
- Stem cell niche and angiogenesis
- NB TIC/SC location - similar cells in primary tumors and in the bone marrow?
- Number of NB TICs/SCs at a given site of prognostic significance?
- Targeting of TICs/SCs

12:25 **Concluding remarks**
David Kaplan and Sven Påhlman

12:30 – 13:00 LUNCH

13:00 – 15:30 Monday June 21st
Hall B/C
Workshop 2 – Contribution of microRNAs to neuroblastoma pathogenesis
Organisers and chairs: Marie Arsenian Henriksson and Angelika Eggert

13:00 **WS11 The neuroblastoma miRNA map, prioritization and functional evaluation of candidate miRNAs** Page 83
Pieter Mestdagh, Belgium

13:30 **WS12 miRNAs regulating neuroblastoma cell differentiation** Page 83
Ray Stallings, Ireland

13:50 **WS13 MYCN-regulated microRNAs repress estrogen receptor-α (ESR1) expression and neuronal differentiation in human neuroblastoma** Page 83
Marie Arsenian Henriksson, Sweden

14:05 BREAK

14:25 **WS14 Assessing the role of miRNAs in neuroblastoma biology – from expression profiling to functional analysis** Page 84
Angelika Eggert, Germany

14:40 **WS15 Bioinformatic analysis of miRNA profiles of a series of neuroblastomas by the R2** Page 84
Rogier Versteeg, Netherlands

15:00 **WS16 Genomic and proteomic study of microRNAs in pediatric cancers** Page 84
Jun Wei, United States

15:20 **Concluding remarks**
Angelika Eggert and Marie Arsenian Henriksson

15:30 – 16:00 Monday June 21st

Hall B/C

Special session – Public web-based analysis tool R2

Chairs: Marie Arsenian Henriksson and Angelika Eggert

SS1 Introduction to practical use of an analysis tool and database for high throughput and clinical data of neuroblastoma series Page 87

Rogier Versteeg, Netherlands

16:00 – 16:30 BREAK

16:30 – 17:45 Monday June 21st
Hall A/B/C
Welcome to Stockholm!
The Road to Stockholm and Beyond 1
Chairs: Bruno De Bernardi and Carol Thiele

PL1 The role of eicosanoids in health and disease
Bengt Samuelsson, Sweden

Dr. Bengt Samuelsson is Professor of Physiological Chemistry at the Karolinska Institute. Dr. Samuelsson's research led to the discovery of various prostaglandins and related substances. Of particular interest are the thromboxanes which are involved in such common, severe thrombotic diseases as strokes and coronary infarcts. He also discovered the leukotrienes, substances that play a role in inflammation and asthma and other allergic diseases. For his discovery of prostanoids and leukotrienes he was awarded the Nobel Prize in Physiology or Medicine in 1982.

Dr. Samuelsson obtained his Doctor of Medical Science degree in biochemistry and later, his M.D. degree, from the Karolinska Institute. He spent a year as a research fellow in the Department of Chemistry at Harvard University, Cambridge, Mass., USA. In 1972, Dr. Samuelsson was appointed professor at the Karolinska Institute. In 1973–1983, he was Chairman of the Department of Chemistry; in 1978–1983, Dean of the Medical Faculty and in 1983–1995, President of the Karolinska Institute.

Dr. Samuelsson has been a member of the Nobel Assembly and the Nobel Committee for Physiology or Medicine at the Karolinska Institute and in 1993–2005, he was Chairman of the Nobel Foundation in Stockholm.

In addition to the Nobel Prize, Dr. Samuelsson has received a number of worldwide awards and honorary academic degrees. These include the Louisa Gross Horwitz Award, the Gairdner Foundation Award and the Albert Lasker Basic Medical Research Award. He is a Foreign Associate of the US National Academy of Sciences and a Foreign Member of the Royal Society, London. He is a member of the Royal Swedish Academy of Sciences, the Royal National Academy of Medicine, Spain, the French Academy of Sciences and the Institute of Medicine, USA.

PL2 Fatty acids as positive and negative regulators in neuroblastoma development Page 88
Per Kogner, Sweden

Per Kogner, MD Karolinska Institutet 1985, Certified Pediatrician 1990, PhD Karolinska Institutet 1993, Postdoc Karolinska Institutet 1993-1994, Full Professor Karolinska Institutet 2005. Present position: Professor of Pediatric Oncology, Childhood Cancer Research Unit, Department of Women's and Children's Health, Karolinska Institutet, and Senior Consultant, Astrid Lindgren Children's Hospital.

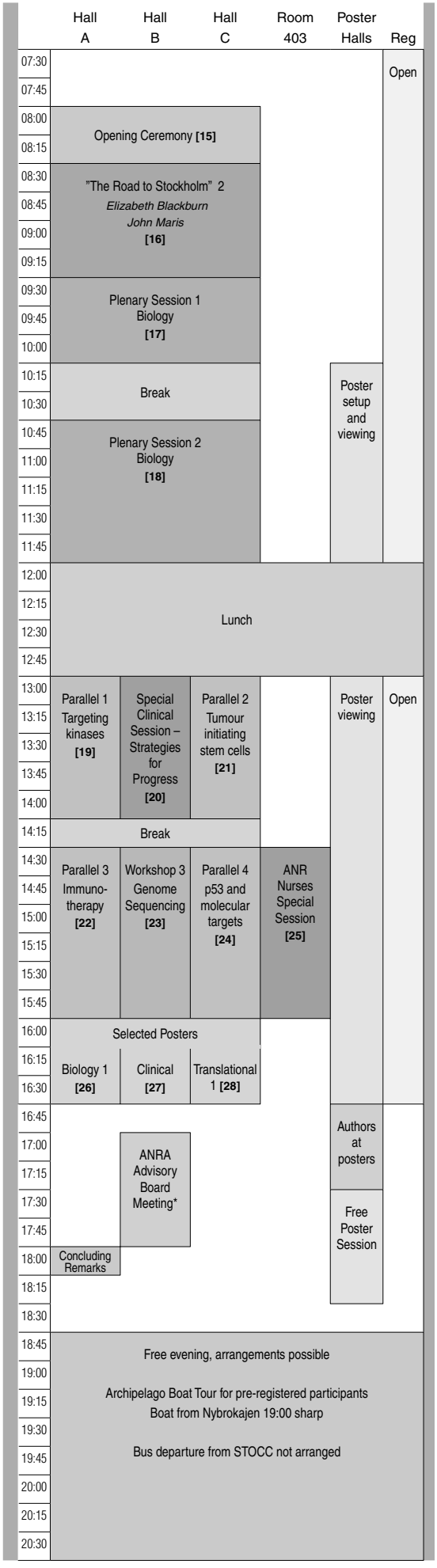
17:45 – 18:30 Monday June 21st
Hall A/B/C
Welcome to Sweden!

The programme includes traditional Swedish music and performance by various artists, and you will get to know Sweden culturally and historically.

Performers:
Ann-Marie Sundberg, birch-bark horn
Elin & Edward Anderzon, Swedish keyed fiddle
Ulrika Gunnarsson, Swedish traditional falsetto song
Skansen Folkdance Team
Children's Choir

The Welcome Programme is followed by a Welcome Reception in Stockholm City Hall.

Programme
Tuesday June 22nd



* By invitation only

Tuesday June 22nd

08:00 – 08:30 Tuesday June 22nd
Hall A/B/C
Opening Ceremony

Professor Per Kogner, ANR 2010 President
Professor Harriet Wallberg-Henriksson, President Karolinska Institutet
Professor Susan L. Cohn, President ANRA
Professor Olle Björk, General Secretary, Swedish Childhood Cancer Foundation
Mrs Filippa Reinfeldt, Health Care Commissioner, Stockholm County Council

08:30 – 09:30 Tuesday June 22nd
Hall A/B/C
The Road to Stockholm and Beyond 2
Chairs: Garrett Brodeur and Victoria Castel

PL3 Telomeres and telomerase: Their implications for human health and disease
Elizabeth Blackburn, United States

Elizabeth Blackburn, Ph.D. is the recipient of the 2009 Nobel Prize in Physiology or Medicine for her discoveries in telomere biology that have uncovered a new understanding of normal cell functioning and given rise to a growing field of inquiry.

Throughout her distinguished career, whether as the editor of high-profile scientific journals, such as Molecular Cancer Research and Molecular Biology of the Cell, or as a current member of over 30 distinct institutional advisory boards or review committees, Dr. Elizabeth Blackburn has spent countless hours in service to her constituency. Further, she has held leadership positions in several scientific societies, including her current appointment as President of the American Association for Cancer Research.

Dr. Blackburn has been recognized for her seminal contribution to the field of telomere biology with numerous prizes, awards, and honorary degrees, including the 2006 Albert Lasker Award for Basic Medical Research and elections to the American Association for the Advancement of Science and the Institute of Medicine. In 2007, Time magazine named her one of the '100 Most Influential People in the World,' and in 2008 she was the North American Laureate for the L'Oreal_ UNESCO For Women In Science. The scientific community bestowed upon her the ultimate recognition of her legacy by honoring Dr. Elizabeth Blackburn with the 2009 Nobel Prize in Physiology or Medicine.

Dr. Blackburn is currently the Morris Herzstein Endowed Chair in Biology and Physiology in the Department of Biochemistry and Biophysics at the University of California, San Francisco. She is also a Non-Resident Fellow of the Salk Institute.

PL4 Neuroblastoma genetics: From the beginning to the end(s) Page 89
John Maris, United States

Dr. John Maris is a tenured Professor in the Department of Pediatrics at the University of Pennsylvania.

He is a physician-scientist who has developed a translational research program broaching the basic genetic mechanisms of childhood cancer initiation to pivotal clinical trials for these same diseases. He currently serves as Chief of the Division of Oncology at the Children's Hospital of Philadelphia (CHOP) and Director of the Center for Childhood Cancer research, also housed at CHOP. He is also Chair of the Children's Oncology Group Neuroblastoma Committee and Director of the Pediatric Oncology Program in the Abramson Cancer Center at Penn. Dr. Maris' major research contributions involve providing key insights into the genetic basis of neuroblastoma initiation and progression.

09:30 – 10:15 Tuesday June 22nd
Hall A/B/C
Plenary session 1 – Biology
Chairs: Frank Speleman and Susan L. Cohn

09:30 **PL5 Copy number variations (CNVs) in neuroblastoma** Page 90

Sharon J. Diskin; Kristopher Bosse; Patrick Mayes; Michael LaQuaglia; Edward F. Attiyeh; Yael P. Mosse; Marci Laudenslager; Maura Diamond; Geoffrey Norris; Cuiping Hou; Kai Wang; Haito Zhang; Cecilia Kim; Wendy London; Marcella Devoto; Hongzhe Li; Hakon Hakonarson; John M. Maris
United States

09:45 **PL6 A potential role for the pluripotency factor LIN28B and Let-7 signalling in neuroblastoma** Page 90

Molenaar, Jan[1]; Ebus, Marli[1]; Koster, Jan[1]; Sluis van, Peter[1]; Schulte, Johannes[2]; Eggert, Angelika[2]; Mestdagh, Pieter[3]; Sompele van de, Jo[3]; Speleman, Frank[3]; Caron, Huib[4]; Versteeg, Rogier[1]
[1]University of Amsterdam, Human Genetics, Amsterdam, Netherlands; [2]University Childrens Hospital Essen, Pediatric oncology, Essen, Germany; [3]Gent University Hospital, CMGG, Gent, Belgium; [4]University of Amsterdam, Pediatric Oncology, Amsterdam, Netherlands

10:00 **PL7 CAMTA1, a 1p36 tumor suppressor candidate, activates differentiation programmes in neuroblastoma cells** Page 91

Kai-Oliver Henrich; Tobias Bauer; Volker Ehemann; Hedwig Deubzer; Sina Gogolin; Matthias Fischer; Manfred Schwab; Frank Westermann
Germany

10:15 – 10:45 BREAK

10:45 – 12:00 Tuesday June 22nd
Hall A/B/C
Plenary session 2 – Biology
Chairs: Akira Nakagawara and Michelle Haber

10:45 **PL8 Whole genome and transcriptome sequencing of ten stage IV primary neuroblastoma tumors: a TARGET project report** Page 91

Olena Morozova [1]; Edward F. Attiyeh [2]; Ryan D. Morin [1]; Martin Hirst [1]; Timothee Cezard [1]; Richard Moore [1]; Cecelia Suragh [1]; Nina Thiessen [1]; Richard Varhol [1]; Yongjun Zhao [1]; Michael D. Hogarty [2]; Shahab Asgharzadeh [2]; Daniela S. Gerhard [2]; Malcolm A. Smith [2]; Javed Khan [2]; Robert C. Seeger [2]; John M. Maris [2]; Marco A. Marra [1]

[1]Canada; [2]United States

11:00 **PL9 Role of a novel inducible dependence receptor UNC5D in spontaneous regression of neuroblastoma: its functional cooperation with p53 and E2f1 for inducing programmed cell death** Page 92

Yuyan Zhu; Yuanyuan Li; Seiki Haraguchi; Meng Yu; Miki Ohira; Atsuko Nakagawa; Eriko Isogai; Haruhiko Koseki; Yohko Nakamura; Hirofumi Arakawa; Akira Nakagawara

Japan

11:15 **PL10 Metastatic neuroblastoma cancer stem cells display a mixed phenotype of tumor and niche origin required for survival** Page 92

Loen Hansford [1]; Olena Morozova [1]; Tatiana Lipman [1]; Kim Blakely [1]; Miki Ohira [2]; Paula Marrano [1]; Paola Angelini [1]; Jason Moffat [1]; Carol Thiele [3]; Paul Thorner [1]; John Dick [1]; Akira Nakagawara [2]; Meredith Irwin [1]; Marco Marra [1]; David Kaplan [1]

[1]Canada; [2]Japan; [3]United States

11:30 **PL11 Cell-cell communication via ion fluxes in control of neuroblastoma cell cycle** Page 93

Hiromi Hiyoshi [1]; Shaimaa Abdelhady [1]; Baldur Sveinbjörnsson [1]; Lova Segerström [1]; Loen Hansford [2]; Meredith Irwin [2]; David Kaplan [2]; Per Kogner [1]; John Inge Johnsen [1]; Michael Andäng [1]; Per Uhlén [1]

[1]Sweden; [2]Canada

11:45 **PL12 *PHOX2B* is essential for peripheral sympathetic neuronal differentiation in the zebrafish** Page 93

William Luther II; Rodney Stewart; John Kanki; A. Thomas Look; Rani E. George

United States

12:00 – 13:00 LUNCH

13:00 – 14:10 Tuesday June 22nd
Hall A
Parallel session 1 – Targeting kinases
Chairs: Huib Caron and Pat Reynolds

13:00 **OR1 The KidsCancerKinome: Validation of Aurora kinases as potential drug targets in neuroblastoma and other pediatric tumors** Page 106
Ellen Westerhout [1]; Marcel Kool [1]; Jan Molenaar [1]; Peter Stroeken [1]; Monique den Boer [1]; Stephanie Segers [1]; Steven Clifford [2]; Olivier Delattre [3]; Magdalena Benetkiewicz [3]; Claudia Lanvers [4]; Ron Pieters [1]; Torsten Pietsch [4]; Marcel Holst [4]; Jane Renshaw [2]; Janet Shipley [2]; Massimo Serra [5]; Katie Scotlandi [5]; Birgit Geoerger [3]; Gilles Vassal [3]; Olivier Degrand [3]; Arnaud Verschuur [1]; Rogier Versteeg [1]; Huib Caron [1]
[1]Netherlands; [2]United Kingdom; [3]France; [4]Germany; [5]Italy

13:10 **OR2 Inhibition of Aurora-A as an approach to control N-Myc levels in neuroblastoma** Page 106
Markus Brockmann [1]; Louis Chesler [2]; Martin Eilers [1]
[1]Germany; [2]United Kingdom

13:20 **OR3 Molecular analysis and therapeutic targeting of the PI3K/AKT/mTOR pathway in paediatric neuroblastoma** Page 106
Paul Wood; David Ashley; Carleen Cullinane; Kathryn Kinross; Gretchen Poortinga; Kerry Ardley; Grant McArthur
Australia

13:30 **OR4 PI3K inhibitors prime neuroblastoma cells for chemotherapy *in vitro* and *in vivo* by shifting the balance towards pro-apoptotic Bcl-2 proteins and increased mitochondrial apoptosis** Page 107
Ariane Bender [1]; Daniela Opel [1]; Ivonne Naumann [1]; Roland Kappler [1]; Lori Friedman [2]; Klaus-Michael Debatin [1]; Simone Fulda [1]
[1]Germany; [2]United States

13:40 **OR5 PLK1 is a novel target for high-risk neuroblastoma therapy** Page 107
Sandra Ackermann; Felix Goeser; Johannes Schulte; Volker Ehemann; Angelika Eggert; Barbara Hero; Frank Berthold; Matthias Fischer
Germany

13:50 **OR6 MicroRNA-184 inhibits neuroblastoma cell proliferation and promotes apoptosis by targeting the serine/threonine kinase *AKT2*** Page 107
Niamh H Foley; Isabella Bray; Derek M Murphy; Jacqueline Ryan; Amanda Tivnan; Patrick Buckley; Stallings Ray
Ireland

14:00 **OR7 Exploring a new therapy for neuroblastoma: silencing of doublecortin-like kinase using RNA-interference** Page 107
Carla S. Verissimo; Jan J. Molenaar; John Meerman; Jordi C. Puigvert; Fieke Lamers; Maarten Rotman; Petra van Kuik-Romeijn; Erik H.J. Danen; Bob van de Water; Rogier Versteeg; Carlos P. Fitzsimons; Erno Vreugdenhil
Netherlands

14:10 – 14:30 BREAK

13:00 – 14:10 Tuesday June 22nd
Hall B
Special Clinical Session – Strategies for Progress
Chairs: Kate Matthay and Frank Berthold

SS2 Current and future strategies to improve outcomes in neuroblastoma: An update from the Children's Oncology Group Neuroblastoma Disease Committee Page 87
John Maris, United States

SS3 Strategies to improve outcome and quality of life in patients with neuroblastoma: Activities of the SIOP European Neuroblastoma Group Page 87
Ruth Ladenstein, Austria

SS4 Recent achievements and future strategies of GPOH to improve outcome for children with neuroblastoma Page 87
Thorsten Simon, Germany

Invited discussants

This session focuses on current and future strategies for clinical studies with special emphasis on areas of priority for international collaboration. The session intends to be interactive, chaired by two experienced experts, including three main speakers, representing three collaborative clinical groups, COG, SIOPEN and GPOH. There will be invited discussants and an active auditorium is expected.

14:10 – 14:30 BREAK

13:00 – 14:10 Tuesday June 22nd

Hall C

Parallel session 2 – Tumour initiating stem cells

Chairs: Sylvain Baruchel and Johannes Schulte

13:00 **OR8 HIF-2α maintains an undifferentiated state in neural crest-like human neuroblastoma tumor-initiating cells** Page 108
Alexander Pietras [1]; Loen M. Hansford [2]; A. Sofie Johnsson [1]; Esther Bridges [1]; Jonas Sjölund [1]; David Gisselsson [1]; Matilda Rehn [1]; Siv Beckman [3]; Rosa Noguera [4]; Samuel Navarro [4]; Jörg Cammenga [1]; Erik Fredlund [1]; David R. Kaplan [2]; Sven Påhlman [1]
[1]Sweden; [2]Canada; [3]Switzerland; [4]Spain

13:10 **OR9 Identification of signaling pathways and drug candidates using primary neuroblastoma cancer stem cells by phosphoproteomics and transcriptome sequencing** Page 108
Milijana Vojvodic; Olena Morozova; Kim Blakely; Natalie Grinshtein; Loen Hansford; Kristen Smith; Jiefie Tong; Paul Taylor; Meredith Irwin; Jason Moffat; Mike Moran; Marco Marra; David Kaplan
Canada

13:20 **OR10 Induced stable neuroblastoma cancer stem cells** Page 108
Naohiko Ikegaki; Paul Regan; Autumn Fox; Joshua Jacobs; Eric Rappaport; Xao Tang
United States

13:30 **OR11 Inhibition of global DNA methylation induces differentiation of human neuroblastoma tumor-initiating cells** Page 109
A. Sofie Johnsson; Alexander Pietras; Caroline Wigerup; Sven Pahlman
Sweden

13:40 **OR12 Exploiting the embryonic environment to reprogram cancer stem cells in neuroblastoma** Page 109
Rachel Carter; Edwin Jesudason; Mike White; Violaine See; Paul Losty; Heather McDowell; Louis Chesler; Barry Pizer; Diana Moss
United Kingdom

13:50 **OR13 Endocrine-gland vascular endothelial growth factor (EG-VEGF) in neuroblastoma tumor initiating cells** Page 109
Elly Ngan [1]; Cynthia Lau [1]; Jana Woo [1]; Wing-Keung Chan [1]; Godfrey Chan [1]; Yu Wang [1]; David Kaplan [2]; Paul Tam [1]
[1]Hong Kong; [2]Canada

14:00 **OR14 Novel cardiac glycoside analogues selectively target neuroblastoma tumor initiating cells (TICs)** Page 109
Paolo De Gouveia [1]; Kristen Smith [1]; Mayumi Fujitani [1]; Murugesapillai Mylvaganam [1]; Jamie La [1]; Shaimaa Abdelhady [2]; Michael Andang [2]; David Uehling [1]; Ahmed Mamai [1]; Rima Al-awar [1]; Clifford Lingwood [1]; David R. Kaplan [1]; Meredith S. Irwin [1]
[1]Canada; [2]Sweden

14:10 – 14:30 BREAK

14:30 – 16:00 Tuesday June 22nd
Hall A
Parallel session 3 – Immunotherapy
Chairs: Holger Lode and Jean Michon

14:30 **OR15 Anti-GD2 murine monoclonal antibody (MoAb) 3F8/Granulocyte-Macrophage colony stimulating factor (GM-CSF) plus 13-Cis-Retinoic acid (13-cis-RA) for consolidation of >2nd complete remission/very good partial remission (CR/VGPR) of neuroblastoma** Page 110
Brian Kushner; Kim Kramer; Shakeel Modak; Karima Yataghene; Nai-Kong Cheung
United States

14:40 **OR16 Combined administration of *in vitro* expanded Vγ9Vγ2+ T cells and bisphosphonate zoledronic acid as preclinical immunotherapeutic approach for neuroblastoma** Page 110
Laura Emionite; Michele Cilli; Paola Bocca; Lizzia Raffaghello; Vito Pistoia; Ignazia Prigione
Italy

14:50 **OR17 Galectin-1 modulates immune response towards a state of tolerance in neuroblastoma** Page 110
Rocio Soldati [1]; Elisa Berger [1]; Ana C Zenclussen [1]; Gerhard Jorch [1]; Mariana Salatino [2]; Gabriel A Rabinovich [2]; Stefan Fest [1]
[1]Germany; [2]Argentina

15:00 **OR18 Lenalidomide activates anti-tumor functions of NK cells and overcomes immune suppression by IL-6 and TGFβ1** Page 110
Yibing Xu; Jianping Sun; Hong-Wei Wu; Michael Sheard; Hung Tran; Zesheng Wan; Cathy Liu; Robert Seeger
United States

15:10 **OR19 Treatment of high risk neuroblastoma with autologous T lymphocytes engineered to recognize GD2** Page 111
Chrystal Louis; Martin Pule; Barbara Savoldo; G Doug Myers; Claudia Rossig; Heidi Russell; Teresita Lopez; Gianpietro Dotti; Enli Liu; Hao Liu; Adrian Gee; Eric Yvon; Cliona Rooney; Helen Heslop; Malcolm Brenner
United States

15:20 **OR20 A novel lentiviral-transduced dendritic cell vaccine targeting the survivin antigen is effective against neuroblastoma** Page 111
Elisa Berger; Holger N Lode; Ana C Zenclussen; Gerhard Jorch; Stefan Fest
Germany

15:30 **OR21 Immunotherapy for neuroblastoma by GD2 specific chimeric antigen receptor** Page 111
John Anderson; Simon Thomas; Nourredine Himoudi; Martin Pule
United Kingdom

15:40 **OR22 NKT cells co-localize with tumor-associated macrophages in neuroblastoma in an innate response to tumor-induced hypoxia** Page 111
Daofeng Liu; Liping Song; Jie Wei; Leonid Metelitsa
United States

15:50 **OR23 Bone marrow response evaluation with a quantitative device identifies prognostic groups in patients over 18 months** Page 112
Inge M. Ambros; Ulrike Pötschger; Andrea Ziegler; Ditha Modritz; Helmut Gadner; Ruth Ladenstein; Peter F. Ambros
Austria

14:30 – 16:00 Tuesday June 22nd

Hall B

Workshop 3 – Next generation seqencing techniques in neuroblastoma genomics

Organisers: Ingrid Öra and Tommy Martinsson

Chairs: Javed Khan and Tommy Martinsson

14:30	**WS17 Next generation sequencing technologies to investigate the cancer genome & Insights into neuroblastoma biology and tumor progression models l earnt from massively parallel sequencing strategies** *Javed Khan, United States*	Page 84
14:45	**WS18 Next generation sequencing of the (small) RNA transcriptome - from catalogisation to quantitative expression profiling** *Johannes H. Schulte, Germany*	Page 85
15:00	**WS19 Next-Generation Sequencing to characterize somatic alterations in neuroblastoma samples** *Olivier Delattre, France*	Page 85
15:15	**WS20 The Neuroblastoma-TARGET project: Plans for sequencing, validation and frequency scans** *John Maris, United States*	Page 85
15:30	**WS21 Finding variant needles in the neuroblastoma haystack: the Ghent NGS approach** *Jo Vandesompele, Belgium*	Page 85
15:45	**Discussion**	

14:30 – 16:00 Tuesday June 22nd
Hall C
Parallel session 4 – p53 and molecular targets
Chairs: Naohiko Ikegaki and Glenn Marshall

14:30 **OR24 Cooperative induction of apoptosis through p53 signaling and mTOR inhibition in neuroblastoma** Page 112
Eveline Barbieri; Zaowen Chen; Eugene Kim; Danielle Patterson; Denae Sikorski; Jason Shohet
United States

14:40 **OR25 Repressed p53 stress responses in normal perinatal cells provides a susceptibility to N-Myc oncogenesis as an initiating event in embryonal malignancy** Page 113
Eric Sekyere [1]; Wayne Thomas [1]; Hongjuan Cui [2]; Joanna Keating [1]; Jin -Biao Chen [1]; Anna Raif [1]; Belamy Cheung [1]; Kacper Jankowski [1]; Neil Davies [1]; Bernard Chen [1]; Margo van Bekkum [1]; Tammy Ellis [1]; Murray Norris [1]; Michelle Haber [1]; Eugene Kim [2]; Jason Shohet [2]; Brandon Wainwright [1]; Han-Fei Ding [2]; Glenn Marshall [1]
[1]Australia; [2]United States

14:50 **OR26 Common and distinct MYC target genes in embryonal tumors** Page 113
Christina Poehler [1]; Daniel Muth [1]; Stephan Gade [1]; Giulio Fiaschetti [2]; Oskar Smrzka [3]; Tim Beissbarth [1]; Dominik Sturm [1]; Stefan Pfister [1]; Alexandre Arcaro [2]; Heinrich Kovar [3]; Matthias Fischer [1]; Manfred Schwab [1]; Frank Westermann [1]
[1]Germany; [2]Switzerland; [3]Austria

15:00 **OR27 Addiction of MYCN amplified neuroblastomas to B-MYB underscores a reciprocal regulatory loop** Page 113
Francesco Gualdrini; Arturo Sala; Arturo Sala
United Kingdom

15:10 **OR28 Combined massively parallel sequencing and synthetic lethal screening identifies multiple druggable targets in neuroblastoma** Page 114
Qing-Rong Chen; David Azorsa; Young Song; Jun Wei; Tom Badgett; Xiang Guo; Peter Johansson; Xinyu Wen; Susan Yeh; Catherine House; John Maris; Javed Khan
United States

15:20 **OR29 Tumor regression and curability of preclinical neuroblastoma models by the novel targeted camptothecin EZN-2208** Page 114
Fabio Pastorino [1]; Monica Loi [1]; Puja Sapra [2]; Pamela Becherini [1]; Michele Cilli [1]; Laura Emionite [1]; Domenico Ribatti [1]; Lee M Greenberger [2]; Ivan D Horak [2]; Mirco Ponzoni [1]
[1]Italy; [2]United States

15:30 **OR30 Selective targeting of neuroblastoma tumor inititating cells by a telomerase inhibitor IMETELSTAT** Page 114
Tatiana Lipman [1]; Mayumi Fujitani [1]; Loen Hansford [1]; Ian Clarke [1]; Calvin Harley [2]; Robert Tressler [2]; David Malkin [1]; Erin Walker [1]; Peter Dirks [1]; Baruchel Sylvain [1]; David Kaplan [1]; Uri Tabori [1]
[1]Canada; [2]United States

15:40 **OR31 ABCC transporters influence multiple aspects of neuroblastoma biology, as well as clinical outcome, independent of cytotoxic drug efflux** Page 115
Michelle Haber [1]; Michelle Henderson [1]; Antonio Porro [2]; Marcia Munoz [1]; Nunzio Iraci [2]; Cheng Xue [1]; Jayne Murray [1]; Claudia Flemming [1]; Janice Smith [1]; Jamie Fletcher [1]; Samuele Gherardi [2]; Alan Kwek [1]; Amanda Russell [1]; Wendy London [3]; Allen Buxton [3]; Lesley Ashton [1]; Alan Sartorelli [3]; Susan L. Cohn [3]; Manfred Schwab [4]; Glenn Marshall [1]; Giovanni Perini [2]; Murray Norris [1]
[1]Australia; [2]Italy; [3]United States; [4]Germany

15:50 **OR32 EZH2 mediates epigenetic silencing of candidate neuroblastoma tumor suppressor gene Casz1** Page 115
Chunxi Wang [1]; Chan-Wook Woo [2]; Zhihui Liu [1]; Jun Wei [1]; Young Song [1]; Lifeng Wang [1]; Victor Marquez [1]; Javed Khan [1]; Kai Ge [1]; Carol Thiele [1]
[1]United States; [2]Republic of Korea

14:30 – 16:00 Tuesday June 22nd
Room 403
ANR Nurses Special Session

Special programme for nurses in pediatric oncology and other interested.
Hosted by ANR 2010 Nurses Committee; Lisa Burström, Karin Enskär, Pernilla Pergert, Eva Turup

16:00 – 16:45 Tuesday June 22nd
Hall A
Selected poster – Biology 1
Chairs: Katarina Ejeskär and Miki Ohira

16:05 **SEL1 Omics analysis and evolution for identification of candidate genes in progression of neuroblastoma** Page 134
Eiso Hiyama; Naomi Kamei; Arata Kamimatsuse; Keiko Hiyama; Yukina Hirai; Tsutomu Masujima
Japan

16:10 **SEL2 Impaired activation of the tumor suppressor p14^{ARF} impedes its oncosuppressive impact in neuroblastoma** Page 134
Daniel Dreidax; Sina Gogolin; Daniel Muth; Marc Zapatka; Jessica Berthold; Matthias Fischer; Manfred Schwab; Frank Westermann
Germany

16:15 **SEL3 The FRAGILOME Project to discover new biomarkers for neuroblastoma** Page 134
Larissa Savelyeva; Anne Blumrich; Sarah Zahedi; Manfred Schwab
Germany

16:20 **SEL4 Expression profiling in neuroblastoma identifies a fourth subgroup with high expression of ERBB3** Page 134
Frida Abel [1]; Daniel Dalevi [1]; Katleen De Preter [2]; Joelle Vermeulen [2]; Raymond Stallings [3]; Per Kogner [1]; John Maris [4]; Staffan Nilsson [1]
[1]Sweden; [2]Belgium; [3]Ireland; [4]United States

16:25 **SEL5 A SP1/MIZ1/MYCN ternary complex induces repression of TRKA and p75NTR neurotrophin receptors and affects neuroblastoma malignancy through inhibition of the cell apoptotic response to NGF** Page 135
Emanuele Valli [1]; Giuliano Della Valle [1]; Nunzio Iraci [1]; Antonio Porro [1]; Daniel Diolaiti [1]; Roberto Bernardoni [1]; Martini Eilers [2]; Giovanni Perini [1]
[1]Italy; [2]Germany

16:30 **SEL6 NCYM, a protein product of an antisense *MYCN* gene co-amplified with *MYCN*, targets MYCN for functional modulation and affects the prognosis of neuroblastoma** Page 135
Suenaga Yusuke; Yoshiki Kaneko; Daisuke Matsumoto; Miki Ohira; Yohko Nakamura; Akira Nakagawara
Japan

16:35 **SEL7 Identification of a new fusion gene on 11q23 in neuroblastoma tumor samples** Page 135
Jan Molenaar [1]; Marli Ebus [1]; Jan Koster [1]; Arjan Lakeman [1]; Johannes Schulte [2]; Angelika Eggert [2]; Ingrid Ora [3]; Ray Stallings [4]; Huib Caron [1]; Rogier Versteeg [1]
[1]Netherlands; [2]Germany; [3]Sweden; [4]Ireland

16:40 **SEL8 Characterization of amplicon junction sequences in genomic regions surrounding the MYCN gene in neuroblastoma tumors; implications for clinical follow-up of high-risk patients** Page 135
Hanna Kryh; Jonas Abrahamsson; Elsa Jegerås; Rose-Marie Sjöberg; Irene Devenney; Per Kogner; Tommy Martinsson
Sweden

16:00 – 16:45 Tuesday June 22nd

Hall B

Selected poster – Clinical

Chairs: Fredrik Hedborg and Thorsten Simon

16:05 **SEL9 Causal inference, a novel approach to disentangle the effects of off-protocol therapy from the primary effects of interest in COG protocol P9462: Topotecan vs. Topotecan+cyclophosphamide in relapsed neuroblastoma** Page 136
Wendy B. London; Christopher N. Frantz; Laura A. Campbell; Robert C. Seeger; Babette A. Brumback; Susan L. Cohn; Katherine K. Matthay; Robert P. Castleberry; Lisa Diller
United States

16:10 **SEL10 Persistence of disease in long-term survivors of high-risk neuroblastoma. Analysis of ENSG5 cooperative trial** Page 136
Lucas Moreno [1]; Sucheta Vaidya [1]; Ross Pinkerton [2]; Ian J Lewis [1]; John Imeson [1]; Caroline Ellershaw [1]; David Machin [1]; Andrew DJ Pearson [1]
[1]United Kingdom; [2]Australia

16:15 **SEL11 High dose MIBG and haploidentical stem cell transplantation with cell therapy in therapy resistant neuroblastoma** Page 136
Jacek Toporski; Dominik Turkiewicz; Jan Tennvall; Michael Garkavij; Josefina Dykes; Katarina Le Blanc; Stig Lenhoff; Gunnar Juliusson; Stefan Scheding; Ingrid Øra; Albert N. Bekassy
Sweden

16:20 **SEL12 Combined radioimmunotherapy and anti-angiogenic therapy for resistant neuroblastoma** Page 137
Shakeel Modak; Brian H. Kushner; Kim Kramer; Neeta Pandit-Taskar; Jorge A. Carrasquillo; Pat Zanzonico; Peter Smith-Jones; Steven Larson; Nai-Kong V. Cheung
United States

16:25 **SEL13 MYCN amplified neuroblastoma differ in clinical features at initial presentation** Page 137
Barbara Hero [1]; Thorsten Simon [1]; Ruediger Spitz [1]; Jessica Theissen [1]; Frank Westermann [1]; Holger Christiansen [1]; Freimut H. Schilling [1]; Felix Niggli [2]; Boris De Carolis [1]; Frank Berthold [1]
[1]Germany; [2]Switzerland

16:30 **SEL14 Diffusion-weighted whole-body imaging with background body suppression (DWIBS) in pediatric oncology patients - a feasibility assessment** Page 137
Throstur Finnbogason; Bo Ehnmark; Hans Jacobsson; Bo Nordell; Linda Guler; Per Kogner; Lena Douglas
Sweden

16:35 **SEL15 Analysis of toxicity and efficacy of high dose chemotherapy with Busulfan and Melphalan followed by stem cell transplantation in high risk neuroblastoma patients: a retrospective study of a large cohort in a single institution** Page 137
Stéphanie Proust-Houdemont; Ellen Benhamou; Christelle Dufour; Gisèle Goma; Nathalie Gaspar; Véronique Minard-Colin; Cormac Owens; Olivier Hartmann; Dominique Valteau-Couanet
France

16:40 **SEL16 Natural history of infantile neuroblastoma under "wait and see" observation — current status of patients after long term follow up for 5 - 15 years** Page 138
Akihiro Yoneda; Masami Inoue; Takaharu Oue; Yasuyuki Mitani; Keisuke Nose; Hiroshi Nakai; Hisayoshi Kawahara; Akio Kubota; Masanori Nishikawa; Masahiro Nakayama; Keisei Kawa
Japan

16:00 – 16:45 Tuesday June 22nd
Hall C
Selected poster – Translational 1
Chairs: Ingrid Öra and Cai Weisong

16:00 **SEL17 Polyamine inhibition blocks initiation and progression of neuroblastoma** Page 138
Michelle Haber [1]; Nicholas F. Evageliou [2]; Annette Vu [2]; Jayne Murray [1]; Ngan Ching Cheng [1]; Xueyuan Liu [2]; Kelly-Ann Corrigan [2]; David Ziegler [1]; Glenn M. Marshall [1]; Murray D. Norris [1]; Michael D. Hogarty [2]
[1]Australia; [2]United States

16:05 **SEL18 Blocking Galectin-1 function reduces growth of aggressive neuroblastoma cells *in vitro* and *in vivo*** Page 138
Alexander Schramm [1]; Sali Timah [1]; Flora Cimmino [2]; Achille Iolascon [2]; Jan Koster [3]; Rogier Versteeg [3]; Johannes Schulte [1]; Angelika Eggert [1]
[1]Germany; [2]Italy; [3]Netherlands

16:10 **SEL19 Bortezomib delays neuroblastoma tumor growth while impairing bone growth, testis development and fertility in a male xenograft mouse model** Page 138
Emma Eriksson; John I. Johnsen; Per Kogner; Lars Savendahl
Sweden

16:15 **SEL20 Evaluation of the effect of acetyl l-carnitine on experimental cisplatin ototoxicity and neurotoxicity** Page 139
Dilek Gunes; Günay Kýrkým; Efsun Kolatan; Enis Alpin Güneri; Candan Özoðul; Bülent Þerbetçioðlu; Osman Yýlmaz; Özlem Tüfekçi; Kamer Mutafoðlu; Zekiye Altun; Safiye Aktaþ; Zübeyde Erbayraktar; Nur Olgun
Turkey

16:20 **SEL21 BTK expression is critical in neuroblastoma tumor initiating cells** Page 139
Erika Currier; Sharon Illenye; Lee Dorf; Lee Honigberg; Giselle Sholler
United States

16:25 **SEL22 Inflammatory prostaglandin E_2 induces neuroblastoma cell proliferation and survival in an autocrine and/or paracrine manner** Page 139
Agnes Rasmuson [1]; Anna Kock [1]; John Inge Johnsen [1]; Ole Martin Fuskevåg [2]; Lena-Maria Carlson [1]; Jaione Simon SantaMaria [2]; Per Kogner [1]; Baldur Sveinbjörnsson [2]
[1]Sweden; [2]Norway

16:30 **SEL23 Inhibition of Fatty Acid Synthase (FASN) as a potential therapy in neuroblastomas with MYCN amplification** Page 139
Patrick Mayes; Sharon Diskin; Edward Attiyeh; John Maris
United States

16:35 **SEL24 Neuroblastoma-mononuclear phagocyte interactions promoting tumor growth are suppressed by lenalidomide** Page 140
Yibing Xu; Jianping Sun; Hong-Wei Wu; Michael Sheard; Hung Tran; Zesheng Wan; Cathy Liu; Robert Seeger
United States

16:00 – 18:30 Tuesday June 22nd and Wednesday June 23rd
Poster session – All posters will be displayed throughout the meeting

Odd numbers/left aligned posters = Presenting authors present at posters Tuesday June 22nd 16:45 – 17:30
Even numbers/indented posters = Presenting authors present at posters Wednesday June 23rd 16:45 – 17:30

Posters – Biology

POB11 Role of ATP and myeloid-derived suppressor cells in neuroblastoma microenvironment Page 150
Giovanna Bianchi; Ignazia Prigione; Laura Emionite; Michele Cilli; Patrizia Pellegatti; Francesco Di Virgilio; Ilaria Marigo; Francesca Simonato; Vincenzo Bronte; Vito Pistoia; Lizzia Raffaghello
Italy

POB12 Non-transcriptional role of MYC and genomic rearrangements in neuroblastoma Page 150
Anne Blumrich; Daniel Muth; Christina Poehler; Stephan Gade; Frank Westermann; Manfred Schwab
Germany

POB13 Two common fragile sites *FRA2Ctel* and *FRA2Ccen* map to the borders of *MYCN* amplicons in neuroblastoma Page 150
Anne Blumrich; Marc Zapatka; Manfred Schwab; Larissa Savelyeva
Germany

POB14 Expression and clinicla relevance of melanoma-associated antigens in neuroblastoma Page 150
Paola Bocca [1]; Ignazia Prigione [1]; Barbara Carlini [1]; Maria Valeria Corrias [1]; Soldano Ferrone [2]; Vito Pistoia [1]; Fabio Morandi [1]
[1]Italy; [2]United States

POB15 Heterogeneous *MYCN* amplification - amplicon, genomic background and genome instability Page 151
Dominik Bogen [1]; Inge M. Ambros [1]; Gabriele Amann [1]; Ekkehard Spuller [1]; Jennie Erichsen [2]; Bettina Brunner [1]; Ruth Ladenstein [1]; Tommy Martinsson [2]; Peter F. Ambros [1]
[1]Austria; [2]Sweden

POB16 Immunocytochemical study of bone marrow in neuroblastoma patients - Polish experience Page 151
Katarzyna Bolek-Marzec; Aleksandra Wieczorek; Walentyna Balwierz
Poland

POB17 *ChIPaway*: A tool for visualization and analysis of high-throughput microarray based immunoprecipitation data Page 151
Kenneth Bryan; Patrick G. Buckley; Derek M. Murphy; Sudipto Das; Raymond L. Stallings
Ireland

POB18 Genome-wide DNA methylation profiling reveals extensive and complex epigenetic alterations in neuroblastic tumors Page 151
Patrick Buckley [1]; Sudipto Das [1]; Kenneth Bryan [1]; Raymond Stallings [1]; Leah Alcock [1]; Rogier Versteeg [2]
[1]Ireland; [2]Netherlands

POB19 Identification of epigenetically regulated genes that predict patient outcome in neuroblastoma Page 152
Helena Carén; Anna Djos; Maria Nethander; Rose-Marie Sjöberg; Per Kogner; Staffan Nilsson; Tommy Martinsson
Sweden

POB20 Factors affecting the outcome of the p53 mediated DNA damage response in neuroblastoma Page 152
Jane Carr-Wilkinson [1]; Rebecca Griffiths [2]; Rebecca Elston [2]; Laura, D Gamble [2]; John Lunec [2]; Deborah A. Tweddle [2]
[1]United Kingdom; [2]United Kingdom

POB21 Characterization of *ALK* rearrangements in neuroblastoma Page 152
Alex Cazes; Valentina Boeva; Agnès Ribeiro; Emmanuel Barillot; Olivier Delattre; Isabelle Janoueix-Lerosey
France

POB22 Clusterin interacts with HSP60: implications in neuroblastoma development Page 152
Korn-Anong Chaiwatanasirikul; Arturo Sala
United Kingdom

POB23 Human mesenchymal stromal cells (hMSCs) enhanced migration and invasion of neuroblastoma cells via SDF-1/CXCR4 and SDF-1/CXCR7 axes Page 153
Godfrey Chi-Fung Chan; Ming Ma
Hong Kong

POB24 Alterations of NDP kinase A/ NM23-H1 deregulate c-myc transcription Page 153
Christina Chang; Kai-Hui Chan; Larry Paris; Lin-Jen Ma; Renn-Shiuan Wei; Choon-Yee Tan
Taiwan

POB25 Overexpression of β1,4-N-acetylgalactosaminyltransferase III suppresses the malignant phenotype of neuroblastoma cells via β1-integrin signaling Page 153
Mei-Ieng Che; Hsiu-Hao Chang; Min-Chuan Huang
Taiwan

POB26 The role of protein tyrosine-phosphatases in neuroblastoma Page 153
Owen Clark; Andrew Stoker
United Kingdom

POB27 Identification of hypoxia signatures in neuroblastoma cell lines by l1-l2 regularization and data filtering Page 154
Andrea Cornero; Paolo Fardin; Massimo Acquaviva; Annalisa Barla; Sofia Mosci; Lorenzo Rosasco; Alessandro Verri; Luigi Varesio
Italy

POB28 Autophagy and its regulation in neuroblastoma Page 154
Sonia Cournoyer [1]; Tina V Imbriglio [1]; Carine Nyalendo [1]; Claire Barelli [1]; Pierre Teira [1]; Michel Duval [1]; Gilles Vassal [2]; Hervé Sartelet [1]
[1]Canada; [2]France

POB29 All-trans retinoic acid induced differentiation of SK-N-BE cells results in extensive DNA methylation alterations of gene promoter regions Page 154
Sudipto Das; Patrick Buckley; Kenneth Bryan; Karen Watters; Niamh Foley; Leah Alcock; Isabella Bray; Raymond Stallings
Ireland

POB30 Chemokines CXCR5-CXCL13 cross-talk between malignant neuroblastoma cells and schwannian stromal cells suggests a role in the inhibition of metastatic dissemination Page 155
Federica Del Grosso; Simona Coco; Paola Scaruffi; Sara Stigliani; Francesca Valdora; Roberto Benelli; Simona Boccardo; Sandra Salvi; Mauro Truini; Michela Croce; Silvano Ferrini; Gian Paolo Tonini
Italy

POB31 N-glycosylation of ALK as a potential target for disruption of prosurvival signaling pathways in neuroblastoma cell lines Page 155
Federica Del Grosso; Marilena De Mariano; Lorena Passoni; Laura Paleari; Gian Paolo Tonini; Luca Longo
Italy

POB32 Discovery of gene regulatory pathways implicated in neuroblastoma pathogenesis through integration of coding and non-coding gene expression and gene copy number data Page 155
Katleen De Preter [1]; Annelies Fieuw [1]; Candy Kumps [1]; Pieter Mestdagh [1]; Bram De Wilde [1]; Alexander Schramm [2]; Johannes Schülte [2]; Rosa Noguera [3]; Angelika Eggert [2]; Jo Vandesompele [1]; Frank Speleman [1]
[1]Belgium; [2]Germany; [3]Spain

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POB89 A biological link between p53 and MYCN/MYC expression in neuroblastoma Page 170
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POB90 Mitochondria-related destabilization of MYC family proteins in neuroblastoma cells by OSU-03012, FCCP and Salinomycin Page 170
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POB98 Galectin-3 binding protein/90 kDa Mac-2 binding protein stimulates interleukin-6 expression in the neuroblastoma microenvironment Page 172
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POB99 TrkAIII isoform expression is associated with aggressive behavior in human neuroblastomas Page 172
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POB100 Evaluation of MCPIP expression patterns and their impact on the survival of neuroblastoma cell lines Page 172
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POB102 Aurora A kinase is a possible target of OSU-03012 to destabilize MYC family proteins Page 173
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POB103 Expression and function of RET in neuroblastoma cell lines Page 173
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POB106 JAGGED1 antogonizes NOTCH2 mediated cell migration Page 174
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POB108 Functional microRNA library screen to identify synthetically lethal interactions in neuroblastoma Page 174
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POB109 Galectin-3 protects MYCN single copy neuroblastoma cells from apoptosis: a mechanism impaired by MYCN Page 175
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POT5 Cytomegalovirus infection in neuroblastoma, high prevalence in tumors and reduced growth *in vivo* and *in vitro* using HCMV targeted therapies Page 179
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POT7 Prognosis approach of one segmental chromosome aberration in neuroblastoma Page 179
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POT11 ALK and PHOX2B mutations in neuroblastic tumours with highly suspected predisposition: Rare events and unexpected clinical features Page 180
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POT16 IL-21-based immunotherapy of neuroblastoma in combination with lymphodepleting antibodies Page 182
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POT17 Characterisation of tumour progression, vascularisation and response to chemotherapy in transgenic mouse models of Neuroblastoma (TH-*MYCN and TH-MYCN/p53ER^{Tam}*) using magnetic resonance imaging Page 182
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POT18 Development and characterization of bioluminescent orthotopic and metastatic neuroblastoma models Page 182
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POT19 The DNA-binding protein, YB-1 is a direct N-Myc target and influences repair and resistance in neuroblastoma cell lines Page 183
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POT20 Comparative interactomics, transcriptomics and proteomics studies of CDK inhibitors roscovitine and CR8 effects on human neuroblastoma SH-SY5Y cells converge to a central role of Myc Page 183
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POT21 ALK and pALK protein levels in NBL cell lines correlate with ALK mutation status and responsiveness to ALK inhibition Page 183
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Josiah Dungwa; Urmila Uparkar; David Bates; Pramila Ramani
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POT26 Analysis of cytotoxic drugs that selectively target cells with MYC overexpression Page 184
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Sweden

POT27 Expression of the neuron-specific protein CHD5 is an independent marker of outcome in neuroblastoma Page 185

Idoia Garcia [1]; Gemma Mayol [1]; Eva Rodríguez [1]; Mariona Suñol [1]; Timothy Gershon [1]; José Ríos [1]; Nai-Kong Cheung [2]; Carmen de Torres [1]; Mark Kieran [2]; Jaume Mora [1]; Cinzia Lavarino [1]
[1]Spain; [2]United States

POT28 Sphingosine-1-phosphate signaling is a mechanism of fenretinide resistance and provides a novel therapeutic target Page 185

Matthew V Ghent; Taylor Chen; Youngleem Kim; Ana Jakimenko; Min Kang; C. Patrick Reynolds
United States

POT29 Omega-3 fatty acid supplementation delays the progression of neuroblastoma *in vivo* Page 185

Helena Gleissman [1]; Lova Segerström [1]; Mats Hamberg [1]; Frida Ponthan [2]; Magnus Lindskog [1]; John Inge Johnsen [1]; Per Kogner [1]
[1]Sweden; [2]United Kingdom

POT30 'BH3 profiles' identify neuroblastomas with exquisite ABT-737/chemotherapy *in vivo* and Bim signaling is a critical determinant sensitivity Page 185

Kelly C. Goldsmith; Michelle Gross; Xueyuan Liu; Susan K. Peirce; Chengyu Prince; Annette Vu; Niel Chen; C. Patrick Reynolds; Michael D. Hogarty
United States

POT31 Cepharanthine reverses multidrug resistance and sensitizes neuroblastoma cells to vincristine-induced cell death Page 186

Regina Graham; John Thompson; James Guest; Keith Webster; Steven Vanni
United States

POT32 *ATM* deletion is a frequent event in neuroblastoma Page 186

F Gumy-Pause [1]; H Ozsahin [1]; M Khoshbeen-Boudal [1]; B Pardo [1]; DR Betts [2]; P Maillet [1]; MD Hogarty [3]; EF Attiyeh [3]; AP Sappino [1]
[1]Switzerland; [2]Ireland; [3]United States

POT33 Computer vision in neuroblastoma: computer-aided prognosis Page 186

Metin Gurcan; Olcay Sertel; Hiroyuki Shimada
United States

POT34 Analysis of aggressive human and mouse ALK neuroblastoma mutations Page 186

Bengt Hallberg; Sattu Kamaraj; Christina Schönherr; Kristina Ruuth; Cecilia Axelsson; Ruth Palmer
Sweden

POT35 Rapamycin upregulates osteoprotegerin and increases time to pathologic fracture in a mouse neuroblastoma bone metastasis model Page 187

Joseph Hartwich; Adrianne Myers; Cathy Ng; Andrew M Davidoff
United States

POT36 The addition of HIF inhibition potentiates the effects of angiogenesis inhibition in mouse neuroblastoma xenografts Page 187

Joseph Hartwich; Yunyu Spence; Cathy Ng; Christopher Morton; Andrew M Davidoff
United States

POT37 Screening at 18 months of age using the new serum marker for reducing the mortality of neuroblastoma: Simulation using Japanese population based cohort study Page 187

Eiso Hiyama; Arata Kamimatsuse; Naomi Kamei; Tsutomu Masujima; Keiko Hiyama; Megu Ohtaki
Japan

POT38 Effect Phosphoinositide-3-Kinase (PI3K) and mTOR dual inhibitors in Human Neuroblastomas Page 187

Ruth Ho; Jane Minturn; Valerie Brown; Radhika Iyer; Cecilia Sheen; Jessica Hulitt; Anisha Simpson; Carly Varela; Jennifer Mangino; Venkatadri Kolla; Garrett Brodeur
United States

POT39 Analysis of molecular interactions between the GD2 ganglioside-specific mouse monoclonal antibody 14G2a and GD2-mimicking peptides Page 188

Irena Horwacik; Aleksandra Kowalczyk; Malgorzata Bzowska; Dominik Czaplicki; Hanna Rokita
Poland

POT40 NLRR2 is a novel regulator of neuroblastoma cell death via ER stress Page 188
Shamim Hossain; Atsushi Takatori; Jesmin Akter; Kamrul Hasan; Akira Nakagawara
Japan

POT41 Positive feedback loop of Mycn-nlrr1-egf/egfr signals in aggressive neuroblastomas to accelerate cell growth Page 188
Shamim Hossain; Atsushi Takatori; Jesmin Akter; Yusuke Suenaga; Toshinori Ozaki; Akira Nakagawara
Japan

POT42 Application of chicken anti-human midkine IgY antibody to neuroblastoma diagnosis Page 188
Shinya Ikematsu; Shoma Tsubota; Chika Yamashiro; Satoshi Kishida; Yukio Yuzawa; Akira Nakagawara; Kenji Kadomatsu
Japan

POT43 Expression and gene status of HER2 in neuroblastic tumors Page 189
Ewa Izycka-Swieszewska [1]; Agnieszka Wozniak [2]; Jacek Kot [1]; Wieslawa Grajkowska [1]; Elzbieta Drozynska [1]; Anna Balcerska [1]; Danuta Perek [1]; Bozena Dembowska [1]; Janusz Limon [1]
[1]Poland; [2]Belgium

POT44 Ki-67 proliferation index is marker of poor prognosis in neuroblastoma especially in patients aged over 18 months Page 189
Ewa Izycka-Swieszewska; Beata Stefania Lipska; Elzbieta Drozynska; Anna Balcerska; Danuta Perek; Wieslawa Grajkowska; Bozena Dembowska; Teresa Klepacka; Wojciech Wozniak; Alicja Chybicka; Janusz Limon
Poland

POT45 MLPA (Multiplex Ligation-dependent Probe Amplification) and FISH comparison/validation for genetic characterization of neuroblastoma Page 190
Marta Jeison; Gili Halevy-Berko; Galina Feinbberg-Gorenshtein; Svetlana Itskovitch; Shifra Ash; Jack Mardouck; Drorit Luria; Jerry Stein; Batia Stark; Smadar Avigad; Isaac Yaniv
Israel

POT46 Prolonged low-dose administration of the cyclooxygenase-2 inhibitor celecoxib enhances the antitumor activity of irinotecan against neuroblastoma xenografts Page 190
Setsuko Kaneko; Michio Kaneko
Japan

POT47 CHD5 is part of the nucleosome remodeling and histone deacetylase (NuRD) complex in neuroblastoma (NB) cell lines Page 190
Venkatadri Kolla; Tiangang Zhuang; Hiroshi Koyama; Koumudi Naraparaju; Mayumi Higashi; Gerd A. Blobel; Peter S. White; Garrett M. Brodeur
United States

POT48 Mechanisms of CHD5 inactivation in neuroblastomas Page 190
Hiroshi Koyama; Tiangang Zhuang; Jennifer E. Light; Venkatadri Kolla; Mayumi Higashi; Wendy B. London; Garrett M. Brodeur
United States

POT49 Neuroblastoma cell lines, phenotype and susceptibility towards natural killer cells Page 191
Kathelijne CJM Kraal; MM Ostaijen ten Dam; LM Ball; MJD van Tol; RM Egeler
Netherlands

POT50 UCHL1-Upregulation correlates with reduction of vital neuroblastoma cells by fenretinide and doxorubicin treatment Page 191
Sandra Kuehnel; Grigore Cernaianu; Kai Stuehler; Helmut Meyer; Albrecht Bufe; Manfred Koeller; Barbara Sitek; Ralf-Bodo Troebs
Germany

POT51 Low dose metronomic (LDM) administration of oral to potecan and pazopanib as an effective preclinical antiangiogenic therapy in neuroblastoma Page 191
Sushil Kumar; Reza Mokhtari; Bing Wu; Libo Zhang; Shan Mann; Robert Kerbel; Herman Yeger; Sylvain Baruchel
Canada

POT52 Clinical significance of TRK family gene expression in neuroblastomas Page 191
Jennifer E. Light; Hiroshi Koyama; Eli Gordin; Jane E. Minturn; Radhika Iyer; Ruth Ho; Anisha M. Simpson; Venkatadri Kolla; Patrick W. McGrady; Wendy B. London; Garrett M. Brodeur
United States

POT53 Cancelled

POT54 Induction of miR-183 via an epigenetic mechanism suppresses neuroblastoma malignancy Page 192
Marco Lodrini; Johannes H. Schulte; Mirco Castoldi; Ina Oehme; Martina Muckenthaler; Olaf Witt; Hedwig E. Deubzer
Germany

POT55 Telomere elongation and chromosomal instability in non-MYCN amplified clinically aggressive neuroblastoma Page 192
Gisela Lundberg [1]; John-Kalle Länsberg [1]; Alexander Pietras [1]; Sven Påhlman [1]; Attila Frigyesi [1]; Victoria Castel [2]; Samuel Navarro [2]; Marta Piqueras [2]; Rosa Noguera, [2]; Tommy Martinsson [1]; David Gisselsson [1]
[1]Sweden; [2]Spain

POT56 Nanoparticle (NP) delivery of the Trk inhibitor lestaurtinib in neuroblastomas Page 192
Jennifer Mangino; Radhika Iyer; Michael Chorny; Ivan Alferiev; Jane Minturn; Ruth Ho; Anisha Simpson; Carly Varela; Audrey Evans; Robert Levy; Garrett Brodeur
United States

POT57 Bio-molecular and histo-pathological characterization of Neuroblastoma in adolescent and young adults (AYA). Italian experience with 33 cases Page 192
Katia Mazzocco; Raffaella Defferrari; Claudio Gambini; Angela Rita Sementa; Alberto Garaventa; Gian Paolo Tonini; Massimo Conte
Italy

POT58 Inhibition of PARP-1 enhances the efficacy of [131I]MIBG/Topotecan combinations in vitro Page 193
Anthony G McCluskey [1]; Annette Sorensen [1]; Mathias Tesson [2]; Robert J Mairs [2]; Marie Boyd [1]
[1]United Kingdom; [2]United Kingdom

POT59 CDK inhibitors Roscovitine and CR8 trigger Mcl-1 Down-regulation and apoptotic cell death in neuroblastoma cells Page 193
Laurent Meijer; Claire Delehouze; Nadège Loaec
France

POT60 Preclinical testing of novel kinase inhibitors in high-risk neuroblastoma Page 193
Ebba Palmberg; Linda Rickardson; Malin Wickstrom; Magnus Lindskog; John Inge Johnsen; Per Kogner
Sweden

POT61 Balance of pro- versus anti-angiogenic splice isoforms of vascular endothelial growth factor as a regulator of neuroblastoma growth Page 193
Maria Peiris [1]; David Owen Bates [1]; Pramila Ramani [2]
[1]United Kingdom; [2]United Kingdom

POT62 Predictive consequences of risk stratification neuroblastoma patients using FISH on TMAP Page 194
Marta Piqueras; Samuel Navarro; Adela Cañete; Victoria Castel; Rosa Noguera
Spain

POT63 The effect of COX-2 expression on celecoxib sensitivity and tumour growth in neuroblastoma Page 194
Frida Ponthan; Emma Bell; C.P.F Redfern
United Kingdom

POT64 Targeting neuroblastoma and neuroblastoma tumour initiating cells with the oncolytic viruses myxoma and vesicular stomatitis virus Page 194
Nicole Redding [1]; Loen Hansford [1]; David Kaplan [1]; Grant McFadden [2]; John Bell [1]; Steve Robbins [1]; Paul Beaudry [1]
[1]Canada; [2]United States

POT77 Deregulation of rho/ras rnd neuronal differentiation pathways is associated with fatal outcome in high-risk disseminated neuroblastoma Page 197

Sara Stigliani [1]; Simona Coco [1]; Stefano Moretti [2]; André Oberthuer [3]; Jessica Theissen [3]; Matthias Fischer [3]; Francesca Valdora [1]; Fabio Gallo [1]; Carla De Vecchi [1]; Alberto Garaventa [1]; Frank Berthold [3]; Stefano Bonassi [1]; Gian Paolo Tonini [1]; Paola Scaruffi [1]
[1]Italy; [2]France; [3]Germany

POT78 Tumor cell detection in autologous stem cell harvests in patients with high risk neuroblastoma Page 198

J Stutterheim [1]; F Vree [1]; B Hero [2]; L Zappeij - Kannegieter [1]; C Voermans [1]; R Schumacher-Kuckelkorn [2]; U Koehl [2]; J H Schulte [2]; F Niggli [3]; M C Fruhwald [2]; M M van Noesel [1]; C M Niemeyer [2]; U Bode [2]; F H Schilling [2]; C Schultz [2]; N Graf [2]; M Nathrath [2]; I Schmid [2]; H N Caron [1]; C E van der Schoot [1]; G A M Tytgat [1]
[1]Netherlands; [2]Germany; [3]Switzerland

POT79 Functional analysis of the p53 pathway in neuroblastoma cells using the small-molecule MDM2 antagonist nutlin-3 Page 198

Tom Van Maerken [1]; Ali Rihani [1]; Daniel Dreidax [2]; Sarah De Clercq [1]; Nurten Yigit [1]; Jean-Christophe Marine [1]; Frank Westermann [2]; Anne De Paepe [1]; Frank Speleman [1]; Jo Vandesompele [1]
[1]Belgium; [2]Germany

POT80 Detection of microRNAs in bone marrow from children with high-risk neuroblastoma predicts survival; a UK CCLG study. Page 198

Virginie Viprey [1]; Maria Corrias [2]; Walter Gregory [3]; Penelope Brock [1]; Susan Burchill [1]
[1]United Kingdom; [2]Italy; [3]United Kingdom

POT81 SKP2-mediated neuroblastoma dedifferentiation is triggered by MYCN through CDK4 induction Page 199

Frank Westermann [1]; Daniel Muth [1]; Daniel Dreidax [1]; Christina Pöhler [1]; Sina Gogolin [1]; Matthias Fischer [1]; Kai Henrich [1]; Volker Ehemann [1]; Paul Gillespie [2]; Manfred Schwab [1]
[1]Germany; [2]United States

POT82 DHA is converted to hydroperoxides and potentiates the cytotoxic effect of chemotherapeutics in neuroblastoma Page 199

Malin Wickström [1]; Helena Gleissman [1]; Rong Yang [2]; Kimberly Martinod [2]; Charles N. Serhan [2]; John Inge Johnsen [1]; Per Kogner [1]
[1]Sweden; [2]United States

POT83 Exploiting cell cycle aberrations in neuroblastoma by targeting checkpoint kinase chk1 using AZD7762 in neuroblastoma with p14^{ARF}/MDM2/p53 defects Page 199

Hong Xu; Xiao Wei; Irene Cheung; Nai-Kong Cheung
United States

POT84 Preoperative analysis of 11q loss of heterozygosity using circulating tumor-released DNAin serum: A novel diagnostic tool for therapy stratification of neuroblastoma Page 199

Shigeki Yagyu; Tomoko Iehara; Takahiro Gotoh; Mitsuru Miyachi; Yoshiki Katsumi; Ken Kikuchi; Kunihiko Tsuchiya; Shinya Osone; Hiroshi Kuroda; Hajime Hosoi
Japan

POT85 Neuroblastoma tumor initiating cells express CD22 making them susceptible to HA22 anti-CD22 immunotoxin induced cell death Page 200

Shuang Yan [1]; Zhijie Li [1]; Amy McKee [1]; Maryalice Stetler-Stevenson [1]; Loen Hansford [2]; David Kaplan [2]; Alan Wayne [1]; Ira Pastan [1]; Carol.J Thiele [1]
[1]United States; [2]Canada

POT86 Genotype-guided neuroblastoma therapy, CP751,871 or Rapamycin Page 200

Libo Zhang; Herman Yeger; Reza Mokhtari; Paula Marrano; Paul Thorner; David Kaplan; Sylvain Baruchel
Canada

POC36 Retrospective analysis of treatment results of high risk neuroblastoma Page 210
Egor Shorikov; Olga Lemesheva; Tatiana Popova; Igor Vyatkin; Grigory Tsaur; Alexander Popov; Leonid Saveliev; Larisa Fechina
Russian Federation

POC37 Comparison of anti-GD2-antibody ch14.18 and 13-cis-retinoic acid as consolidation therapy for high-risk neuroblastoma. Results of the German NB97 trial Page 210
Thorsten Simon; Barbara Hero; Rupert Handgretinger; Martin Schrappe; Thomas Klingebiel; Michael C Fruehwald; Guenther Henze; Frank Berthold
Germany

POC38 Metachronous neuroblastoma in an infant with constitutional unbalanced translocation t(2;16)(p23;p13.3) involving *ALK* Page 210
Shui Yen Soh; Dimitri Stavropoulos; Sarah Bowdin; Paul Thorner; Sylvain Baruchel; David Malkin; M. Stephen Meyn; Meredith Irwin
Canada

POC39 Neuroblastomas with non-avid I^{123}-MIBG scan and negative urinary catecholamine secretion: A single institute's experience Page 211
Shui Yen Soh; Sylvain Baruchel; Meredith Irwin
Canada

POC40 Efficacy of tandem high-dose chemotherapy and autologous stem cell rescue in patients with high-risk neuroblastoma: a preliminary report of NB 2004 study at Samsung Medical Center Page 211
Ki Woong Sung; Heewon Cheuh; Soo Hyun Lee; Keon Hee Yoo; Hong Hoe Koo; Juyoun Kim; Eun Joo Cho; Kun Soo Lee
Republic of Korea

POC41 Measurement of tyrosine hydroxylase transcripts in bone marrow using biopsied tissue instead of aspirate for neuroblastoma Page 211
Ki Woong Sung; Seung-Tae Lee; Yeon Lim Suh; Young-Hyeh Ko; Chang-Seok Ki; Hee-Jin Kim; Jong-Won Kim; Sun-Hee Kim; Heewon Chueh; Soo Hyun Lee; Keon Hee Yoo; Hong Hoe Koo
Republic of Korea

POC42 Reduced-intensity allogeneic stem cell transplantation in children with neuroblastoma who have failed a prior tandem autologous stem cell transplantation Page 211
Ki Woong Sung; Heewon Cheuh; Soo Hyun Lee; Keon Hee Yoo; Hong Hoe Koo; Juyoun Kim
Republic of Korea

POC43 Neuroblastoma detected after ending of mass screening at 6 months of age in Japan Page 212
Tatsuro Tajiri; Ryota Souzaki; Yoshiaki Kinoshita; Sakura Tanaka; Yuhki Koga; Aiko Suminoe; Toshiro Hara; Tomoaki Taguchi
Japan

POC44 Whole-body diffusion-weighted MR imaging is useful to detect bone/bone marrow metastasis of neuroblastoma and monitor response to therapy Page 212
Yoshiyuki Takahashi; Nobuhiro Nishio; Hideki Muramatsu; Akira Shimada; Asahito Hama; Masafumi Ito; Kenichiro Kaneko; Hisami Ando; Hisroshi Fukatsu; Seiji Kojima
Japan

POC45 Identification of a therapy-sensitive subtype and stratification of progressive risk in advanced neuroblastomas Page 212
Takeo Tanaka; Yohko Kyo; Kunihiko Hayashi; Tomoko Iehara; Hajime Hosoi; Tohru Sugimoto; Minoru Hamasaki; Michio Kaneko; Tadashi Sawada
Japan

POC46 ^{18}F-FDOPA PET scan is still useful in the presence of ^{123}I-MIBG and ^{18}F-FDG for neuroblastoma imaging Page 212
Kai-Yuan Tzen; Meng-Yao Lu; Hsiu-Hao Chang; Wen-Ming Hsu; Tsai-Yueh Luo; Lie-Hang Shen
Taiwan

POC47 Molecular imaging with ^{18}F-FDOPA PET in the early detection of new metastatic neuroblastoma in bone marrow Page 213
Kai-Yuan Tzen; Meng-Yao Lu; Hsiu-Hao Chang; Wen-Ming Hsu
Taiwan

Posters – Late Breakers

POLB10 Autochthonous TNF alpha as an inducer of immune resistance and survival of neuroblastoma Page 216

Elian Rakhmanaliev [1]; Jinxia Ma [1]; Victor Levitsky [1]; Murray Norris [2]; Jelena Levitskaya [1]
[1]United States; [2]Australia

POLB11 Identifying lesions in translational control of gene expression in neuroblastoma by mRNA polysomal profiling and information-intensive computational integration Page 217

Angela Re; Erik Dassi; Viktoryia Sidarovich; Toma Tebaldi; Paola Scaruffi; Gian Paolo Tonini; Alessandro Quattrone
Italy

POLB12 An integrative bioinformatics approach in neuroblastoma identifies converging alterations in protein networks related to mitotic spindle assembly and splicing Page 217

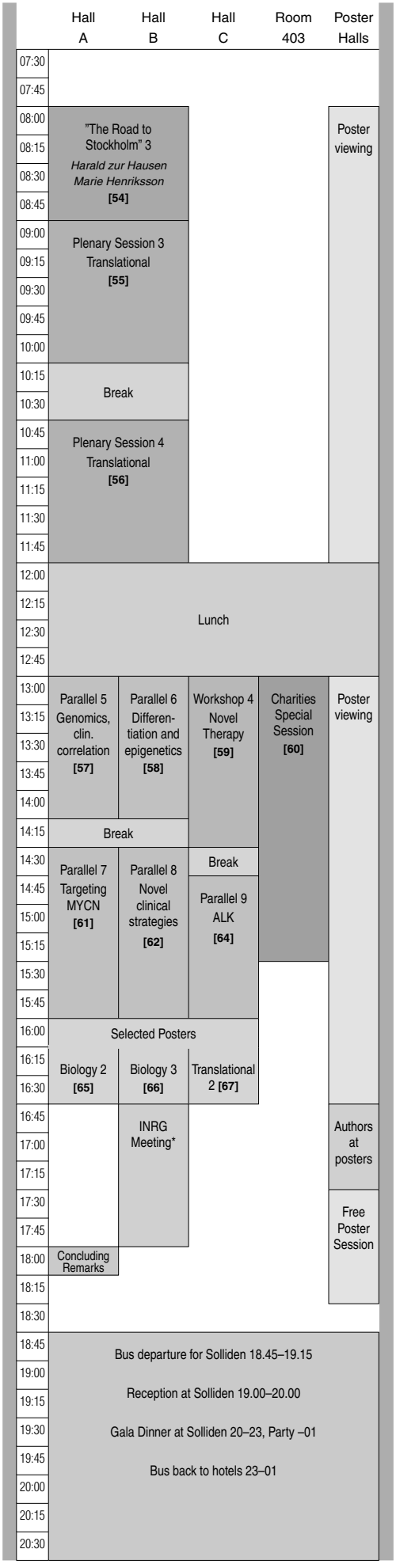
Ooi Wen Fong; Angela Re; Natalia Arseni; Valentina Canella; Giulia Guarguaglini; Patrizia Lavia; Paola Scaruffi; Gian Paolo Tonini; Alessandro Quattrone
Italy

POLB13 Induction of human embryonic stem cells into sympathoadrenal cells Page 217

Michael D Hadjidaniel; Shahab Asgharzadeh
United States

18:00 – 18:15 Tuesday June 22nd
Hall A
Concluding remarks by Garrett Brodeur and Akira Nakagawara

Programme
Wednesday June 23rd



* By invitation only

Wednesday June 23rd

08:00 – 09:00 Wednesday June 23rd
Hall A/B
The Road to Stockholm and Beyond 3
Chairs: Manfred Schwab and Frank Berthold

PL13 Infections linked to human cancers
Harald zur Hausen, Germany

Harald zur Hausen was born on March 11, 1936 in Gelsenkirchen-Buer, Germany. He studied Medicine at the Universities of Bonn, Hamburg and Düsseldorf and received his M.D. in 1960. After his internship he worked as postdoc at the Institute of Microbiology in Düsseldorf, subsequently in the Virus Laboratories of the Children's Hospital in Philadelphia where he was later appointed as Assistant Professor. After a period of 3 years as a senior scientist at the Institute of Virology of the University of Würzburg, he was appointed in 1972 as Chairman and Professor of Virology at the University of Erlangen-Nürnberg. In 1977 he moved to a similar position to the University of Freiburg. From 1983 until 2003 he was appointed as Scientific Director of the Deutsches Krebsforschungszentrum (German Cancer Research Center) in Heidelberg. He retired from this position in 2003.

He had and has a number of special appointments, among them being the Chairman of several Scientific Price-Committees, between 1989-1991 Chairman of the Association of National Research Centers (Großforschungseinrichtungen) in Germany and from 1993 to 1996 President of the Organization of European Cancer Centers (OECI).

Zur Hausen received a number of national and international awards, among them the Robert-Koch-Price, the Charles S. Mott Price of the General Motors Cancer Research Foundation, the Federation of the European Cancer Societies Clinical Research Award, the Paul-Ehrlich-Ludwig Darmstätter-Price, the Jung-Price, Hamburg, the Charles Rudolphe Brupbacher Price, Zürich, the Prince Mahidol Award, Bangkok, the Raymond Bourgine Award, Paris, the Coley-Award, New York, the Life Science Achievement Award of the American Association for Cancer Research, San Diego, and the Nobel-Prize for Medicine, 2008. He received honorary doctorates from the Universities of Chicago, USA, Umeå, Sweden, Prague, Czech Republic, Salford, UK, Helsinki, Finland, Erlangen-Nürnberg and Würzburg, both Germany, Buenos Aires, Argentina and Ferrara, Italy.

He is an elected member of various academies (LEOPOLDINA, Heidelberg Academy of Sciences, Polish Academy of Sciences, Venezuela National Academy of Medicine, American Philosophical Society, Institute of Medicine of the National Academy of Sciences (USA), Foreign member of the US National Academy of Sciences and research organizations (EMBO, HUGO), and became an Honorary Member of a number of biomedical scientific societies. A large number of Special Lectures and Visiting Professorships, Memberships in Editorial Boards and active involvements in the organization of international meetings complement his curriculum.

From 2000 until 2009 zur Hausen was Editor-in-Chief of the International Journal of Cancer and from 2003 until 2009 Vice-President of the German National Academy for Natural Sciences and Medicine LEOPOLDINA in Halle.

Since 2006 he has been a Member of the Board of Directors of the International Union against Cancer (UICC), and a Member of the National Science Tansfer and Development Agency in Bangkok, Thailand.

PL14 The Yin and Yang functions of the Myc oncoprotein in cancer development and as targets for therapy Page 94
Marie Arsenian Henriksson, Sweden

Marie Arsenian Henriksson, BSc Uppsala University 1985, PhD Karolinska Institutet 1993, Postdoc Hannover Medical School, Germany 1993–1996, Associate Professor Karolinska Institutet 2001. Present position: Department Head MTC (Department of Microbiology, Tumor and Cell Biology) Karolinska Institutet.

09:00 – 10:15 Wednesday June 23rd
Hall A/B
Plenary session 3 – Translational
Chairs: Rogier Versteeg and Klaus Beiske

09:00 **PL15 Identification of selective inhibitors of neuroblastoma stem cells - Targeting the kinome** Page 95
Natalie Grinshtein; Kristen Smith; David Uehling; Michael Prakesch; Methvin Isaac; Meredith Irwin; Alessandro Datti; Jeff Wrana; Rima Al-awar; David Kaplan
Canada

09:15 **PL16 A new Aurora kinase inhibitor (CCT241736) regulates Mycn protein expression and prevents neuroblastoma growth *in vitro* and *in vivo*** Page 95
Lynsey Vaughan; Elizabeth Cullis; Karen Barker; Yann Jamin; Spiros Linardopoulos; Vassilios Bavetsias; Butrus Atrash; Julian Blagg; Andrew Pearson; Simon Robinson; Louis Chesler
United Kingdom

09:30 **PL17 Mycn as a critical target of PI3K/mTOR inhibitors in neuroblastoma; paracrine effects on tumor vasculature** Page 96
Yvan Chanthery [1]; Chris Hackett [1]; Melissa Itsara [1]; Matt Grimmer [1]; Louis Chesler [2]; Katherine Matthay [1]; William Weiss [1]
[1]United States; [2]United Kingdom

09:45 **PL18 Exploitation of ALK as a therapeutic target in neuroblastoma** Page 96
Andrew Wood; Marci Laudenslager; Elizabeth Haglund; Joshua Courtright; Jeffrey Plegaria; Erica Carpenter; Sharon Diskin; Edward Attiyeh; Kristina Cole; Yana Toporovskaya; Bruce Pawel; Huaqing Zhao; Junghui Zhang; Patrick Reynolds; Patrick McGrady; Wendy London; Michele McTigue; Tami Marrone; Christensen James; John Maris; Yael Mosse
United States

10:00 **PL19 An RNAi screen of the protein kinome identifies CHK1 as a therapeutic target in neuroblastoma** Page 97
Kristina Cole; Jonathan Huggins; Michael Laquaglia; Chase Hulderman; Edward Attiyeh; Cynthia Winter; Sharon Diskin; Kristopher Bosse; Patrick Mayes; Jayanti Jagganathan; Geoffrey Norris; Yael Mosse; John Maris
United States

10:15 – 10:45 BREAK

10:45 – 12:00 Wednesday June 23rd
Hall A/B
Plenary session 4 – Translational
Chairs: John Maris and Gudrun Schleiermacher

10:45 **PL20 Recruitment of histone deacetylase 2 by N-Myc and c-Myc to a transrepressor complex is a general therapeutic target in Myc-driven cancer** Page 97
Glenn Marshall [1]; Samuele Gherardi [2]; Zillan Neiron [1]; Toby Trahair [1]; Pei Liu [1]; Kacper Jankowski [1]; Nunzio Iraci [2]; Michelle Haber [1]; Murray Norris [1]; Fabio Stossi [3]; Benita Katzenellenbogen [3]; Andrew Biankin [1]; Giovanni Perini [2]; Tao Liu [1]
[1]Australia; [2]Italy; [3]United States

11:00 **PL21 Genome-wide mapping of MYCN binding sites in neuroblastoma reveals e-box motif frequencies and associations with regions of DNA hypermethylation** Page 98
Derek Murphy; Patrick Buckley; Kenneth Bryan; Sudipto Das; Leah Alcock; Niamh Foley; Suzanne Prenter; Isabella Bray; Karen Watters; Higgins Desmond; Raymond L. Stallings
Ireland

11:15 **PL22 Accurate prediction of neuroblastoma outcome based on miRNA expression profiles** Page 98
Stefanie Schlierf [1]; Johannes Schulte [1]; Benjamin Schowe [1]; Pieter Mestdagh [2]; Lars Kaderali [1]; Prabhav Kalaghatgi [1]; Joelle Vermeulen [2]; Bent Brockmeyer [1]; Kristian Pajtler [1]; Theresa Thor [1]; Frank Speleman [2]; Katharina Morik [1]; Angelika Eggert [1]; Jo Vandesompele [2]; Alexander Schramm [1]
[1]Germany; [2]Belgium

11:30 **PL23 Individual survival time prediction from gene-expression and/or global genomic data of neuroblastoma patients using CASPAR** Page 99
Andre Oberthuer; Prabhav Kalaghatgi; Yvonne Kahlert; Barbara Hero; Frank Berthold; Benedikt Brors; Roland Eils; Matthias Fischer; Lars Kaderali
Germany

11:45 **PL24 Identification of multiple protein disrupting mutations in stage 4 neuroblastoma using next generation sequencing transcriptome analysis** Page 99
Thomas Badgett; Xiang Guo; Jun S Wei; Young K Song; Peter Johansson; Xinyu Wen; Qingrong Chen; Catherine Tolman; Susan Yeh; Javed Khan
United States

12:00 – 13:00 LUNCH

13:00 – 14:10 Wednesday June 23rd

Hall A

Parallel session 5 – Genomics, clinical correlation

Chairs: Tommy Martinsson and Wendy London

13:00 **OR33 Discovering epistatic genetic interactions associated with high-risk neuroblastoma** Page 116
Mario Capasso [1]; Kristopher Bosse [2]; Sharon Diskin [2]; Yael Mosse [2]; Achille Iolascon [1]; Marcella Devoto [2]; John Maris [2]
[1]Italy; [2]United States

13:10 **OR34 Accumulation of segmental alterations determines progression in neuroblastoma** Page 116
Gudrun Schleiermacher; Isabelle Janoueix-Lerosey; Agnes Ribeiro; Jerzy Klijanienko; Jerome Couturier; Gaelle Pierron; Veronique Mosseri; Alexander Valent; Nathalie Auger; Dominique Plantaz; Herve Rubie; Dominique Valteau-Couanet; Franck Bourdeaut; Valerie Combaret; Christophe Bergeron; Jean Michon; Olivier Delattre
France

13:20 **OR35 Improved outcome prediction of children with neuroblastoma using a miRNA signature** Page 116
Pieter Mestdagh [1]; Katleen De Preter [1]; Joëlle Vermeulen [1]; Arlene Naranjo [2]; Isabella Bray [3]; Victoria Castel [4]; Caifu Chen [2]; Angelika Eggert [5]; Michael D Hogarty [2]; Wendy B London [2]; Rosa Noguera [4]; Alexander Schramm [5]; Johannes Schulte [5]; Raymond Stallings [3]; Rogier Versteeg [6]; Geneviève Laureys [1]; Nadine Van Roy [1]; Frank Speleman [1]; Jo Vandesompele [1]
[1]Belgium; [2]United States; [3]Ireland; [4]Spain; [5]Germany; [6]Netherlands

13:30 **OR36 Gene expression-based classification improves risk estimation of neuroblastoma patients** Page 117
Andre Oberthuer [1]; Barbara Hero [1]; Frank Berthold [1]; Dilafruz Juraeva [1]; Andreas Faldum [1]; Yvonne Kahlert [1]; Shahab Asgharzadeh [2]; Robert Seeger [1]; Paola Scaruffi [3]; Gian Paolo Tonini [3]; Isabelle Janoueix-Lerosey [4]; Olivier Delattre [4]; Gudrun Schleiermacher [4]; Jo Vandesompele [5]; Joëlle Vermeulen [5]; Frank Speleman [5]; Rosa Noguera [6]; Marta Piqueras [6]; Jean Bénard [4]; Alexander Valent [4]; Smadar Avigad [7]; Isaac Yaniv [7]; Axel Weber [1]; Holger Christiansen [1]; Richard G. Grundy [8]; Katharina Schardt [1]; Manfred Schwab [1]; Roland Eils [1]; Patrick Warnat [1]; Lars Kaderali [1]; Thorsten Simon [1]; Boris DeCarolis [1]; Jessica Theissen [1]; Frank Westermann [1]; Benedikt Brors [1]; Matthias Fischer [1]
[1]Germany; [2]United States; [3]Italy; [4]France; [5]Belgium; [6]Spain; [7]Israel; [8]United Kingdom

13:40 **OR37 Genomic portrait of tumor progression using next-generation sequencing** Page 117
Jun Wei; Peter Johansson; Xiang Guo; Tom Badgett; Young Song; Xinyu Wen; Catherine House; Susan Yeh; Javed Khan
United States

13:50 **OR38 A multi-locus technique for risk evaluation of patients with neuroblastoma** Page 117
Inge M. Ambros [1]; Bettina Brunner [1]; Clare Bedwell [2]; Klaus Beiske [3]; Jean Bénard [4]; Nick Bown [2]; Valerie Combaret [4]; Jerome Couturier [4]; Raffaella Defferrari [5]; Nicole Gross [6]; Marta Jeison [7]; John Lunec [2]; Barbara Marques [8]; Tommy Martinsson [9]; Katia Mazzocco [5]; Rosa Noguera [10]; Gudrun Schleiermacher [4]; Frank Speleman [11]; Ray Stallings [12]; Gian Paolo Tonini [5]; Deborah A Tweddle [13]; Alexander Valent [4]; Ales Vicha [14]; Nadine Van Roy [11]; Eva Villamon [10]; Andrea Ziegler [1]; Günther Schreier [1]; Gerhard Aigner [1]; Mario Drobics [1]; Ruth Ladenstein [1]; Gabriele Amann [1]; Jan Schouten [15]; Ulrike Pötschger [1]; Peter F Ambros [1]
[1]Austria; [2]United Kingdom; [3]Norway; [4]France; [5]Italy; [6]Switzerland; [7]Israel; [8]Portugal; [9]Sweden; [10]Spain; [11]Belgium; [12]Ireland; [13]United Kingdom; [14]Czech Republic; [15]Netherlands

14:00 **OR39 *Detecting the cutting edges.* Highly sensitive and absolute specific detection of *MYCN* amplified neuroblastoma cells by amplicon-fusion-site (AFS) PCR** Page 118
Axel Weber; Sylvia Taube; Sven Starke; Eckhard Bergmann; Nina Merete Christiansen; Holger Christiansen
Germany

14:10 – 14:30 BREAK

13:00 – 14:10 Wednesday June 23rd

Hall B

Parallel session 6 – Differentiation and epigenetics

Chairs: Sven Påhlman and Godfrey C.F. Chan

13:00 **OR40 The homeobox transcription factor HoxC9, a key regulator of development, suppresses tumourigenicity of neuroblastoma** Page 118

Hayriye Kocak; Sandra Ackermann; Barbara Hero; Yvonne Kahlert; Jessica Theissen; Volker Ehemann; Frank Westermann; Margarete Odenthal; André Oberthuer; Frank Berthold; Matthias Fischer

Germany

13:10 **OR41 Identification of miRNAs implicated in neuronal development and neuroblastoma oncogenesis using fetal neuroblast miRNA profiles** Page 119

Sara De Brouwer [1]; Pieter Mestdagh [1]; Nicky D'Haene [1]; Johannes Schulte [2]; Angelika Eggerts [2]; Alexander Schramm [2]; Rosa Noguera [3]; Claire Hoyoux [1]; Geneviève Laureys [1]; Jo Vandesompele [1]; Katleen De Preter [1]; Frank Speleman [1]

[1]Belgium; [2]Germany; [3]Spain

13:20 **OR42 Neuroblastoma Phox2b variants stimulate proliferation and de-differentiation of immature sympathetic neurons** Page 119

Tobias Reiff; Konstantina Tsarovina; Afsaneh Majdazari; Mirko Schmidt; del Pino Isabel; Hermann Rohrer

Germany

13:30 **OR43 Multidrug resistance-associated protein 4 regulates cAMP-dependent differentiation in neuroblastoma and represents a target for therapeutic inhibition** Page 119

Murray Norris [1]; Marcia Munoz [1]; Claudia Flemming [1]; Fujiko Watt [1]; Anasuya Vishvanath [1]; Michelle Henderson [1]; Antonio Porro [2]; Glenn Marshall [1]; Giovanni Perini [2]; Michelle Haber [1]

[1]Australia; [2]Italy

13:40 **OR44 Versatile *in vivo* roles for caspase-8 in neuroblastoma tumorigenesis** Page 119

Tal Teitz [1]; Madoka Inoue [1]; Marcus Valentine [1]; Kejin Zhu [1]; Manrong Jiang [1]; Jerold E. Rehg [1]; Razqallah Hakem [2]; William A. Weiss [1]; Jill M. Lahti [1]

[1]United States; [2]Canada

13:50 **OR45 Histone deacetylase 8 in neuroblastoma tumorigenesis** Page 120

Ina Oehme [1]; Hedwig E. Deubzer [1]; Dennis Wegener [1]; Diana Pickert [1]; Jan-Peter Linke [1]; Barbara Hero [1]; Annette Kopp-Schneider [1]; Frank Westermann [1]; Scott M. Ulrich [2]; Andreas von Deimling [1]; Matthias Fischer [1]; Olaf Witt [1]

[1]Germany; [2]United States

14:00 **OR46 Genome-wide DNA methylation profiling reveals extensive and complex epigenetic alterations in neuroblastic tumors** Page 120

Patrick Buckley [1]; Sudipto Das [1]; Kenneth Bryan [1]; Karen Watters [1]; Leah Alcock [1]; Rogier Versteeg [2]; Raymond Stallings [1]

[1]Ireland; [2]Netherlands

14:10 – 14:30 BREAK

13:00 – 14:30 Wednesday June 23rd

Hall C

Workshop 4 – Future directions in targeted therapy for neuroblastoma

Organisers and chairs: John Inge Johnsen and Carol Thiele

13:00 **Introduction**

13:05 **WS22 Perspectives in immunotherapy of neuroblastoma** Page 85
Holger Lode, Germany

13:17 **WS23 Second generation GD2-targeted immunotherapy and future perspectives** Page 86
Alice Yu, United States

13:29 **Discussion neuroblastoma immunotherapy**

13:33 **WS24 Targeting Signal Transduction Pathways – Taking aktion!** Page 86
Carol Thiele, United States

13:47 **WS25 Targeting apoptosis pathways in neuroblastoma** Page 86
Simone Fulda, Germany

14:01 **WS26 Omega-3 fatty acids in cancer: The protectors of good and the killers of evil?** Page 86
Helena Gleissman, Sweden

14:15 **WS27 Optimizing drug development for neuroblastoma by close integration with adult oncology** Page 86
Pat Reynolds, United States

14:28 **Closing remarks**

14:30 – 14:45 BREAK

13:00 – 15:30 Wednesday June 23rd
Room 403
Charities special session

Meeting's for charities representatives and other interested
Hosted by the Swedish Children's Cancer Foundation.

14:30 – 16:00 Wednesday June 23rd
Hall A
Parallel session 7 – Targeting MYCN
Chair: Nicole Gross and Kenji Kadomatsu

14:30 **OR47 SIRT1 enhances N-Myc protein stability in a positive feedback loop which converts N-Myc expression from a low to high level** Page 120
Glenn Marshall [1]; Pei Liu [1]; Samuele Gherardi [2]; Chris Scarlett [1]; Antonio Bedalov [3]; Ning Xu [1]; Nunzio Iraci [2]; Margo van Bekkum [1]; Eric Sekyere [1]; Kacper Jankowski [1]; Toby Trahair [1]; Michelle Haber [1]; Murray Norris [1]; Andrew Biankin [1]; Giovanni Perini [2]; Tao Liu [1]
[1]Australia; [2]Italy; [3]United States

14:40 **OR48 Identification of therapeutic targets for MYCN-amplifed neuroblastoma by functional genomics** Page 121
Masafumi Toyoshima; Julie Park; Carla Grandori
United States

14:50 **OR49 MYCN transcriptionally controls the expression of the Nijmegen Brekage Syndrome gene product p95 nibrin/NBS1** Page 121
Marialaura Petroni; Massimiliano Mellone; Sonia Albini; Veronica Veschi; Isabella Massimi; Isabella Screpanti; Luigi Frati; Doriana Fruci; Beatrice Cardinali; Alberto Gulino; Giuseppe Giannini
Italy

15:00 **OR50 Bmi1 is a MYCN target gene and regulates tumorigenesis via repression of KIF1Bβ and TSLC1 in neuroblastoma** Page 121
Hidemasa Ochiai; Hisanori Takenobu; Atsuko Nakagawa; Yoko Yamaguchi; Miki Ohira; Yuri Okimoto; Yoichi Kohno; Akira Nakagawara; Takehiko Kamijo
Japan

15:10 **OR51 The interplay between Mycn, microRNAs and estrogen receptor-α during differentiation of the post-migratory sympathetic nervous system** Page 121
Jakob Lovén [1]; Nikolay Zinin [1]; Therese Wahlström [1]; Inga Müller [1]; Petter Brodin [1]; Erik Fredlund [1]; Ulf Ribacke [2]; Andor Pivarcsi [1]; Sven Påhlman [1]; Marie Henriksson [1]
[1]Sweden; [2]United States

15:20 **OR52 Targeting MYCN by modulation of the fate of its mRNA: a new potential therapeutic approach for neuroblastoma** Page 122
Viktoryia Sidarovich; Valentina Adami; Pamela Gatto; Gian Paolo Tonini; Alessandro Quattrone
Italy

15:30 **OR53 Bortezomib and HDAC inhibitor PCI-24781 show synergistic activity in neuroblastoma *in vitro* and *in vivo* models, inducing ROS and depressing MYCN** Page 122
Erika Currier; Sharon Illenye; Jennifer Libous; Jeffrey Bond; Pamela Lescault; Giselle Sholler
United States

15:40 **OR54 Re-activation of CLUSTERIN by epigenetic drugs as a therapeutic approach for MYCN tumourigenesis** Page 122
Daisy Corvetta [1]; Olesya Chayka [1]; Samuele Gherardi [2]; Emanuele Valli [2]; Sandra Cantilena [1]; Izabela Piotrowska [1]; Giovanni Perini [2]; Arturo Sala [1]
[1]United Kingdom; [2]Italy

15:50 **OR55 Identification of small molecules inhibiting Myc oncoprotein function** Page 122
Karin Ridderstråle; Qinzi Yan; Siti Mariam Zakaria; Per Hydbring; Lars-Gunnar Larsson
Sweden

14:30 – 16:00 Wednesday June 23rd
Hall B
Parallel session 8 – Novel clinical strategies and follow up
Chairs: Barbara Hero and Geneviéve Laureys

14:30 **OR56 The role of dietary restriction in the mechanisms of differential cellular protection: a strategy to enhance the efficacy of chemotherapy in the treatment of neuroblastoma** Page 123
Giovanna Bianchi [1]; David Lee [2]; Fernando Safdie [2]; Laura Emionite [1]; Vito Pistoia [1]; Valter Longo [2]; Lizzia Raffaghello [1]
[1]Italy; [2]United States

14:40 **OR57 Fenretinide (4-HPR) orally formulated in Lym-X-Sorb™(LXS) lipid matrix or as an intravenous emulsion increased 4-HPR systemic exposure in patients with Recurrent or Resistant Neuroblastoma. A new approaches to neuroblastoma therapy (NANT) consortium trial** Page 123
Min H. Kang; Araz Marachelian; Judith G. Villablanca; John M. Maris; Matthew M. Ames; Joel M. Reid; Katherine K. Matthay; C. Patrick Reynolds; Barry J. Maurer
United States

14:50 **OR58 Phase II trial of meta-iodobenzylguanidine (mIBG) with intensive chemotherapy and Autologous Stem Cell Transplant (ASCT) for high risk neuroblastoma. A New Approaches to Neuroblastoma Therapy (NANT) study** Page 123
Gregory Yanik; Brian Weiss; John Maris; Judy Villablanca; Barry Shulkin; Araz Marachelian; Howard Katzenstein; Raymond Hutchinson; Ken Koral; David Hubers; Daphne Haas-Kogan; Susan Groshen; Rajen Mody; Adi Lewinson; Shelli Anuszkiewicz; Beth Hasenauer; Katherine Matthay
United States

15:00 **OR59 A phase IIa trial of ultratrace (no-carrier added) iobenguane I-131 (MIBG): A New Approaches to Neuroblastoma Therapy (NANT) study** Page 124
Katherine Matthay; Brian Weiss; Judith G. Villablanca; John Maris; Greg Yanik; Susan Groshen; Hollie Jackson; Randall Hawkins; Fariba Goodarzian; Ashok Panigrahy; Steven Dubois; James Stubbs; John Barrett; John Babich; Alexander Towbin; Norman LaFrance
United States

15:10 **OR60 Characteristics of relapsing localized neuroblastoma: A preliminary report of the second SIOPEN study (LNESG2, localized neuroblastoma European study group 2)** Page 124
Maja Beck Popovic [1]; Emma Garcia [1]; Nicole Gross [1]; Valérie Combaret [2]; Peter Ambros [3]; Klaus Beiske [4]; Alessandro Jenkner [5]; Anne-Sophie Défachelles [2]; Adela Cañete [6]; Bénédicte Brichard [7]; Walentyna Balwierz [8]; Vassilios Papadakis [9]; Shifra Ash [10]; Ellen Ruud [4]; Ruth Ladenstein [3]; Ingrid Ora [11]; Keith Holmes [12]; Bruno De Bernardi [5]; Jean Michon [2]; Véronique Mosseri [2]
[1]Switzerland; [2]France; [3]Austria; [4]Norway; [5]Italy; [6]Spain; [7]Belgium; [8]Poland; [9]Greece; [10]Israel; [11]Sweden; [12]United Kingdom

15:20 **OR61 Outcome for stage 3 neuroblastoma: A report from the Children's Oncology Group** Page 124
Julie Park [1]; Wendy London [1]; Mary Lou Schmidt [1]; David Baker [2]; Susan Kreissman [1]; Judith Villablanca [1]; Hiroyuki Shimada [1]; Edward Attiyeh [1]; Michael Hogarty [1]; John Maris [1]; Katherine Matthay [1]; Susan L. Cohn [1]
[1]United States; [2]Australia

15:30 **OR62 Do relapsed high-risk neuroblastoma patients have a second chance? Results of the German neuroblastoma trials** Page 125
Thorsten Simon; Frank Berthold; Arndt Borkhardt; Bernhard Kremens; Boris De Carolis; Barbara Hero
Germany

15:40 **OR63 Anti-GD2 murine monoclonal antibody (MoAb) 3F8 for consolidation of first complete/very good partial remission of high risk stage 4 neuroblastoma** Page 125
Nai-Kong Cheung; Brian H. Kushner; Kim Kramer; Shakeel Modak; Suzanne L. Wolden; Michael P. La Quaglia
United States

15:50 **OR64 Changes over three decades in the prognostic influence of age in patients with neuroblastoma: A report from the International Neuroblastoma Risk Group Project**

Veronica Moroz [1]; David Machin [1]; Andreas Faldum [2]; Barbara Hero [2]; Tomoko Iehara [3]; Veronique Mosseri [4]; Ruth Ladenstein [5]; Bruno De Bernardi [6]; Hervé Rubie [4]; Frank Berthold [2]; Katherine K. Matthay [7]; Tom Monclair [8]; Peter F. Ambros [5]; Andrew D.J. Pearson [1]; Susan L. Cohn [7]; Wendy B. London [7]

[1]United Kingdom; [2]Germany; [3]Japan; [4]France; [5]Austria; [6]Italy; [7]United States; [8]Norway

14:45 – 15:55 Wednesday June 23rd
Hall C
Parallel session 9 – ALK
Chair: Isabelle Janouiex-Lerosey and Gian Paolo Tonini

14:45 **OR65 Skewed distribution and oncogenic properties of ALK hotspot mutations in neuroblastoma** Page 126
Candy Kumps [1]; Sara De Brouwer [1]; Piotr Zabrocki [1]; Michaël Porcu [1]; Ellen Westerhout [2]; Arjan Lakeman [2]; Jo Vandesompele [1]; Jasmien Hoebeeck [1]; Tom Van Maerken [1]; Anne De Paepe [1]; Geneviève Laureys [1]; Johannes Schulte [3]; Alexander Schramm [3]; Joëlle Vermeulen [1]; Nadine Van Roy [1]; Klaus Beiske [4]; Marleen Renard [1]; Rosa Noguera [5]; Olivier Delattre [6]; Isabelle Janouiex-Lerosey [6]; Per Kogner [7]; Tommy Martinsson [7]; Akira Nakagawara [8]; Miki Ohira [8]; Huib Caron [2]; Karin Verstraeten [1]; Ann De Bondt [1]; Jan Cools [1]; Jorge Vialard [1]; Angelika Eggert [3]; Rogier Versteeg [2]; Katleen De Preter [1]; Frank Speleman [1]
[1]Belgium; [2]Netherlands; [3]Germany; [4]Norway; [5]Spain; [6]France; [7]Sweden; [8]Japan

14:55 **OR66 High ALK receptor tyrosine kinase expression precedes ALK mutation as a determining factor of an unfavorable phenotype in primary neuroblastoma** Page 126
Johannes Schulte [1]; Bent Brockmeyer [1]; Hagen Bachmann [1]; Sandra Nowacki [1]; Benedikt Brors [1]; Yvonne Kahlert [1]; Andre Oberthur [1]; Katleen de Preter [2]; Kristian Pajtler [1]; Jessica Theissen [1]; Frank Westermann [1]; Jo Vandesompele [2]; Frank Berthold [1]; Barbara Hero [1]; Angelika Eggert [1]; Alexander Schramm [1]; Matthias Fischer [1]
[1]Germany; [2]Belgium

15:05 **OR67 Risk stratification of neuroblastoma by genomic signature including *ALK* abnormality** Page 126
Miki Ohira; Yohko Nakamura; Toshio Kojima; Junko Takita; Motohiro Kato; Seishi Ogawa; Shigeyuki Oba; Shin Ishii; Takehiko Kamijo; Akira Nakagawara
Japan

15:15 **OR68 Analysis of human ALK neuroblastoma mutations in *Drosophila melanogaster*** Page 127
Therese Eriksson; Christina Schönherr; Kristina Ruuth; Bengt Hallberg; Ruth Palmer
Sweden

15:25 **OR69 Role of ALK and its ligands in neuroblastoma** Page 127
Fabienne Munier; Marie Regairaz; Céline Renauleaud; Estelle Daudigeos-Dubus; M. Luis Mir; Birgit Geoerger; Gilles Vassal
France

15:35 **OR70 Effects of selective ALK inhibitors to neuroblastoma** Page 127
Junko Takita; Jun Ohkubo; Riki Nishimura; Kentaro Ohki; Naoki Uchisaka; Yuyan Chen; Masashi Sanada; Akira Kikuchi; Takashi Igarashi; Yasuhide Hayashi; Seishi Ogawa
Japan

15:45 **OR71 Therapeutic targeting of ALK on neuroblastoma cells by systemic delivery of GD_2-targeted liposomes entrapping small interfering RNA** Page 127
Daniela Di Paolo; Chiara Ambrogio; Fabio Pastorino; Chiara Brignole; Roberta Carosio; Monica Loi; Gabriella Pagnan; Michele Cilli; Domenico Ribatti; Roberto Chiarle; Mirco Ponzoni; Patrizia Perri
Italy

16:00 – 16:45 Wednesday June 23rd
Hall A
Selected poster – Biology 2
Chairs: Michael Hogarty and Frida Abel

16:05 **SEL25 Identification and molecular characterization of human neuroblastoma tumor-initiating cells** Page 140
Aurelie Coulon; Marjorie Flahaut; Annick Muhlethaler-Mottet; Julie Liberman; Gregor Kiowski; Lukas Sommer; Nicole Gross
Switzerland

16:10 **SEL26 Synergy of targeted GMCSF and IL2 to tumor microenvironments is mediated by an adaptive anti-neuroblastoma immune response** Page 140
Lode, Holger[1]; Bleeke, Matthias[1]; Reisfeld, Ralph[2]; Siebert, Nicolai[1]
[1]University of Greifswald, Pediatric Hematology and Oncology, Greifswald, Germany; [2]TSRI, Immunology, La Jolla, United States

16:15 **SEL27 Opposite roles of distinct caspase-10 isoforms in death receptor apoptotic pathway** Page 140
Annick Mühlethaler-Mottet; Katia Balmas Bourloud; Katya Nardou; Nicole Gross
Switzerland

16:20 **SEL28 The tumor suppressor candidate gene *APITD1/CENP-S* on chromosome 1p36 is involved in chromosome segregation and DNA damage repair** Page 141
Cecilia Krona [1]; Hanna Kryh [1]; Samantha Zeitlin [2]; Dan Foltz [2]; Don Cleveland [2]; Katarina Ejeskär [1]; Rose-Marie Sjöberg [1]; Helena Carén [1]; Tommy Martinsson [1]
[1]Sweden; [2]United States

16:25 **SEL29 Conditional MYCN knockdown using shRNAs encoded by lentivirus vectors** Page 141
Jørn Remi Henriksen; Bjørn Helge Haug; Jochen Buchner; Cecilie Løkke; Trond Flægstad; Christer Einvik
Norway

16:30 **SEL30 Integration of genome-wide ChIP-data of MYCN/MYC and histone marks with gene expression** Page 141
Filip Pattyn [1]; Christina Pöhler [2]; Daniel Muth [2]; Stephan Gade [2]; Tim Beißbarth [2]; Frank Speleman [1]; Manfred Schwab [2]; Jo Vandesompele [1]; Frank Westermann [2]
[1]Belgium; [2]Germany

16:35 **SEL31 *RUNX3*, mapped to chromosome 1p36, is a tumor suppressor functionally regulating p53 and MYCN in neuroblastoma** Page 142
Tomoki Yokochi [1]; Wei Gao [1]; Fan Yu [1]; Chizu Yamada [1]; Toshinori Ozaki [1]; Miki Ohira [1]; Yohko Nakamura [1]; Ken-ichi Inoue [2]; Yoshiaki Ito [2]; Atsuko Nakagawa [1]; Akira Nakagawara [1]
[1]Japan; [2]Singapore

16:40 **SEL32 The p53 target Wig-1 is a novel regulator of N-Myc at the mRNA level** Page 142
Anna Vilborg; Cinzia Bersani; Margareta Wilhelm; Weng-Onn Lui; Klas Wiman
Sweden

16:00 – 16:45 Wednesday June 23rd
Hall B
Selected poster – Biology 3
Chairs: Sue Burchill and Mirco Ponzoni

16:05 **SEL33 Neurocristopathy-associated Phox2b mutations cause Sox10 dysregulation and affects self-renewal, proliferation and differentiation of autonomic neural progenitors** Page 142
Hideki Enomoto; Mayumi Nagashimada; Hiroshi Ohta; Teruhiko Wakayama; Kazuki Nakao
Japan

16:10 **SEL34 *In vivo* analysis of human neuroblastoma cell lines in a human embryonic stem cell derived microenvironment - Impact of cues from the microenvironment** Page 142
Jessica Cedervall; Seema Jamil; Isabell Hultman; Rouknuddin Ali; Lena Kanter; Abiel Orrego; Bengt Sandstedt; Baldur Sveinbjörnsson; John Inge Johnsen; Per Kogner; Lars Ährlund-Richter
Sweden

16:15 **SEL35 Tenascin-C^{+}/Oct-4^{+} perivascular neuroblastoma cells serve as progenitors of tumor-derived endothelial cells** Page 143
Annalisa Pezzolo; Federica Parodi; Danilo Marimpietri; Lizzia Raffaghello; Claudia Cocco; Angela Pistorio; Manuela Mosconi; Claudio Gambini; Michele Cilli; Silvia Deaglio; Fabio Malavasi; Vito Pistoia
Italy

16:20 **SEL36 NVP-BEZ235 a dual PI3K/mTOR inhibitor destabilises Mycn *in vitro* and is growth inhibitory in the TH-*MYCN* murine neuroblastoma model** Page 143
Lynsey Vaughan [1]; Elizabeth Cullis [1]; Karen Barker [1]; Yann Jamin [1]; Simon Robinson [1]; Andrew Pearson [1]; Carlos Garcia-Echeverria [2]; Michel Maira [2]; Louis Chesler [1]
[1]United Kingdom; [2]Switzerland

16:25 **SEL37 Development of novel therapeutic strategy for neuroblastoma: Reactivation of the p53 tumor suppressor function by small molecules** Page 143
Elisabeth Hedström; Yao Shi; Mikhail Burmakin; Galina Selivanova
Sweden

16:30 **SEL38 Modeling neuroblastomagenesis from neural crest stem cells *in vitro* and *in vivo*** Page 143
Johannes Schulte [1]; Anna Bohrer [1]; Sven Lindner [1]; Katleen de Preter [2]; Frank Speleman [2]; Jo Vandesompele [2]; Jan Molenaar [3]; Rogier Versteeg [3]; Kristian Pajtler [1]; Jochen Maurer [1]; Hubert Schorle [1]; Alexander Schramm [1]; Angelika Eggert [1]
[1]Germany; [2]Belgium; [3]Netherlands

16:35 **SEL39 FOXO3/FKHRL1 is activated in high-risk neuroblastoma and contributes to chemotherapy-resistance and angiogenesis** Page 144
Kathrin Geiger [1]; Judith Hagenbuchner [1]; Martina Rupp [1]; Christina Salvador [1]; Bernhard Meister [1]; Consolato Sergi [2]; Petra Obexer [1]; Michael Ausserlechner [1]
[1]Austria; [2]Canada

16:40 **SEL40 Segmental chromosome aberrations and ploidy in localized neuroblastomas without MYCN amplification – Report from the SIOP Europe Neuroblastoma (SIOPEN) Group on the LNESG I Trial** Page 144
Inge M Ambros [1]; Gian Paolo Tonini [2]; Jerome Couturier [3]; Klaus Beiske [4]; Jean Benard [3]; Maria Boavida [5]; Nick Bown [6]; Huib Caron [7]; Valerie Combaret [3]; Raffaella Defferrari [2]; Nicole Gross [8]; Marta Jeison [9]; John Lunec [6]; Tommy Martinsson [10]; Katia Mazzocco [2]; Rosa Noguera [11]; Gudrun Schleiermacher [3]; Alexandre Valent [3]; Nadine Van Roy [12]; Andrew DJ Pearson [13]; Ruth Ladenstein [1]; Veronique Mosseri [3]; Bruno De Bernardi [2]; Jean Michon [3]; Peter F Ambros [1]
[1]Austria; [2]Italy; [3]France; [4]Norway; [5]Portugal; [6]United Kingdom; [7]Netherlands; [8]Switzerland; [9]Israel; [10]Sweden; [11]Spain; [12]Belgium; [13]United Kingdom

16:00 – 16:45 Wednesday June 23rd
Hall C
Selected poster – Translational 2
Chairs: Meredith Irwin and Rochelle Bagatell

16:05 **SEL41 Segmental chromosome abnormalities and age over 36 months at diagnosis are associated with increased risk of relapse in localised unresectable neuroblastoma without MYCN amplification - A preliminary report from the SIOP Europe Neuroblastoma (SIOPEN) Biology Group** Page 144
R Defferrari; K Mazzocco; IM Ambros; PF Ambros; C Bedwell; C Beiske; J Benard; N Bown; V Castel; V Combaret; J Couturier; B De Bernardi; A Garaventa; N Gross; R Haupt; J Kohler; M Jeason; R Ladenstein; J Lunec; B Marques; T Martinsson; R Noguera; S Parodi; H Rubie; G Schleiermacher; F Speleman; A Valent; N Van Roy; A Vicha; E Villamon; GP Tonini for the SIOPEN Biology Group

16:10 **SEL42 Drug-induced senescence in *MYCN*-amplified neuroblastoma - gene expression profiling and functional consequences** Page 145
Sabine Taschner-Mandl [1]; Agata Kowalska [1]; Heidemarie Binder [1]; Dietmar Rieder [1]; Zlatko Trajanoski [1]; Javed Khan [2]; Frank Speleman [3]; Inge M Ambros [1]; Peter F Ambros [1]
[1]Austria; [2]United States; [3]Belgium

16:15 **SEL43 Parvovirus H1 induces oncolytic effects on human neuroblastoma cells *in vitro* and in neuroblastoma xenograft-bearing nude rats** Page 145
Jeannine Lacroix; Barbara Leuchs; Georgi Hristov; Junwei Li; Hedwig E. Deubzer; Jean Rommelaere; Olaf Witt; Jörg R. Schlehofer
Germany

16:20 **SEL44 Targeting MYCN in neuroblastoma with small molecules *in vitro* and *in vivo*** Page 145
Hanna Zirath; Lova Segerström; Anna Frenzel; Per Kogner; Marie Henriksson
Sweden

16:25 **SEL45 Protein interactions of the *PHOX2B* variants identified in patients with neuroblastoma** Page 145
Wenchao Wang; Quan Zhong; William Luther II; A. Thomas Look; David Hill; Marc Vidal; Rani E. George
United States

16:30 **SEL46 Targeted therapeutics in chemotherapy-refractory neuroblastoma** Page 146
W. Clay Gustafson [1]; Benjamin Houseman [1]; Louis Chesler [2]; Melissa itsara [1]; Kevan Shokat [1]; William A Weiss [1]
[1]United States; [2]United Kingdom

16:35 **SEL47 Validation of Survivin as a therapeutic target in neuroblastoma** Page 146
Fieke Lamers; Linda Schild; Ida van der Ploeg; Marli Ebus; Jan Koster; Rogier Versteeg; Huib Caron; Jan Molenaar
Netherlands

16:40 **SEL48 Rituximab is a novel neuroblastoma therapy with efficacy against neuroblastoma tumor initiating cells *in vitro* and *in vivo*** Page 146
Paola Angelini; Loen Hansford; David Kaplan; Meredith Irwin
Canada

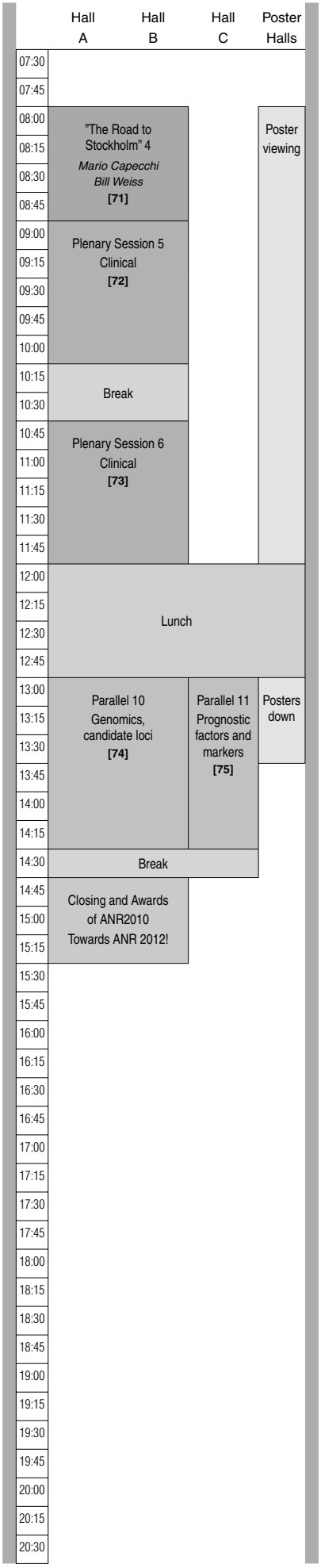
16:00 – 18:30 Wednesday June 23rd

Poster session – All posters will be displayed throughout the meeting

Odd numbers/left aligned posters = Presenting authors present at posters Tuesday June 22nd 16:45 – 17:30
Even numbers/indented posters = Presenting authors present at posters Wednesday June 23rd 16:45 – 17:30

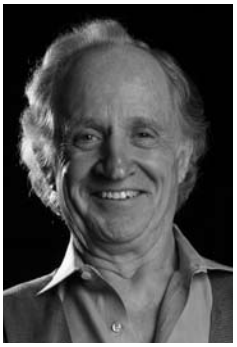
18:00 – 18:15 Wednesday June 23rd
Hall A
Concluding remarks by Robert Seeger and Angelika Eggert

Programme
Thursday June 24th



08:00 – 09:00 Thursday June 24th
Hall A/B
The Road to Stockholm and Beyond 4
Chairs: Olivier Delattre and Murray Norris

PL25 Modeling neuropsychiatric disorders in the mouse
Mario Capecchi, United States



Mario Capecchi graduated from George School and received his B.S. in chemistry and physics from Antioch College in 1961 and his Ph.D. in biophysics from Harvard University in 1967. He completed his thesis work under the guidance of Dr. James D. Watson, co-discoverer of the structure of DNA. Mario advanced quickly through the ranks at Harvard Medical School but he was looking for something different. He sought an environment that believed in long-term investment, one that would allow him to address the big questions. Dr. Capecchi began his research at the University of Utah, 1973. Thirty-four years later, on December 10, 2007, Dr. Capecchi received the highest honor in his field, the Nobel Prize, for his work in molecular biology. His pioneering work in gene targeting of mouse embryo-derived stem cells has set a new standard for research worldwide. This renowned discovery holds endless possibilities for development of treatments and ultimately cures for every known human disease. Dr. Capecchi believes we, as citizens of the world, we have many challenges that face us. Health issues and many diseases that plague us… but also global concerns such as war, equal opportunity for all people, the world economy and most importantly global warming. Millions of lives are in peril. We must begin now to address these issues and make a difference while we still can.

PL26 Genetic and developmental therapeutic studies in a transgenic mouse model for high-risk neuroblastoma Page 100
Bill Weiss, United States

William A. Weiss MD, PhD is a Professor of Neurology, Pediatrics, and Neurosurgery at UCSF, oversees child neurology at San Francisco General Hospital, codirects the pediatrics malignancies program in UCSF's cancer center, is associate editor of Cancer Research and of the NeuroOncology Journals, and advises brain tumor programs at the Children's Hospital of Los Angeles, Mayo Clinic, Saint Jude Children's Research Hospital, and the University of Calgary. Dr. Weiss' lab has developed murine models of glioma, medulloblastoma and neuroblastoma based on recapitulating cardinal genetic abnormalities in transgenic mice and is using theese mice to investigate both Mycn and EGFR/PI3K/mTOR signaling pathways. Dr. Weiss organized two past international meetings of scientists and physicians modeling neural tumors in the mouse. He received graduate degrees from Stanford University, completed residency training at Boston Children's and UCSF, and postdoctoral training with J. Michael Bishop, MD.

09:00 – 10:15 Thursday June 24th
Hall A/B
Plenary session 5 – Clinical
Chairs: Tom Monclair and Mattias Fischer

09:00 **PL27 Widespread dysregulation of miRNAs by MYCN amplification and chromosomal imbalances in neuroblastoma: Association of miRNA expression with survival** Page 101
Isabella Bray [1]; Kenneth Bryan [1]; Suzanne Prenter [1]; Patrick G Buckley [1]; Niamh H Foley [1]; Derek M Murphy [1]; Leah Alcock [1]; Pieter Mestdagh [2]; Jo Vandesompele [2]; Frank Speleman [2]; Wendy B London [3]; Patrick W McGrady [3]; Desmond G Higgins [1]; Anne O'Meara [1]; Katleen De Preter [2]; Maureen O'Sullivan [1]; Raymond L Stallings [1]
[1]Ireland; [2]Belgium; [3]United States

09:15 **PL28 Evaluation of *PHOX2B*, tyrosine hydroxylase (TH), *GD2* and *ELAVL4* expression for minimal residual disease (MRD) detection in neuroblastoma patients** Page 101
Alexander Druy; Grigory Tsaur; Alexander Popov; Tatyana Verzhbitskaya; Egor Shorikov; Leonid Saveliev; Larisa Fechina
Russian Federation

09:30 **PL29 QRT-PCR for TH and Phox2B mRNA in peripheral blood and bone marrow from children with high risk neuroblastoma predicts overall survival; a SIOPEN molecular monitoring group study** Page 102
Virginie Viprey [1]; Maria Corrias [2]; Andrei Tchirkov [3]; Katrien Swerts [4]; Ales Vicha [5]; Sandro Dallorso [2]; Walter Gregory [6]; Roberto Luksch [2]; Penelope Brock [6]; Dominique Valteau-Couanet [3]; Genevieve Laureys [4]; Josef Malis [5]; Vassilios Papadakis [7]; Pavel Bician [8]; Ruth Ladenstein [9]; Susan Burchill [1]
[1]United Kingdom; [2]Italy; [3]France; [4]Belgium; [5]Czech Republic; [6]United Kingdom; [7]Greece; [8]Slovakia; [9]Austria

09:45 **PL30 Analyses of mIBG scoring as a prognostic indicator in patients with stage 4 neuroblastoma. A Children's Oncology Group (A3973) report** Page 102
Gregory Yanik; Marguerite Parisi; Barry Shulkin; Arlene Naranjo; Susan Kreissman; Wendy London; Judy Villablanca; Patrick McGrady; Katherine Matthay
United States

10:00 **PL31 Characterization of neuroblastoma imaging studies using F-18-DOPA PET/CT** Page 103
Kai-Yuan Tzen; Meng Yao Lu; Hsiu-Hao Chang; Kai-Hsin Lin; Shiann-Tarng Jou; Yung-Li Yang; Dong-Tsamn Lin; Wen-Ming Hsu
Taiwan

10:15 – 10:45 BREAK

10:45 – 12:00 Thursday June 24th
Hall A/B
Plenary session 6 – Clinical
Chairs: Ruth Ladenstein and Julie Park

10:45 **PL32 Clinical and biological features predictive of survival after relapse of neuroblastoma: A study from the International Neuroblastoma Risk Group (INRG) Database** Page 103
Victoria Castel [1]; Kate K Matthay [2]; Tom Monclair [3]; Andrew D Pearson [4]; Susan L. Cohn [2]; Wendy B London [2]
[1]Spain; [2]United States; [3]Norway; [4]United Kingdom

11:00 **PL33 Topotecan-vincristine-doxorubicin in metastatic neuroblastoma failing to respond to rapid COJEC. Preliminary results of a Siopen Group Study** Page 104
Loredana Amoroso [1]; Guy Makin [2]; Ruth Ladenstein [3]; Genevieve Laureys [4]; Roberto Luksch [1]; Victoria Castel [5]; Peppy Brock [2]; Caroline Thomas [6]; Dominique Valteau-Couanet [6]; Alberto Garaventa [1]
[1]Italy; [2]United Kingdom; [3]Austria; [4]Belgium; [5]Spain; [6]France

11:15 **PL34 Suppression of human anti-mouse antibody response by rituximab plus cyclophosphamide permits continuation of anti-GD2 immunotherapy** Page 104
Shakeel Modak; Brian H. Kushner; Kim Kramer; Irene Y. Cheung; Nai-Kong V. Cheung
United States

11:30 **PL35 Long term outcome: the price of treatment for surviving high-risk neuroblastoma** Page 105
Barbara Hero; Simon Thorsten; Dagmar Dilloo; Bernhard Kremens; Lorenz Grigull; Hans-Gerhard Scheel-Walter; Frank Berthold
Germany

11:45 **PL36 Long-term toxicity in survivors of ENSG5 trial for children with high-risk neuroblastoma** Page 105
Lucas Moreno [1]; Sucheta Vaidya [1]; Ross Pinkerton [2]; Ian J Lewis [1]; John Imeson [1]; Caroline Ellershaw [1]; David Machin [1]; Andrew DJ Pearson [1]
[1]United Kingdom; [2]Australia

12:00 – 13:00 LUNCH

13:00 – 14:30 Thursday June 24th
Hall A/B
Parallel session 10 – Genomics, candidate loci
Chairs: Peter Ambros and Frank Westermann

13:00 **OR72 A regulatory BCL2 promoter polymorphism (-938C>A) is associated with outcome in neuroblastoma** Page 128
Bent Brockmeyer; Hagen Bachmann; Annette Kunkele; Kristian Pajtler; Winfried Siffert; Angelika Eggert; Alexander Schramm; Johannes Schulte
Germany

13:10 **OR73 Identification of critical domains that mediate the transcriptional and growth-inhibiting functions of the neuroblastoma tumor suppressor gene CASZ1** Page 128
Ryan Virden; Zhihui Liu; Carol Thiele
United States

13:20 **OR74 KIF1Bβ tumor suppressor, identified from the homozygous deletion at chromosome 1p36.2, interacts with YME1L1 metalloprotease to induce apoptosis through mitochondrial morphogenesis and cytochrome c release** Page 128
Koji Ando; Kiyohiro Ando; Tomoki Yokochi; Sonja Kramer; Akira Mukai; Toshinori Ozaki; Akira Nakagawara
Japan

13:30 **OR75 Coordinate expression of Let-7 family members in neuroblastoma and their dysregulation by DNA copy number loss** Page 128
Fernandez Raquel; Bryan Kenneth; Patrick G Buckley; Isabella Bray; Leah Alcock; Raymond L Stallings
Ireland

13:40 **OR76 A genome-wide association study (GWAS) of neuroblastoma** Page 129
John Maris [1]; Sharon Diskin [1]; Kristopher Bosse [1]; Le Nguyen [1]; Robert Schnepp [1]; Edward Attiyeh [1]; Yael Mosse [1]; Mario Capasso [2]; Cynthia Winter [1]; Maura Diamond [1]; Marci Laudenslager [1]; Kai Wang [1]; Haitao Zhang [1]; Cuiping Hou [1]; Cecilia Kim [1]; Joseph Glessner [1]; Wendy London [1]; Nazneen Rhaman [3]; Hongzhe Li [1]; Marcella Devoto [1]; Hakon Hakonarson [1]
[1]United States; [2]Italy; [3]United Kingdom

13:50 **OR77 Acquired segmental copy number changes in relapsed neuroblastoma** Page 129
David Cobrinik; Irene Y. Cheung; Nai-Kong V. Cheung
United States

14:00 **OR78 Identification and characterization of somatic rearrangements in neuroblastoma cell lines using genome-wide massively parallel sequencing** Page 129
Isabelle Janoueix-Lerosey; Valentina Boeva; Stéphanie Jouannet; Romain Daveau; Alex Cazes; Gudrun Schleiermacher; Valérie Combaret; Emmanuel Barillot; Olivier Delattre
France

14:10 **OR79 Genome/transcriptome analysis of metastatic neuroblastoma, reveals an increase of structural aberrations and deregulation of rho/ras and telomerase pathways associated with poor patients outcome** Page 130
Simona Coco [1]; Jessica Theissen [2]; Paola Scaruffi [1]; Sara Stigliani [1]; Stefano Moretti [3]; André Oberthuer [2]; Barbara Hero [2]; Matthias Fischer [2]; Stefano Bonassi [1]; Fabio Gallo [1]; Carla De Vecchi [1]; Frank Berthold [2]; Gian Paolo Tonini [1]
[1]Italy; [2]Germany; [3]France

14:20 **OR80 Irregular chromosome segregation by tripolar divisions; mechanisms for heterogeneity of karyotypes in neuroblastoma** Page 130
Fumio Kasai [1]; Hirofumi Kobayashi [1]; Willem Rens [2]; Malcolm A. Ferguson-Smith [3]; Yasuhiko Kaneko [1]
[1]Japan; [2]United Kingdom; [3]United Kingdom

14:30 – 14:45 BREAK

13:00 – 14:30 Thursday June 24th
Hall C
Parallel session 11 – Prognostic factors and markers
Chairs: Kurkure Purna and Dominique Valteau-Couanet

13:00 **OR81 Is subtotal resection sufficient for treatment of ganglioneuroma and localized ganglioneuroblastoma intermixed?** Page 130
Boris De Carolis; Thorsten Simon; Ivo Leuschner; Dietrich von Schweinitz; Thomas Klingebiel; Rudolf Erttmann; Lothar Schweigerer; Peter Kaatsch; Frank Berthold; Barbara Hero
Germany

13:10 **OR82 Survival variability by race and ethnicity in neuroblastoma: A Children's Oncology Group (COG) Study** Page 131
Navin Pinto; Tara Henderson; Smita Bhatia; Wendy London; Patrick McGrady; Catherine Crotty; Can-Lan Sun; Susan L. Cohn
United States

13:20 **OR83 Stable incidence of neuroblastoma during 28 years in Sweden with significant sex differences and improved survival, in particular for children with high-risk disease with MYCN amplification** Page 131
Catarina Träger; Åsa Vernby; Helena Caren; Hanna Kryh; Fredrik Hedborg; Tommy Martinsson; Göran Gustafsson; Per Kogner
Sweden

13:30 **OR84 Long term outcome and impact of biology within risk adapted treatment strategies: The Austrian neuroblastoma trial A-NB94** Page 131
Ruth Ladenstein; Inge Ambros; Ulrike Poetschger; Christian Urban; Georg Ebetsberger; Bernhard Meister; Gabriele Amann; Ekkehart Spuller; Karin Dieckmann; Ernst Horcher; Bettina Brunner; Andrea Ziegler; Peter Ambros
Austria

13:40 **OR85 Exon-Level gene expression analyses of primary neuroblastoma improves risk prediction and identifies MYCN status as major determinant of alternative transcript use** Page 132
Alexander Schramm [1]; Benjamin Schowe [1]; Tobias Marschall [1]; Marcel Martin [1]; Joelle Vermeulen [2]; Jo Vandesompele [2]; Jessica Theissen [1]; Barbara Hero [1]; Theresa Thor [1]; Katharina Morik [1]; Sven Rahmann [1]; Angelika Eggert [1]; Johannes Schulte [1]
[1]Germany; [2]Belgium

13:50 **OR86 High expression of KIF1Bβ-interacting protein MAP1A and its family member MAP1B significantly correlates with favourable prognosis of neuroblastoma** Page 132
Sonja Kramer [1]; Miki Ohira [1]; Tomoki Yokochi [1]; Koji Ando [1]; Akira Mukai [1]; Angelika Eggert [2]; Akira Nakagawara [1]
[1]Japan; [2]Germany

14:00 **OR87 Determination of 17q gain in neuroblastoma patients by analysis of circulating DNA** Page 132
Valerie Combaret; Stephanie Brejon; Isabelle Iacono; Gudrun Schleiermacher; Gaelle Pierron; Agnes Ribeiro; Christophe Bergeron; Aurelien Marabelle; Alain Puisieux
France

14:10 **OR88 Phox2B but not TH mRNA detected by QRT-PCR in peripheral blood stem cell harvest predicts time to relapse in randomised children with high risk neuroblastoma; a SIOPEN molecular monitoring group study** Page 133
Sandro Dallorso [1]; Maria Corrias [1]; Virginie Viprey [2]; Ales Vicha [3]; Katrien Swerts [4]; Andrei Tchirkov [5]; Walter Gregory [2]; Roberto Luksch [1]; Penelope Brock [2]; Josef Malis [3]; Genevieve Laureys [4]; Dominique Valteau-Couanet [5]; Ruth Ladenstein [6]; Susan Burchill [2]
[1]Italy; [2]United Kingdom; [3]Czech Republic; [4]Belgium; [5]France; [6]Austria

14:20 **OR89 Clinical utility of minimal residual disease marker panel during sequential phases of a multi-modality treatment of high-risk neuroblastoma** Page 133
Irene Cheung; Brian Kushner; Kim Kramer; Shakeel Modak; Nai-Kong Cheung
United States

14:30 – 14:45 BREAK

14:45 – 15:30 Thursday June 24th
Hall A/B
Closing and Awards of ANR 2010
Towards ANR 2012!
Chairs: Michelle Haber and Susan L. Cohn

Abstract Book

Neuroblastoma update course C1–C12

C1

Using genome-wide strategies to discover new gene aberrations

Speleman, Frank
Belgium

What is needed to successfully combat cancer? For certain childhood cancer entities conventional chemotherapy nowadays achieves high cure rates, e.g. above 80% for acute lymphoblastic leukemias. However, treatment with DNA damaging drugs can cause serious side effects due to lack of specificity, an issue which is of particular importance when treating children. Also, treatment may ultimately fail due to intrinsic and acquired drug resistance. Recent successes in 'targeted therapeutics' have risen the hope that an increasing number of highly effective drugs specifically targeting a genetic defect in cancer cells with far less side effects will become available. Few cancer types however carry a common 'driver' mutation thus precluding a simple 'one tumor - one target' strategy. Many tumors carry complex genetic alterations impeding the definition of clear mechanistic principles to develop targeted therapeutic strategies. Moreover, even with a well-defined molecular target and highly efficient therapeutic compound, drug resistance occurs and therefore a strategy based on targeting multiple pathways or multiple vulnerable nodes in a key pathway (or both) is warranted. Present genome-wide technologies are allowing a deeper probing into the cancer genome with unprecedented speed for detecting DNA copy number alterations with resolution up to a few kb or less, genome wide expression profiling at the exon level and high throughput sequencing of entire cancer genomes, chromatin marks and methylated regions and RNA sequencing. These new platforms open the perspective of achieving molecular portraits of individual cancer genomes, a prelude to personalized medicine in cancer treatment. In order to be able to rationally use this rapidly increasing amount of genetic information, we are in desperate need for equally performant assays that allow determining the functional relevance of mutations or differentially expressed genes. At the same time, we will need a systems biology approach in order to link the various levels of genomic information and track down connections and nodes of the complex regulatory networks that are perturbed in cancer. In this presentation I will give an overview of the current state-of-the art of genome-wide technologies for studying cancer as well as emerging fields and future challenges.

C2

Biological and clinical relevance of ALK mutations

George, Rani[1]; Mosse, Yael[2]
[1]Dana-Farber Cancer Institute, Harvard Medical School, Department of Pediatric Oncology, Boston, United States; [2]Children's Hospital of Philadelphia, Department of Oncology, Philadelphia, United States

No abstract available

C3

International Neuroblastoma Risk Group (INRG): Next steps

Pearson, A D J[1]; London, W B[2]; Cohn, S L[3]; and the INRG Task Force, [4]
[1]Institute of Cancer Research, Royal Marsden Hospital, United Kingdom; [2]Harvard Medical School, Children's Hospital Boston, Boston, United States; [3]University of Chicago, Section of Hematology / Oncology, Chicago, United States; [4]

The International Neuroblastoma Risk Group (INRG) classification system was developed to establish a consensus approach for pre-treatment risk stratification. The statistical and clinical significance of 13 potential prognostic factors were analyzed in a cohort of 8,800 children diagnosed with neuroblastoma between 1990-2002 from North America and Australia, Europe, and Japan. Factors prognostic of event-free survival were identified using survival tree regression analyses. Stage, age, histological category, grade of tumour differentiation, the status of the MYCN oncogene, chromosome 11q status, and DNA ploidy were the most highly statistically significant and clinically relevant factors and these were utilised in the classification system, published in 2009.
The INRG is now being utilized in prospective co-operative clinical studies. Consensus documents on biological features, staging, and detection of minimal residual disease, MIBG scanning and entry and response criteria for phase II studies have been published or are in preparation. Twelve retrospective studies have been undertaken utilizing the INRG data base. The INRG has been a significant success, but the challenge is now to build on this system and develop a mores precise classification system - the second INRG. Already the prognostic impact of the presence of or absence of segmental chromosomal alterations is being employed in therapeutic decisions. The next INRG should utilise extensively genomic data, both DNA and mRNA and possibly data from methylation, micro RNA and genome-wide association studies. Incorporating data relating to aberrations of the ALK pathway is also a focus. It is anticipated that inclusion of genomic data may allow some clinical data for example age not be needed to describe the population. The current INRG cohort has been stratified into 16 clinically and statistically distinct pre-treatment groups on the basis of factors prognostic of event free survival. The goal in the future would be to have fewer pre- treatment groups and these to be closely linked to therapy which is required for the maximal chance of cure. Data from approximately 4,000 patients in studies recently accepted for publication will be available for analysis, permitting a total data set of nearly 16, 000 patients. New data items which will be added will include:
- gender, race, ethnicity, information on late effects (non-tumour related deaths and second malignancy) and more precise data on therapy.
To enable the next INRG to be developed, it will be essential that INRG data is linked with biobank or genomic databases. In order to do this it is proposed to develop a web-based Interactive INRG Database Network with technology that will support the linkage of INRG patient information with host and tumour biological data in biobank databases. The Interactive INRG Database Network would have three components:
- a core database (comprising biological data, phenotypic measures, clinical outcome and available tumor/host banked samples); a middle layer (middleware) and applications.
It is proposed that to ensure that the Interactive INRG Database Network has the capacity to support the development of the Second INRG, as well as the needs of the broad community of neuroblastoma researchers, a needs assessment will be performed, before it is developed.
In conclusion, the substantial international effort expended in establishing the INRG can be utilised as an invaluable base to develop a new version of the INRG which will be utilise predominantly genomic data with the objective of providing a more precise classification system of neuroblastoma.

C4

Using PET and MIBG to evaluate disease and response

Sharp, Susan
Cincinnati Children's Hospital Medical Center, Department of Radiology, Cincinnati, United States

Functional imaging plays an important role in neuroblastoma assessment. I-123-MIBG remains the most frequently used functional imaging agent with a high sensitivity and specificity for neuroblastoma. I-123-MIBG is useful in depicting disease extent at diagnosis, following therapy response, and localizing residual/recurrent disease. Use of FDG PET is increasing and studies continue to clarify its role. FDG has been shown to be especially useful in neuroblastomas which weakly accumulate MIBG and should be considered when MIBG demonstrates less disease than suspected by conventional imaging or clinical symptoms. The spatial resolution of FDG PET/CT also aids in disease delineation, especially in the chest, abdomen, and pelvis.

C5

The evolving role of minimally invasive surgery in treatment of neuroblastoma

Nuchtern, Jed
Texas Children's Hospital/ Baylor College of Medicine, Pediatric Surgery United States

Minimally invasive surgery, both thoracoscopy and laparoscopy, have emerged as important therapeutic modalities over the past two decades. These techniques have many advantages over traditional open approaches including better visualization, decreased postoperative pain, earlier return to normal activities, and improved cosmesis. These gains are balanced by persistent challenges, which include monoscopic vision, decreased manipulative degrees of freedom, and lack of tactile feedback. Surgery remains a key modality in diagnosis and treatment of patients with neuroblastoma. The practice of minimally invasive surgery in the treatment of neuroblastoma continues to evolve as technical capabilities improve and the overall role of surgery adapts to our current therapeutic strategy for this disease.
As the importance of biological tumor characteristics in risk stratification has increased, it has become increasingly crucial to obtain adequate tumor tissue at initial presentation. Both image-guided needle biopsy and videoscopic incisional biopsy are useful techniques that can decrease the morbidity associated with tumor acquisition for histologic and molecular studies. A team approach among surgeons, radiologists, pathologists and oncologists is crucial to assure that adequate tissue is obtained and the appropriate studies are performed.
Thoracoscopy and laparoscopy are likely to become more prevalent in definitive resection of primary neuroblastoma tumors in the coming years. These techniques are ideally suited to dissection of well-localized tumors that do not invade or encase adjacent vital structures. In fact, there is significant complementarity between the absence of image-defined risk factors (IDRF), a key component of the new INRG staging system, and the likelihood of a successful minimally invasive surgery.
The two areas that continue to be challenging for application of minimally invasive surgery are: resection of extensive, infiltrative retroperitoneal and thoracic masses, and thorough lymph node dissection. In the former situation, current minimally invasive techniques are neither delicate nor efficient enough to accomplish these procedures. Although laparoscopy can be used successfully for retroperitoneal lymph node sampling, this technique is not generally used for resection of the bulky nodal disease that accompanies large infiltrative retroperitoneal tumors.
The barriers to increased utilization of minimally invasive surgery for treatment of neuroblastoma are both technical and educational. If previous trends continue, it is reasonable to expect that equipment will continue to improve in the coming years. It is important to assure that training programs focus on increasing the skills of residents and fellows to keep pace with these technical improvements.

C6

Use of the genomic profile for treatment stratification in neuroblastoma

Schleiermacher, Gudrun[1]; Michon, Jean[1]; Janoueix-Lerosey, Isabelle[2]; Delattre, Olivier[2]
[1]Institut Curie, Department of Pediatrics, Paris, France; [2]Institut Curie, U830 INSERM, Paris, France

In neuroblastome a large number of recurrent genetic somatic alterations have been described. Ploidy variations are frequently observed, with DNA indexes in the pseudo-triploid range associated with a better prognosis. Amplification of the MYCN oncogene is associated with a poor outcome. Segmental chromosome alterations such as deletion of chromosomes 1p,3p,11q, and gain of 1q,2p and 17q are frequently observed, and many have also been shown to be of prognostic impact in univariate analyses. It has been demonstrated, using pangenomic techniques such as chromosomal or more recently array-CGH, that these genetic alterations combine to define distinct genomic profiles associated with different clinical presentations and evolution. Tumors with numerical chromosome alterations only are frequently observed in young patients with localized disease having a good outcome, whereas tumors with any segmental alteration and tumors with MYCN amplification frequently occur in older children with advanced stages of disease presenting a poor outcome. Furthermore, it has recently been shown that patients with tumors harboring both numerical and segmental alterations share the poor outcome of those with segmental alterations only. More recent data show that a higher number of chromosome breakpoints is correlated with advanced age at diagnosis, advanced stage of disease, with a higher risk of relapse and a poorer outcome. In case of relapse or progression, new segmental alterations can be observed in tumor samples obtained at relapse, compared to diagnostic samples, indicating a role of segmental alterations in tumor progression. In multivariate analyses, taking into account the genomic profile, but also previously described individual genetic and clinical markers with prognostic significance, a genomic profile characterized by the presence of segmental alterations is the strongest predictor of relapse, rather than individual genetic markers. Thus, the analysis of the overall genomic pattern, which probably unravels particular genomic instability mechanisms, rather than the analysis of individual markers, is important to predict relapse in NB patients. The genomic profile adds critical prognostic information to conventional clinical markers and will be helpful for further treatment stratification, especially in patients with low and intermediate-risk NB. Indeed, a favourable genomic profile might be used to identify patients with low or intermediate-risk neuroblastome in whom it can be deemed safe to propose controlled treatment reduction, whereas for patients with an unfavourable profile, an upfront increase in treatment could be justified.

C7

When should we use a "wait and see" approach?

Berthold, Frank
Childrens Hospital, University of Cologne, Pediatric Oncology and Hematology, Kerpener Str. 62, Cologne, Germany

The ability of spontaneous tumor regression is estimated to up to 54 % of neuroblastoma patients by clinical and up to 56 % by molecular criteria. Although events like significant tumor progression may be observed after diagnosis in those patients, the outcome is excellent regardless the used treatment modalities (3 year overall survival > 95%). Current experience from prospective studies may be summarized as follows: 1. A "wait and see" approach can be safely applied to infants with MYCN non-amplified neuroblastoma of the stages 1,2,3 and 4S. 2. Gene expression signatures appear to define regression biology more accurate than clinical categories. 3. Approximately 25 % of the patients present symptoms requiring chemotherapeutic intervention. It is sufficient to treat until relief of symptoms and not necessary to treat until tumor disappearance. 4. Tumor progression with and without threatening symptoms may be observed in up to 40% of patients. Almost all of those patients can be rescued by surgical and/or chemotherapeutic intervention. 5. The begin of clinical regression is seen in one third of patients beyond the 1. year of life. 6. Regression may be observed in patients with 1p aberration and with unfavorable histology.

C8

Anti-GD2 immunotherapy plus isotretinoin: A new standard of care for high risk neuroblastoma

Yu, Alice[1]; Gilman, Andrew[2]; Ozkaynak, Fevzi[3]; London, Wendy[4]; et al. on behalf of Children's, Oncology Group, USA[5]
[1]University of California in San Diego, Pediatrics, 200 W. Arbor Drive, San Diego, CA 92103-8447, United States; [2]Levine Children¡¦s Hospital, Pediatric Hematology Oncology, Charlotte NC, United States; [3]New York Medical College,, Pediatric Hematology Oncology, Valhalla, NY, United States; [4]Dana Farber Cancer Institute and Children¡¦s Hospital Boston, Children¡¦s Oncology Group Statistics and Data Center, Boston MA, United States; [5]

Failure to eliminate minimal residual disease (MRD) is the major obstacle to the cure of high risk neuroblastoma. Immunotherapy is an attractive option for the treatment of these chemotherapy refractory MRD. A surface glycolipid molecule, disialoganglioside (GD2), which is uniformly expressed by neuroblastoma with limited expression in normal tissues, is an ideal antigen target for immunotherapy of neuroblastoma. Three monoclonal antibodies targeting GD2 have been investigated for immunotherapy of neuroblastoma: 1) mAb 14G2a, a murine IgG2a anti-GD2 antibody, 2) mAb ch14.18, a human-mouse chimeric anti-GD2 monoclonal antibody that contains H gene of human IgG1 and V gene of 14G2a, and 3) mAb3F8, a murine anti-GD2. Initial clinical trials of these 3 anti-GD2 mAbs have documented their toxicity profile and anti-tumor activities. The most common toxicity is neuropathic pain associated with antibody infusion, which is controllable, reversible and dependent on the mAb dose and rate of infusion. Other side effects include fever, urticaria, blood pressue changes, nausea, vomiting, hyponatremia, hypokalemia and facial edema. Subsequent studies of mAb 14G2a in combination with IL-2, and ch14.18 + GM-CSF in patients with recurrent/refractory neuroblastoma further demonstrated the feasibility of combining anti-GD2 with cytokines and anti-tumor activities of such combinations. A pivotal phase III randomized trial in high risk neuroblastoma, COG ANBL0032 has been conducted under the auspice of Chilren's Oncology Group. High risk neuroblastoma patients who responded to induction therapy and stem cell transplant were randomized to 6 cycles of isotretinoin or isotretinoin with 5 concomitant cycles of ch14.18 combined with alternating cycles of GM-CSF and IL2. A recent analysis of 226 randomized patients demonstrated a significantly improved outcome with 20% reduction in relapses in the group receiving ch14.18 + cytokines + isotretinoin. Immunotherapy was associated with significant pain, vascular leak syndrome and hypersensitivity reactions. This is the first clinical trial to show a substantive increase in cure rate in well over a decade for this dreadful disease. The 20% improvement in prevention of relapse for children with neuroblastoma receiving the experimental immunotherapy makes this therapy the new standard of care.

C9

Targeted radiotherapy for neuroblastoma using ^{131}I-metaiodobenzylguanidine (^{131}I-mIBG)

Matthay, Katherine
UCSF, Pediatrics, San Francisco, United States

Neuroblastoma is a tumor of the sympathetic nervous system and the most common pediatric extra-cranial solid cancer. Nearly half of patients have high risk disease due to metastases or unfavorable biology, with less than 40% long term survival despite intensive chemotherapy followed by myeloablative therapy and treatment of minimal residual disease. MIBG is actively transported into neuroblastoma cells by the norepinephrine transporter (hNET). This tumor is characterized by mIBG avidity in 90% of cases, prompting the use of radiolabeled mIBG for staging and for targeted radiotherapy in these tumors. Clinical studies of ^{131}I-mIBG in patients with relapsed or refractory neuroblastoma have identified myelosuppression as the main dose-limiting toxicity, necessitating stem cell reinfusion at higher doses. Most studies report a response rate of 30-40% with single agent ^{131}I-mIBG in this population. More recent studies focusing on the use of ^{131}I-mIBG in combination with chemotherapy or myeloablative regimens have been successful for refractory disease. Phase I and II studies in refractory patients of topotecan or irinotecan combined with mIBG have shown tolerability and activity. Phase I and II studies of mIBG combined with myeloablative carboplatin, etoposide and melphalan (CEM) have also shown promise, with a new clinical trial opening in the Children's Oncology Group to pilot the feasibility of this regimen in newly diagnosed high-risk neuroblastoma. Initial studies of no-carrier added mIBG suggest the possibility of a higher therapeutic ratio. We will review the recently completed, current and planned studies of mIBG for neuroblastoma in the USA, as well as some ongoing pre-clinical studies. New studies, using mIBG in combination with other molecularly targeted therapies or radiosensitizers may further optimize the role of this targeted radionuclide for high risk neuroblastoma.

C10

Late Outcomes after Treatment for Neuroblastoma

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Few data are available on the the longterm outcomes in children who have been treated for neuroblastoma. Reductions in therapy for low and intermediate risk neuroblastoma should be expected to be associated with fewer late effects. However, recent improvements in therapy for high-risk neuroblastoma along with more widespread use of stem cell transplantation suggest an need for significant research in delineating the medical, social and psychological outcomes in survivors of aggressive chemoradiotherapy. This presentation will review available published data from the Childhood Cancer Survivor Study and other large cohort studies, as well as from small clinical case series of survivors. Late mortality risk and the causes of late mortality will be discussed. The risk of musculoskeletal and growth problems will be reviewed, as well as the risks of hearing loss, endocrinopathy, secondary leukemias and solid tumors. At the Dana-Farber Cancer Institute/Children's Hospital, we reviewed the longterm outcomes in 95 patients treated for high risk neuroblastoma in the time period 1994-2007 to determine the prevalence of hypothyroidism, insulin resistance, short stature, hearing loss, ovarian failure, cardiomyopathy, school problems and dental agenesis. These unpublished data will be reported. The role of radiation compared with other therapies in the induction of late effects will be discussed.

C11

Clinical and biological features predictive of survival after relapse of neuroblastoma: A study from the International Neuroblastoma Risk Group (INRG) database

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Background: In NB, most patients (pts) who relapse eventually die. Prognostic factors are used to stratify treatment at diagnosis, but typically not at the time of relapse. Our goals were to determine a) which factors were predictive of time to death post-relapse; b) if time from diagnosis until relapse/progression has a predictive role.
Methods: Retrospective analysis included INRG pts with first event of relapse, progressive disease, or secondary malignancy (excluding pts whose first event was death). Time from diagnosis until event ("time-to-first-event") was calculated and analyzed as <1 year (yr) vs ≥1 yr. 5-yr estimates of overall survival (OS ± standard error), time from first event until death or last contact, are presented (lifetable methods). Time-to-first-event was tested in a multivariable Cox model (adjusting for nonproportional hazards) with clinical and biologic factors; hazard ratios (HR) for increased risk of death post-relapse were calculated.
Results: From 8,800 INRG pts, 2,266 experienced a non-death first event. Median time to relapse was 13.2 months (mo) (range: 1 day to 11.4 yrs). The 5-yr OS after first event was 20%±1%. Time-to-first-event (HR=1.8), age >18 mo (HR=2.3), INSS stage 4 (HR=3.4), *MYCN* amplified (HR=2.8), diploidy (HR=1.6), high MKI (HR=2.0), undifferentiated grade (HR=1.6), and 1p aberration (HR=1.7) were significantly predictive of death after relapse (p<0.0001), but not 11q aberration. Compared to pts whose first event occurred <6 mo from diagnosis, pts who relapsed 6-<18 mo from diagnosis had increased risk of death, while relapses ≥18 mo from diagnosis had *decreased* risk of death. Shorter time-to-first-event was not independently predictive of death after adjustment for undifferentiated grade, high MKI, *MYCN* amplification, or diploidy.
Conslusions: Time to first relapse is a significant predictor of time to death after relapse; however, the risk of death is higher for pts who relapse within 6-<18 mo, but lower for pts who relapse ≥18 mo from diagnosis. Stratification of relapsed NB pts according to the timing of first relapse, age, stage, *MYCN*, and MKI, and diploidy is important in retrieval study designs.

C12

Molecularly-targeted therapy in Neuroblastoma

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Improvements in survival rates for children's cancers have stagnated within the last decade, and have been achieved in large part through intensified dosing of standard chemotherapeutic agents. This has resulted in incremental increases in drug toxicity, secondary malignancy and long-term disability for paediatric patients. Clearly, improved therapeutics and approaches are required. In theory, molecularly-targeted therapeutics that selectively inhibit the activity of a single molecule can be synthesized. Ideally the targeted molecule should play an essential role in the genesis or maintenance of the cancer of interest, such that partial or complete inhibition is cytotoxic to tumour cells and results in tumour regression in the absence of any secondary, "off-target" effects. In practice, very few molecules with such ideal characteristics have been identified and successfully "drugged" in adult cancer to date, and arguably no molecule with a function restricted to a paediatric cancer has been rationally targeted with a selective drug. Nevertheless, a variety of compelling targets with favourable therapeutic profiles have emerged lately, through the application of enhanced sequencing and coordinated "omics" approaches. In Neuroblastoma, discrete molecular targets exist and are being identified which are of substantial therapeutic interest by virtue of their unique functions, pharmaceutical accessibility and significant association with clinical outcome. Amplification of the MYCN gene is among the first known oncogenic mutations associated with a paediatric cancer, and has been used to stratify therapy for many years. Expression of the Mycn oncoprotein is largely restricted to tumour tissue and can initiate spontaneous tumours in transgenic mice, making it an ideal therapeutic target. Nevertheless no drug exists to date targeting this critical oncoprotein. Although attempts to inhibit the dimerisation function of this transcription factor have failed, recent publications point to indirect approaches that could stimulate destabilisation of Mycn oncoprotein, and would be predicted to impact progression of "MYC-addicted" tumours. Several potential therapeutics are in development that could address this mechanism. Somatic tumour and germline point mutations in the anaplastic lymphoma kinase (ALK) gene have been identified in a significant proportion of Neuroblastoma patients, implying a critical role for ALK in the initiation of this disease. Efforts are underway to establish the oncogenic role of ALK through murine transgenic modeling. Initial clinical evaluation of an ALK-targeted agent is in progress. Further efforts will likely be required to develop agents more specifically targeted to individual, etiologic ALK mutations as these are unravelled. Finally, aberrant expression of critical molecular signalling pathways occurs in discrete clinical subgroups of Neuroblastoma, (i.e.: WNT pathway in high-risk patients without MYCN gene amplification) providing additional opportunities for development of pathway-targeted agents. Taken together, these findings suggest additional approaches by which we could incorporate molecularly-targeted therapeutics into existing treatment strategies for discrete subgroups of high-risk neuroblastoma patients.

Workshops
WS1–WS27

WS1

Tumor initiating/stem cells, hypoxia and vascularization - what are the connections?

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Hypoxia and hypoxia inducible factor (HIF)-induced expression of VEGF and other proangiogenic factors are major driving forces behind phenotypic tumor heterogeneity and tumor angiogenesis. As we demonstrated previously, hypoxia dedifferentiates cultured neuroblastoma (NB) and breast cancer cells towards neural crest- and stem cell-like phenotypes, respectively. Furthermore, low stages of tumor cell differentiation as well as high HIF-2a protein levels are associated with advanced disease in both NB and breast cancer. We recently identified HIF-2α as a marker of immature, neural crest-like NB cells located in perivascular niches in tumor specimens and of neural crest-like NB tumor-initiating/stem cells (TICs) isolated from bone marrow of patients with aggressive NB. We have further shown that TICs have high HIF-2a levels and express *VEGF* at normoxic (21%) oxygen levels. Knockdown of HIF-2α in the TICs, which have few or no large-scale genomic aberrations by SNP-array analysis, induced sympathetic neuronal differentiation *in vitro* and to a greater extent *in vivo* in xenograft tumors. These NB TICs have stem cell characteristics, as epigenetic modification together with relevant growth factor combinations could drive these cells towards ganglionic, glial/Schwann and melanocytic lineages. Presumably due to the reduced *VEGF* expression, tumors of HIF-2α-silenced cells were poorly vascularized, widely necrotic and resembled the bulk of tumor cells in clinical NBs by expressing sympathetic neuronal markers including tyrosine hydroxylase, while control tumors were immature, well-vascularized and stroma-rich. We conclude that HIF-2α is required to maintain the undifferentiated phenotype of NB TICs and that NB TICs might initiate and support tumor vascularization. As similar data for human primary glioma stem cells (high HIF-2a levels, VEGF expression, perivascular localization, immature phenotype) were recently reported, important links between pseudo-hypoxic states, tumor aggressiveness, stem cell-like phenotypes and tumor vascularization appear to be common denominators of neurally derived tumors and possibly also of other malignancies.

WS2

Development of the autonomic nervous system: a molecular view

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The development of sympathetic neurons from neural crest progenitors is elicited by Bone Morphogentic Proteins (BMPs), which are secreted from endothelial and smooth muscle cells of the dorsal aorta. BMPs induce a group of transcription factors, i.e Ascl1, Phox2b, Phox2a, Gata3, Hand2 and Insm1 that, in turn, control the expression of terminal differentiation genes, e.g. TH, DBH. More recently it became apparent that these transcription factors, in addition to their essential functions in sympathetic neuron differentiation, are also involved in the control of neurogenesis. Elimination of Hand2, Insm1, Ascl1 and Gata3 results in decreased sympathetic neuron generation, whereas Phox2b displays an antiproliferative effect. The characterization of the molecular links to cell cycle control of progenitors and immature sympathetic neurons is essential for our understanding of neurogenesis in sympathetic ganglia and may provide insights into mechanisms of neuroblastoma tumor initiation.

WS3

Tumor initiating cells from bone marrow of high-stage neuroblastoma patients

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No abstract available

WS4

TICs from MYCN amplified neuroblastomas

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We have isolated Tumor Initiating Cells from several MYCN amplified neuroblastoma tumors and bone marrow samples with a success rate of about 30%. The isolation methods were described before and consisted of filter isolation and maintenance in neural stem cell medium containing bFGF and EGF. For all TIC lines we have corresponding tumor samples and normal DNA available. Using array CGH we showed that TIC lines were related to the primary tumor samples. Some TIC lines seemed to be grossly identical to the primary tumors while other TIC lines and corresponding primary tumors did show genomic variability. Affymetrix mRNA expression analysis showed interesting characteristics of neural stem cells in TIC lines that were maintained in neural stem cell medium for a prolonged period. A strong increase in PROM1 positivity was seen. We were able to subcutaneously xenograft all TICs in NMRI nu/nu mice. Growth characteristics of the xenografts were initially very diverse but upon maintained passaging from mouse to mouse we could create lines that showed less variation in growth in vivo. These new TIC models are of value for further neuroblastoma research.

WS5

The miRNAome of TICs in relation to tumor cells and fetal neuroblasts

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Recent studies have demonstrated the important role of miRNAs in normal development, stem cells and cancer. Therefore, we decided to investigate the possible involvement of miRNA deregulation in neuroblastoma. To achieve this goal we have profiled a total of 450 miRNAs using a robust real-time quantitative PCR platform (Mestdagh et al., 2008). In total we analyzed 100 tumors as well as 10 tumor initiating cell lines (TICs), 7 embryonal stem cell lines, one neuronal progenitor cell line and 7 fetal neuroblast samples. Analysis of a stem cell activity score (deduced from published stem cell miRNA studies) of the profiled samples showed a markedly high activity score for the embyronal stem cell lines and the TIC cell lines, in keeping with their presumed stem cell phenotype. Moreover, differential expression analysis of the TICs versus the tumor samples and the normal neuroblast samples highlighted a number of potentially important miRNAs contributing to the tumor (stem cell) phenotype which are currently under study. In conclusion, this study uncovered the deregulated miRNA landscape of neuroblastoma tumor cells and comparison with various normal cell types including embryonal stem cells and fetal neuroblasts has allowed to pinpoint several strong candidate miRNAs for further study.

WS6

Identification and molecular characterization of human neuroblastoma tumor-initiating cells

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Background: Neuroblastoma (NB) displays a cellular heterogeneity within the tumor. There is increasing evidence that at the top of this observed tumor cell hierarchy, there is a sub-population of tumor-initiating cells (TICs), responsible for initiation and maintenance of the tumor. Candidates TICs have been isolated in a variety of adult solid tumors, representing a powerful potential therapeutic target. However this population has not yet been identified nor characterized in NB.
NB is the most common extracranial childhood solid tumour originates from neural crest-derived malignant sympathoadrenal cells. We have identified cells within primary NB tissues and cell lines that express markers of neural crest stem cells and their derivatives, leading us to postulate the existence of TICs in NB tumour that recapitulate the properties of sympathetic precursor cells.
Method: We proposed a novel approach to identify and characterize NB TICs by prospectively identifying their self-renewal properties. From a very aggressive stage 4 NB sample, we selected self-renewing putative TICs by their sphere-forming capacity and analyzed their gene expression profiles by time-course micro-array analysis.
Results: Supervised and unsupervised analyses provided a list of sphere markers genes involved in embryogenesis and nervous system development (*CD133, EDNRA/B, NOTCH1/3, GPR177…*), and drug resistance (*MDR1, ABCA1*). To determine whether the sup-populations selected in spheres correspond to TICs, their tumorigenic potential was assayed by *in vivo* tumor growth analyses using subcutaneous and orthotopic (adrenal glands) implantations of tumor cells into nude mice. Tumors derived from the sphere cells were significantly more frequent and were detected earlier compared to whole tumor cells. In addition, a more detailed study of the potential NB-TICs revealed a phenotypic heterogeneity in the sphere sub-populations based on the exclusive expression of CD133 and MDR1.
Conclusion: In our study, we identified new NB-TICS specific markers and we characterized heterogeneous sphere sub-populations that will be individually analyzed by functional assays.

WS7

Vascular mimicry in human neuroblastoma: identification of the progenitors of tumor-derived endothelial cells

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Vascular mimicry is a phenomenon whereby cancer cells are incorporated in vascular structures where they may acquire features typical of "professional" endothelial cells (EC). We previously demonstrated for the first time that primary NB-associated endothelial microvessels (EM) may be lined by tumor-derived endothelial cells (TEC) and, accordingly, TEC lined EM were identified in tumors formed in immunodeficient mice by human *MYCN* amplified and *MYCN* non-amplified neuroblastoma (NB) cell lines. In this study we have investigated whether the embryonal stem cell marker Oct-4 could identify NB cells acting as TEC progenitors. Both primary and metastatic NB cells as well as NB cell lines were found to express Oct-4. Tenascin-C (TNC), a huge protein of the extracellular matrix expressed by most tumor cells and involved in tumor growth, metastasis and angiogenesis was consistently expressed on NB cell surface and found to mark exclusively Oct-4$^+$ NB cells. The availability of a surface marker allowing positive selection of Oct-4$^+$ NB cells made it possible to perform functional studies *in vitro* and *in vivo* proving that Oct-4$^+$, TNC$^+$ are TEC progenitors. These results will be discussed in the frame of the potential role of TEC in chemoresistance and tumor relapse.

WS8

Identification *in vitro* and *in vivo* of tumoral glial precursor cells in neuroblastic tumors

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Background: Neuroblastic tumors (NBT) are derived from multipotential neural crest stem cells, and composed by a neuroblastic component and Schwannian-like (glial) stroma. A correlation has been established between the degree of differentiation of the neuroblastic subtype, the amount of glia and patient outcome. However, the physiological relationship between Schwannian-like stromal cells and neuroblastic cells has not been clarified. We reported lineage specificity of membrane GD2 for the neuroblastic component and the nuclear membrane S100A6 for glial cells (Acosta S, 2009). The aim of the present study was to explore *in vivo* the existence of cells that express simultaneously neuronal (GD2) and glial (S100A6) cell lineage marker proteins, possible bipotential cells, that could embody a potential NBT precursor or initiating cell.
Methods: Double immunofluorescence (IF) for GD2 and S100A6 was performed in 29 primary NBT and 8 metastasic bone marrow specimens from patients diagnosed and uniformly treated at Hospital Sant Joan de Déu, Barcelona. The proportion of GD2$^+$/S100A6$^+$ neuroblasts was assigned a score from 0 to 3: 0 = <1%; 1 = 1-10%; 2 = 10-25%; 3 = >25%. The potential existence of S100A6 positive endothelial cells localized in the GD2$^+$/S100A6$^+$ stromal bundles was further investigated by performing double immunostaining with S100A6 and CD34 antibodies. FACS analysis was performed in 7 enzymatically disaggregated tumor samples. To further characterise the GD2/S100A6 subpopulation as a potential cancer stem cell fraction, CD133 staining was performed in 4 samples in parallel with GD2/S100A6 staining. MYCN amplification was used as a molecular marker amongst the sorted cell subpopulation isolated from a MYCN amplified NB tumor. The I-type cell line SK-N-Be2C differentiated with 1μM ATRA was used to model GD2$^+$/S100A6$^+$ cells in vitro.
Results: In all the neuroblastoma tumor samples analyzed (n=23), intense cell membrane GD2 expression was observed in all the cells morphologically identified as neuroblastic. Conversely, no specific staining was detected in primary ganglioneuroma or ganglioneuro- blastoma tumors. S100A6 expression was detected in the nuclear membrane of all the cells which make up the Schwannian stroma and also in the cells morphologically constituting the blood vessels. Moreover, S100A6 nuclear expression was observed in isolated, sparse undifferentiated neuroblasts. On the basis of these results, GD2 and S100A6 double immunostaining was performed in 14 primary non-treated diagnostic neuroblastoma samples and 9 post-treatment specimens. Concomitant expression of both markers was observed in a subpopulation of neuroblasts (< 25% of the total) in 12 (85%) of the 14 samples obtained at diagnosis. In those tumors, the majority of the GD2$^+$/S100A6$^+$ neuroblasts within the tumor were found isolated and surrounded by GD2$^+$/S100A6$^-$ neuroblasts. Six (66%) of the 9 post-treatment neuroblastoma samples analyzed contained undifferentiated/poorly differentiated neuroblasts. In all of these samples, a variable percentage of neuroblastic cells displayed GD2$^+$/S100A6$^+$. The presence of co-staining cells was, in average, higher than in diagnostic samples and distributed in non-necrotic areas where blood vessels were found. Surprisingly, GD2$^+$/S100A6$^+$ double stained cells morphologically Schwannian-like, were also identified in primary tumors at diagnosis in the stromal bundles and in some of the blood vessels. A significant proportion of GD2$^+$/S100A6$^+$ neuroblasts were distributed around the tumoral blood vessels, suggesting that GD2$^+$/S100A6$^+$ neuroblasts are not distributed randomly, but either arise from or tend to populate near the stromal regions. All bone marrow specimens showed GD2$^+$/S100A6$^+$ representing less than 10% of the total. By FACS analysis, the percentage of GD2$^+$ cells ranged from 15-89% of the total nucleated cells, and thus potentially are all tumor cells. On the other hand, 3-53% of these cells were S100A6 positive. Double stained cells represented percentages ranging from 11-53% of cells, distributed both in a large and small subpopulations. A small percentage of CD133$^+$ cells were identified, 0.1-0.7% of the total viable population. The proportion of CD133$^+$ cells did not correspond to that of GD2$^+$/S100A6$^+$. For one MYCN amplified tumor, all FACS-sorted GD2/S100A6 differentially stained subpopulations showed MYCN amplification. During *in vitro* neuronal induced differentiation of I-type NB cells, rare GD2$^+$ neuroblastic cells with concomitant S100A6$^+$ staining appeared. Subsequently, these GD2$^+$/S100A6$^+$ cells changed morphology, displaying flat and enlarged cytoplasms, distinctive features of the S-like type cell phenotype.
Conclusions: Our results show, in primary neuroblastoma tumor samples, the presence of a morphologically undistinguishable tumoral subpopulation of neuroblasts that has features of both neuronal and glial lineage. The clinical correlations, however, suggested that these co-expressing cells were most likely cells in the process of differentiation towards glial lineage. *In vitro*, we were able to recapitulate the differentiation stages which give rise to these bipotential cells, and demonstrate how some neuroblastic tumor cells give rise to other cellular components of the tumor.

WS9

Exploiting the embryonic environment to reprogram cancer stem cells in neuroblastoma

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Neuroblastoma (NB) arises from the embryonic neural crest (NC). Within the embryo, NC cells migrate along clearly defined pathways and respond to a precise sequence of spatial and temporal developmental factors. The cells' developmental potential is progressively restricted, until they eventually form peripheral structures including the sympathetic ganglia (SG) and adrenal glands. NB tumours form as a result of genetic mutation and/or changes in the timing, sequence or location of the epigenetic factors responsible for the correct programming of the NC cells. Propagation of such tumours may be attributed to a rare population of tumour initiating cells (TICs), which due to their relatively undifferentiated stem cell-like state, may be sensitive to the factors that program NC cells. Coupled with the knowledge that NB tumours can spontaneously regress, this has led us to propose that TICs may be reprogrammed following transplantation into the NC and differentiate into a benign phenotype. In contrast, we expect cells to proliferate when located in parts of the embryo lacking these signals.
The aim is to compare the fate of GFP-labelled Kelly cells transplanted into the chick embryo NC immediately prior to migration, with cells injected intravenously at embryonic day 3 and 6 (E3 and E6). Preliminary data suggests that cells transplanted to the NC migrate with NC cells as expected, while the location of cells injected at E3 and E6 and analysed at E10 varies. At E3 they preferentially localise to the SG, tail and gut; all of which contain cells derived from the NC. Following E6 injection, when the embryo is further developed and many early developmental signals have been turned off, cells were more widespread throughout the chick, although very few were found in the SG. Preliminary data using markers to investigate the fate of cells in the different locations will be discussed.

WS10

Low dose metronomic anti-angiogenic (LDM) oral topotecan and pazopanib as a potential model for maintenance therapy neuroblastoma

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Background: Angiogenesis plays a critical role in neuroblastoma (NB) growth and metastasis. Low dose metronomic (LDM) chemotherapy, combining with VEGF pathway inhibitors is an emerging treatment strategy having increased efficacy with reduced toxicity. This strategy can potentially target tumor hypoxic zone which are more susceptible to develop drug resistance.
Objectives: Pharmacokinetic/ pharmacodynamic markers and efficacy of LDM topotecan (TP) with/without pazopanib (PZ) an oral antiangiogenic tyrosine kinase inhibitor (TKI) was evaluated in xenograft and metastatic and TICs NB mouse model.
Methods: In vitro IC50 was established using SK-N-BE(2) and SH-SY5Y cell lines. NOD-SCID mice model was used for both subcutaneous primary tumour and metastatic experiments . Mice were randomized into four groups: control group, LDM TP (1.0mg/Kg) ,PZ(30mg/kg and 150mg/Kg) and the combination (1.0mg/Kg TP +150mg/Kg PZ). For the localized tumor model, the animals were treated daily till 56 days; while for metastatic model, animals were treated until death.. Micro vessel density, Angiogenic makrers, circulating Endothelial cells (CECs) and circulating Endothelial Progenitor cells (CEPs) were determined by flow cytometry. Pharmacokinetic studies were conducted to determine the plasma concentration-time profiles of both the drugs.
Results: IC 50 of topotecan on SK-N-BE(2) and SH-SY5Y cells was 125.9ng/ml and 4.0ng/ml respectively. Pazopanib did not induce cytotoxicity in NB cell up to 10µg/ml.
In SK-N-BE(2) xenograft model, a statistically significant efficacy (tumor size till the end of treatment) was observed for single agent (TP or PZ) and combination. combination (p=0.0002) > LDM TP (p=0.0008)> than PZ >than control< In the SK-N-BE(2) metastatic model, all the three treatment regimens significantly prolonged animal overall survival compared to control group. PZ TP and PZ+TP., LDM PZ+ TP (P=0.001) was superior than single agent PZ or TP . No toxicity was observed in any of the groups. This was correlated with CECs CEPs and microvessel density The Cmax of PZ in single agent and combination group was 130.5µg/ml and 125.6µg/ml, respectively. PZ plasma concentration was maintained above the optimal concentration for up to 18 h.. The combination of LDM TP and PZ reduced the levels of viable CEP (P = 0.125) and CEC (P = 0.005) after 7 days treatment.
Conclusion: Daily LDM TP and PZ and combination are effective and safe regimens in both localized and metastatic neuroblastoma mouse models. The reduction in microvessel density and CEC/CEP levels supports the anti-angiogenic activity of these drugs.Correlation between HilF1 alpha and response as well as TICs mouse model related studies are ongoing

WS11

The neuroblastoma miRNA map, prioritization and functional evaluation of candidate miRNAs

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MicroRNAs are tiny regulators of coding gene expression. While a growing body of evidence implicates deregulated microRNA expression in human cancers, insights in global microRNA function remain limited. For neuroblastoma, a number of candidate miRNAs, including miR-34a, miR-17-92, miR-184 and miR-9, have been established and, most likely, many others await identification. To streamline the selection of candidate miRNAs, we present the microRNA body map, an interactive compendium and mining tool of high-dimensional microRNA expression profiles and functional annotation inferred through integrative transcriptomics. This combination of miRNA expression and function greatly enhances miRNA prioritization and might prove to be a valuable tool in completing the neuroblastoma miRNA map. Following prioritization of candidate miRNAs, functional evaluation is crucial to understand their role in neuroblastoma biology. The miR-17-92 miRNAs are among the top candidates for neuroblastoma, however, insights in miR-17-92 function are limited. To fully elucidate miR-17-92 function in neuroblastoma, we measured protein-response upon miR-17-92 activation using quantitative mass spectrometry and found miR-17-92 miRNAs to be implicated in multiple hallmarks of the tumourigenic program. Most importantly, miR-17-92 was identified as a potent inhibitor of TGFβsignaling. By functioning both upstream and downstream of pSMAD2, miR-17-92 activation triggers a targeted clampdown of TGFâ-signaling by downregulation of multiple key effectors along the signaling cascade as well as through direct inhibition of TGFβresponsive genes. Of interest, several of the empirically identified miR-17-92 functions could also be inferred through the miRNA body map, demonstrating its great potential in miRNA prioritization.

WS12

MicroRNAs regulating neuroblastoma cell differentiation

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MicroRNAs are non-coding RNAs which function as complex negative regulators of post-transcriptional gene expression during normal development. Their dysregulation contributes to the pathogenesis of many cancers, including neuroblastoma. Here, we identify miRNAs that contribute in a major way to the process of all-trans-retinoic acid (ATRA) induced in vitro differentiation of neuroblastoma SK-N-BE cells. We demonstrate that ectopic over-expression of two of the most significantly up-regulated miRNAs in response to ATRA, miR-10a and miR-10b, results in neurite outgrowth, over-expression of neuronal differentiation markers GAP43 and TUBB3, and a decrease in both MYCN expression and cell growth, which are characteristic of ATRA induced differentiation. The reduction in MYCN is through a secondary effect, as neither miRNA is predicted to target the 3' UTR of the MYCN mRNA. Ectopic up-regulation of miR10a/b also directly decreases the abundance of a large set of mRNAs through targeting their 3' UTRs, as evidenced by statistically significant enrichment for miR-10a/b seed sites among down-regulated genes identified through expression profiling with microarrays. Gene ontology analysis of the down-regulated, putative direct targets of miR-10a/b indicate significant enrichment for genes involved with transcriptional regulation, explaining why miR-10a/b ectopic up-regulation also results in a major cascade of secondary transcriptional alterations, such as MYCN down-regulation. We further experimentally demonstrate that miR-10a/b recapitulates many elements of an ATRA induced differentiated phenotype through direct targeting of the nuclear receptor co-repressor 2 (NCOR2), a gene known to inhibit neurite out-growth. siRNA mediated knockdown of NCOR2 by itself completely recapitulates the effects of miR-10a/10b over-expression. Although the biological effects of miR-10a and miR-10b are indistinguishable using in vitro models, they have remarkably different patterns of expression in primary neuroblastoma tumors of differing genetic subtypes, with under-expression of miR-10a having a greater negative impact on patient survival in the 11q- disease subtype. We conclude that miR-10a/b contributes to the process of in vitro neuroblastoma cell differentiation, producing a differentiated phenotype that is remarkably similar to that induced by ATRA, through direct targeting of NCOR2. The anti-proliferative effects of these miRNAs indicate that they could be of potential benefit for miRNA mediated therapeutics if targeted delivery is achievable.

WS13

MYCN-regulated microRNAs repress estrogen receptor-α (ESR1) expression and neuronal differentiation in human neuroblastoma

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MYCN, a proto-oncogene normally expressed in the migrating neural crest, is in its amplified state a key factor in the genesis of human neuroblastoma (NB). However, the mechanisms underlying *MYCN*-mediated NB progression are poorly understood. The impact of deregulated miRNA expression in NB has just begun to emerge reinforcing the importance of miRNA biology in NB-associated tumorigenesis. We have described a MYCN-mediated miRNA signature involving the activation and down-regulation of several miRNA genes from paralogous clusters. In line with previous reports, we showed that MYCN transcriptionally activates oncogenic miRNAs from the miR-17~92 cluster and its paralogs miR-106a~363 and miR- 106b~25. Expression analysis in NB tumors confirmed increased levels of these microRNAs in *MYCN*-amplified samples. In particular, we demonstrated that miR-18a and miR-19a from the miR-17¡92 cluster target and repress the expression of estrogen receptor-alpha (ESR1), a ligand-inducible transcription factor implicated in neuronal differentiation. Importantly, we demonstrated expression of ESR1 in human fetal sympathetic ganglia, suggesting a role for ESR1 during sympathetic nervous system development. Restoration of ESR1 in NB cells led to marked growth arrest and neuronal differentiation. Moreover, lenti-viral inhibition of miR-18a in *MYCN* amplified NB cells resulted in severe growth retardation followed by robust morphological and biochemical differentiation. ESR1 represents a previously undescribed MYCN target in NB and our data demonstrate a novel oncogenic circuitry in which the repression of ESR1 through MYCN-regulated miRNAs may play a fundamental role in NB tumorigenesis. We propose that *MYCN* amplification may disrupt estrogen-signaling sensitivity in primitive sympathetic cells through deregulation of ESR1, thereby preventing the normal induction of neuroblast differentiation. We are currently investigating putative interacting growth factors and downstream targets involved in the regulation of differentiation by ESR1 in NB cells.

WS14

Assessing the role of miRNAs in neuroblastoma biology – from expression profiling to functional analysis

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MiRNAs (miRNAs) constitute a family of small RNA species that regulate translation and stability of mRNA. Soon after their discovery, miRNAs were found to act as tumor suppressor genes by blocking the translation of oncogenes and act as oncogenes by inhibiting the translation of tumor suppressor genes. Most interestingly, miRNAs can be therapeutically targeted using Agomirs to restore or Antagomirs to inhibit miRNA function in vivo. We have extensively analyzed miRNA expression in neuroblastoma using real-time PCR, microarray and next generation sequencing approaches. MiRNA signatures predictive of clinical course have been identified, as well as miRNAs of functional importance in neuroblastoma tumor biology. These miRNAs include the MYCN-regulated miRNA Cluster 17-92, the MYCN-regulated miR-9 and miR-221, as well as the tumor suppressive miR-34a/b/c, miR-542 and miR-628. The main future challenges include exploring miRNA signatures as prognostic factors in a clinical setting and comparing these signatures to mRNA predictors. Furthermore, comprehensive identification of mRNA targets of microRNAs is an essential step towards miRNA targeted therapies. Strategies for target identification include bioinfomatic analysis, proteomics and RNA-Immunoprecpitation. Finally, the physiological role of miRNAs as well as their role in tumor biology has to be explored in vivo using xenograft and transgenic / knock-out mouse models.

WS15

Bioinformatic tools to integrate and analyse high throughput neuroblastoma data

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High throughput mRNA profiling is an efficient tool to identify expression levels for any gene in series of tumors and cell lines. Many tools have been developed for analysis of such data. However, most of them require specialist bioinformatic support or are time consuming or are not integrated in one user-friendly platform. We developed a tool for basic and advanced analysis of such data, called R2. This web-based tool enables swift and user-friendly analyses by any interested researcher. We have generated Affymetrix expression profiles of a series of 88 neuroblastoma tumors, as well as from a limited number of ganglioneuromas and ganglioneuroblastomas. The series is annotated for clinical and molecular parameters. R2 enables to analyse expression values for any gene, establish its prognostic value and find correlating expression patterns with other genes. It also permits global expression analysis and prognostic ordering for gene sets, which can be formed by KEGG pathways, functional groups, etc. Differentially expressed genes can efficiently be identified within annotated parameters, like age, stage, histology etc. Any resulting gene list can be analysed for pathway or gene ontology enrichment, or be visualized in heat maps. Finally, the R2 tool and database includes Affymetrix expression profiles of over 20,000 normal and tumor samples from other tissues, grouped per tumor or tissue type. This enables quick searches for expression profiles of specific genes over a wide range of tissues. The web-based R2 analysis tool and database will be made publicly available at the ANR.

WS16

Genomic and proteomic study of microRNAs in pediatric cancers

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MicroRNAs (miRNAs) are an important class of gene expression regulators that play a critical role in cancer biology. To understand the importance of microRNAs in the tumorigenesis and tumor progression, we took a genomic approach to study the global expression of microRNAs in pediatric tumors by performing parallel microRNAs and mRNA profiling on 57 tumor xenografts and cell lines representing 10 different pediatric solid tumors using microarrays. We found that pediatric cancers express cancer-specific microRNAs. Of the fourteen microRNAs differentially expressed between rhabdomyosarcoma and neuroblastoma, 8 of them were validated in independent patient tumor samples. Exploration of the expression of microRNAs in relationship with their host genes showed that the expression for 43 of 68 (63%) microRNAs located inside known coding genes was significantly correlated with that of their host genes. Among these 43 microRNAs, 5 of 7 microRNAs in the OncomiR-1 cluster correlated significantly with their host gene MIRHG1. In addition, the expression level of MIRHG1 could predict the outcome of neuroblastoma patients independently from the current neuroblastoma risk-stratification in two independent patient cohorts, indicating important oncogenic functions of this microRNA cluster in neuroblastoma biology. With the advent of the massive parallel sequencing technologies which miRNA molecules can be directly sequenced and counted, we applied the next-generation sequencing technologies to characterize miRNA expression in high-risk neuroblastoma. We initially sequenced a small-RNA pool of four stage 4, MYCN-amplified neuroblastoma tumors. This sequencing run yielded 4.1 million high-quality reads. Sequence analysis revealed the expression levels of 525 known miRNAs (mature and minor miR*) and 6 novel miRNA genes. Two miRNAs, let-7f and let-7a, had extremely high expression levels, accounting for ~50% of the annotated miRNA reads in the samples. These miRNAs have been implicated in other cancers with important roles in carcinogenesis. The high expression of let-7f and let-7a was confirmed by quantitative RT-PCR. We also validated six novel miRNAs. In parallel to the expression studies, we attempted to understand the function of an important microRNA, miR-34a, located on 1p36 which is commonly lost of heterozygosity in neuroblastoma and other tumors. Transactivation of miR-34a and its other family members (miR-34b and c) by p53 has been shown to be critical to p53 function. In addition, we and others have reported that miR-34a could directly target important oncogenes such as MYCN and E2F3. Furthermore, miR-34a caused significant suppression of cell growth through increased apoptosis and growth arrest in tumor cells. Therefore, miR-34a is a bona fide tumor suppressor. In order to identify the downstream targets of miR-34a globally, we utilized a proteomic approach, namely Isotope Coded Affinity Tags (ICAT), to detect protein changes in neuroblastoma cells transfected with miR-34a. Consistent with the working mechanism of microRNAs, miR-34a had a little effect on the global transcript levels measured by microarray; whereas a much bigger overall effect on the protein expression levels measure by ICAT. Among the affected proteins, 192 were down regulated and 143 were up regulated (≥2 folds). Gene ontology analysis showed that proteins involving in cell cycle, transcription and translation were significantly enriched among the down regulated genes; whereas proteins involving in apoptosis and development were over-represented in the up regulated gene list. Several important pathways such as caspase 3, NF-κB, and YY1 were clearly altered by miR-34a in a network analysis, indicating that these are the potential key downstream pathways that miR-34a regulates. Therefore, ICAT study of global protein changes provides an insight of the biology and therapeutic potential of miR-34a-based therapies.

WS17

Next generation sequencing technologies to investigate the cancer genome & insights into neuroblastoma biology and tumor progression models learnt from massively parallel sequencing strategies

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United States

For the first half of my talk I will discuss the next generation sequencing (NGS) technologies for investigating the cancer genome. NGS technology directly identifies billions of nucleic acid species in parallel in a single experiment. Different from the Sanger method of traditional sequencing, the massively parallel DNA sequencing technology not only generates sequence information for each nucleic acid strand, but also determines the abundance of each nucleic acid species due its large capacity, resulting in a digital readout of levels for any sequence, even those at low levels beyond the detection sensitivity of hybridization-based technologies. I will discuss this technology and its wide-ranging applications for both DNA and RNA studies. For DNA it is possible to sequence an entire cancer genome in one month, a staggeringly short time considering that it took 13 years to sequence a handful of human genomes by the Human Genome Project. There are also hybridization based methods for pulling down the DNA from protein coding exons or a defined genomic region (termed 'genome-partitioning') for targeted resequencing. With these methods it is possible to detect every single nucleotide variant, mutation, genomic rearrangement, profile the whole "methylome", and determine copy number alteration at the base pair level of a given genome. For RNA studies it is possible to determine the gene expression level of every gene, identify every splice variants, novel transcripts, single nucleotide variants and mutations for the expressed genome. It will also identify novel gene rearrangements that result in chimeric fusion gene products. Finally I will discuss how next and next-next generation sequencing has and will revolutionize the field of cancer genomics. Neuroblastoma is an extremely heterogeneous disease in which the outcome can range from spontaneous regression of the tumor to relentless progression leading to the death of the patients. In the second half of the talk I will discuss the applications of next generation sequencing of neuroblastoma transcriptomes and genomes and its use for deciphering the biology of this enigmatic malignancy, identifying novel transcripts, splice variants, and single nucleotide variants and targets for therapy. I will also discuss its application for determining tumor progression models in this disease.

WS18

Next generation sequencing of the (small) RNA transcriptome - from catalogisation to quantitative expression profiling

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With next generation sequencing (NGS), the unbiased global analysis of transcript structure and abundance is now feasible. This allows an unprecedented view of the transcriptome, uncovering allele specific expression, posttranscriptional RNA modifications including RNA editing, splicing and terminal additions / deletions, as well as single nucleotide variations (SNV), which include SNPs and somatic mutations. To achieve optimal transcriptome coverage, the whole RNA is fractionated to separately sequence small, intermediate and large RNAs. In addition, the latter ones require diverse sequencing studies, including short reads, long reads and paired end sequencing reads, to uncover complex splice variants and potential fusion and read-through transcripts. Technical challenges include (i) mapping of the often ambiguous reads, (ii) detection and quantification of posttranscriptional editing events as well as splicing patterns, (iii) normalization of quantitative expression data, (iv) integration with genomic and epigenomic information and finally (v) presentation and interpretation of the respective data. We will discuss results of our most recent small RNA NGS study, as well as a general perspective for transcriptome sequencing in neuroblastoma.

WS19

Next-generation sequencing to characterize somatic alterations in neuroblastoma samples

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Background: The genetic alterations of neuroblastoma (NB) cell lines and tumors have been, up to now, characterized using conventional strategies including cytogenetic and molecular methods, providing a picture of genomic rearrangements at a quite low resolution. Next-Generation Sequencing technologies now offer the possibility to identify all somatic mutations of all classes in individual cancer genomes.
Methods: We use several strategies in order to characterize genomic alterations in NB samples including paired-end sequencing of mate-paired libraries, RNA-Sequencing of a normalized random primed cDNA library and whole genome sequencing (30x coverage).
Results: For two cell lines, almost 60 millions of pairs were obtained from the mate-paired librairies and aligned against the reference genome. Various criteria were applied in order to identify aberrant links with the highest relevance, including both inter and intra-chromosomal rearrangements. The majority of the unbalanced translocations previously detected by spectral karyotyping and/or array-CGH were detected amongst the inter-chromosomal rearrangements. A high number of intra-chromosomal rearrangements was also identified in both cell lines. For one of these two samples, RNA-Sequencing generated ~ 500 000 reads of around 400 pb. Expression levels obtained by RNA-Sequencing were compared to those measured using Affymetrix arrays. Analysis is ongoing to search for chimaeric transcripts and mutations. Finally, we recently started whole genome sequencing with a high depth in order to detect all types of mutations in a NB patient.
Conclusions: Genome-wide massively parallel sequencing provides a more exhaustive and precise characterization of rearrangements in tumor cells as compared to conventional strategies. It highlights the diversity of somatic rearrangements and allows to characterize structural variants to the base-pair level. It therefore represents a powerful approach to get insights into the mechanims that underlie NB oncogenesis.

WS20

The Neuroblastoma-TARGET project: Plans for sequencing, validation and frequency scans

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The neuroblastoma TARGET (Therapeutically Applicable Research to Generate Effective Treatments) collaborative research initiative aims to discover novel therapeutic targets and genomic predictors of outcome in an unbiased systematic fashion. In year one of the project, we defined high resolution DNA copy number alterations and loss of heterozygosity using Illumina SNP arrays, and genome-scale mRNA expression signatures using Affymetrix HuEx arrays. In the second phase of the project, we are performing a comprehensive resequencing of the high-risk neuroblastoma genome. Two hundred samples will undergo solution phase exon capture followed by paired end resequencing (N=100 Illumina; N=100 AB Solid), and an additional 10 samples will undergo full genome resequencing using Illumina paired-end read technology. All samples will have paired germline DNA sequencing in parallel. Finally, at least 110 of the samples will also have tumor RNA sequencing performed to detect expressed somatic variations. The first 100 exomes and 10 full genomes should be complete by July 2010, with the next 100 exomes to follow closely. RNAseq work is ongoing. Validation and frequency scans in up to 1500 cases (all risk groups) are being planned, as will integration with our genome wide association study to determine if any of the discovered germline variations may be predisposition alleles. Taken together, this project should define the mutational landscape of neuroblastoma and provide a rich resource for the international community of neuroblastoma investigators.

WS21

Finding variant needles in the neuroblastoma haystack: the Ghent approach

Vandesompele, Jo
behalf of all co-workers at CMGG, NXTGNT, NRC, Center for Medical Genetics, Ghent, Belgium

Massively parallel sequencing has heralded a new era in genomics in which sky is expected to be the limit. While throughput and cost per base almost exponentially grow and decrease, respectively, the data deluge and haze pose serious challenges. Especially in cancer genome resequencing projects, interpreting the sequence variants in order to separate the wheat from the chaff is a formidable task. Instead of performing whole genome resequencing or more focused exome sequencing, we chose to pursue alternative and more cost-effective approaches to find cancer genes that play a role in neuroblastoma.
Preliminary results will be presented for a PCR based targeted resequencing study on a cohort of 125 matched tumor/normal samples in which all human microRNA loci and a substantial set of prioritized coding genes will be screened. Innovative features of the study are the gene prioritization strategy based on fitSNP scores and validated on resequencing data from other cancer types; the 3D sample pooling design and the low DNA input requirements through whole genome sample pre-amplification. In a second study, we evaluate RNA-seq on normalized cDNA libraries from neuroblastoma cell lines with at least one known mutation as a more powerful and focused alternative to DNA based exome sequencing to find sequence variants and structural aberrations such as fusion genes and splice isoforms. In a third and final study, we apply ChIP-seq on neuroblastoma cell lines to create a transcription factor genome binding map and to explore the possibility of finding sequence variants in the enriched genome loci.

WS22

Perspectives in immunotherapy of neuroblastoma

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Immunotherapy of neuroblastoma has gained momentum as a result of efficacy reports in clinical trials. Three targeted approaches and expected added value to the treatment of this challenging disease will be discussed. First, the hallmark for immunotherapy in neuroblastoma combining passive immunotherapy using anti-GD2 antibodies with cytokines opened a new venue to further improve this approach with enhanced antibodies. Immunocytokines combine the targeting capacity of an anti-GD2 monoclonal antibody with the immunostimulatory activity of a cytokine in one molecule. Superior preclinical efficacy and activity in clinical trials provide an important base line for this approach and opens new options also in combination with the following immunotherapeutic strategies. Second, cell based strategies are well under way to strengthen the effector arm of the immunesystem against neuroblastoma. Here the implementation of blood stem cell transplantation to provide for a new immune system and activated natural killer cells is a promising strategy. Such approaches can be refined by genetic modification of immune effector cells with chimeric receptors (CARs). CARs consist of an antibody-derived single chain Fv domain linked to the cytoplasmic signaling domain of the T cell receptor (TCR) βchain and thereby retarget cellular activation pathways to tumor surface antigens. G_{D2}-specific CARs have been used to redirect T cells to neuroblastoma and have induced tumor regressions in patients with relapsed or refractory neuroblastoma. Future efforts aim at augmenting in vivo persistence and activity of G_{D2}-redirected immune effector cells to provide sustained immune control of residual neuroblastoma cells.
Third, neuroblastoma is not MHC class I negative. Thus, active vaccination approaches to induce long lasting and persistent immunity against neuroblastoma associated antigens is the ultimate goal in a phase of immune reconstitution after blood stem cell transplantation. Here the use of DNA vaccination to create B-cell vaccines (anti-idiotypic vaccination; GD2 mimotopes) and T-cell vaccines (Tyrosine hydroxylase, MYCN) are promising preclinical approaches.
The final goal is to implement these strategies along a time line of immune reconstitution after blood stem cell transplantation and thereby create a most effective neuroblastoma immunotherapy protocol in the future.

WS23

Second generation GD2-targeted immunotherapy and future perspectives

Yu, Alice
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With the recent demonstration by the Children's Oncology Group (COG) that ch14.18 + cytokine significantly improved outcome of patients with high risk neuroblastoma, an ongoing COG phase III trial to collect comprehensive toxicity profile for regulatory approval of ch14.18 is now underway. However, immunotherapy with ch14.18 + cytokines is associated with significant toxicities, including pain, hypersensitivity reaction, acute vascular leak syndrome. There are several second generation GD2-targeted immunotherapeutic agents on the horizon with the potentials for reduced toxicities and/or enhanced efficacy. Hu14.18-IL2 is a fusion protein of humanized anti-GD2 antibody (hu14.18) and IL-2. Twenty-seven pediatric patients with recurrent/refractory neuroblastoma and one with melanoma were treated with escalating doses of hu14.18-IL-2. The maximal tolerated dose was determined to be 12 mg/m2/d, approximately 50% of ch14.18. Clinical toxicities were similar to those reported with IL-2 and anti-GD2 mAbs. There were no measurable complete or partial responses to hu14.18-IL2 in this study; however, evidence of antitumor activity was noted in three patients (Neal, Yang et al. 2004). A phase II study of this immunocytokine is being planned. Hu14.18K332A is a humanized ch14.18 that shares identical C regions of IgG1-k as ch14.18 with the exception of a mutation to alanine at lysine 322 that limits its ability to fix complement and thereby reduces pain associated with ch14.18. While the ability of hu14.18K322 to activate complement was reduced, its ADCC capabilities were retained. Preclinical studies in rats confirmed that hu14.18K322 elicited significantly less allodynia than ch14.18 (Sorkin et al, Pain 2010). A phase I clinical trial of hu14.18K322 is ongoing at St. Jude Children¡¦s Hospital in Memphis, TN, USA. mAb1A7 is an anti-idiotype antibody directed against a murine anti-GD2, 14G2a. Anti-idiotype antibodies are antibodies to anti-tumor antibodies, and thereby mimic the original tumor antigen to which antibodies were developed and act as a surrogate tumor antigen. Active immunotherapy with anti-idiotype antibodies should give rise to a gradual increase in humoral anti-tumor immunity. Thus, it may have the advantage of lower toxicity than passive immunotherapy with the relatively rapid infusion of high concentrations of mAbs. A pilot clinical trial of mAb 1A7 as a surrogate GD2 vaccine was conducted in patients with high risk neuroblastoma (Yu, Eskenazi et al. 2001). Thirty one patients with high risk neuroblastoma who achieved first or subsequent complete response (CR) or very good partial response (VGPR) were entered into this trial. The treatment was well tolerated with only transient local reactions, transient fever and chills in 4 patients and serum sickness in 1. There was no systemic toxicity, such as neuropathic pain which is often seen with infusion of anti-GD2. Despite prior intensive treatment including stem cell transplantation in all but 4 patients, all 31 patients generated anti-mAb 1A7. More importantly, immune sera from some patients displayed CDC and ADCC activities. Many of those patients who enrolled during first remission have no evidence of disease progression while only 1 of 10 patients who enrolled during 2nd remission remains progression free. These findings indicated that mAb1A7 vaccine has little toxicities and is effective in inducing biologically active anti-GD2 in heavily pre-treated neuroblastoma patients, and therefore may be useful in controlling MRD. In light of the observed and anticipated low toxicity profile of mAb 1A7, and hu14.18K332A, respectively, it may be possible to replace ch14.18 with one of these products. Future clinical trials of mAb 1A7 vaccine or the mutant anti-GD2 mAb to document their therapeutic efficacy are warranted.

WS24

Targeting signal transduction pathways – Taking aktion!

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A number of receptor tyrosine kinase signaling pathways have been implicated in the biologic characteristics of neuroblastoma tumors from patients with a poor prognosis. These signaling pathways contribute to their unregulated growth (ALK, ERBB) enhanced vascularity (TrkB, VEGFR), and metastatic capability (TrkB, Met) as well as their resistance to chemotherapy (TrkB, TrkAIII, IGFR, PDGFR). Targeted therapies to mutated tyrosine kinase genes such as ALK hold tremendous promise for patients whose tumors harbor these mutations. While one approach is to target individual receptors with highly selective agents, this may select for the emergence of alternative receptors that serve a similar function. Another approach is to inhibit downstream of these individual receptors to common downstream intermediaries or key intracellular signaling nodes that mediate these functions. We have focused on the PI3kinase/AKT pathway, which is a key mediator of growth, survival and the angiogenic and metastatic activities of these tyrosine kinase receptors in NB tumor cells. Consistent with the pre-clinical models, NB patients with elevated levels of activated AKT have a worse prognosis. By focusing on this common downstream intermediary we have found that inhibition using Perifosine or an allosteric inhibitors of the PH domain of AKT inhibits NB cell survival, decrease VEGF production and enhances sensitivity to cytotoxic chemotherapeutics. Moreover, inhibition of AKT inhibits growth even in ALK mutated cells. Delineation of the signaling paths deranged or mutated in individual NB tumors will be important in determining the most efficacious combination of inhibitors to utilize.

WS25

Targeting apoptosis pathways in neuroblastoma

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Evasion of apoptosis, the cell's intrinsic death program, is a hallmark of human cancers including neuroblastoma. Also, failure to undergo apoptosis may cause treatment resistance, since the cytotoxic activity of anticancer therapies commonly used in the clinic, e.g. chemotherapy, βirradiation or immunotherapy, is predominantly mediated by triggering apoptosis in tumor cells. Therefore, a better understanding of the signaling pathways and molecules that govern apoptosis in neuroblastoma cells is expected to open new avenues for the design of molecular targeted therapies for neuroblastoma.

WS26

Omega-3 fatty acids in cancer: The protectors of good and the killers of evil?

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Omega-3 fatty acids have been implicated in cancer prevention and treatment. Conventional chemotherapeutics are considered "double-edged swords", as they kill the cancer cells but also strike the healthy cells causing severe morbidity and sometimes also mortality. Could omega-3 fatty acids in this setting work as a "sword and shield" instead, by being cytotoxic to cancer cells, but at the same time protect healthy cells from these deleterious effects? In addition, may our current diet with decreased omega-3/omega-6 ratio contribute to the increased cancer incidence, and could an omega-3 enriched diet be used as a preventive measure against cancer? Here, our data concerning the toxicity of the omega-3 fatty acid docosahexaenoic acid in neuroblastoma, both *in vitro* and *in vivo*, will be summarized. Mechanisms behind the observed effects will be described as well as the intracellular metabolome of DHA. In addition, the potential use of omega-3 fatty acid in combination with conventional chemotherapy will be discussed.

WS27

Optimizing drug development for neuroblastoma by close integration with adult oncology

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Developing new drugs for neuroblastoma ultimately requires obtaining regulatory approval to enable routine and continual access to an active drug by the largest number of patients. While new agents targeting only neuroblastoma have been successfully developed, the current economic climate diminishes such opportunities for the foreseeable future. The substantial costs required for drug development and for insuring ongoing clinical supplies of a new agent will be surveyed. Developmental pathways will be reviewed for agents successfully incorporated into standard of care for neuroblastoma (isotretinoin + ch14.18), and for selected agents currently in clinical development (MIBG, BSO, fenretinide, safingol, ABT-751, hu14.18-IL2, ALK inhibitors). New drugs that have potential adult oncology indications likely will undergo more rapid development as the adult data informs pediatric clinical trials and funding is more accessible. Drugs that obtain regulatory approval in adult indications can be made available for treating neuroblastoma without conducting neuroblastoma drug registration studies, which are costly in time, money, and patients. Thus, future new agent development for neuroblastoma should, whenever possible, involve early collaborations with adult oncology investigators and should seek to maximally leverage recent legislation requiring pediatric drug development plans.

Special sessions
SS1–SS5

SS1

A public web-based analysis tool and database for high throughput and clinical data of neuroblastoma: introduction to practical use of R2

Koster, Jan; van Sluis, Peter; Ora, Ingrid; Caron, Huib; Molenaar, Jan; Versteeg, Rogier
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High throughput mRNA profiling is an efficient tool to identify expression levels for any gene in series of tumors and cell lines. Many tools have been developed for analysis of such data. However, most of them require specialist bioinformatic support or are time consuming or are not integrated in one user-friendly platform. We developed a tool for basic and advanced analysis of such data, called R2. This web-based tool enables swift and user-friendly analyses by any interested researcher. We have generated Affymetrix expression profiles of a series of 88 neuroblastoma tumors, as well as from a limited number of ganglioneuromas and ganglioneuroblastomas. The series is annotated for clinical and molecular parameters. R2 enables to analyse expression values for any gene, establish its prognostic value and find correlating expression patterns with other genes. It also permits global expression analysis and prognostic ordering for gene sets, which can be formed by KEGG pathways, functional groups, etc. Differentially expressed genes can efficiently be identified within annotated parameters, like age, stage, histology etc. Any resulting gene list can be analysed for pathway or gene ontology enrichment, or be visualized in heat maps. Finally, the R2 tool and database includes Affymetrix expression profiles of over 20,000 normal and tumor samples from other tissues, grouped per tumor or tissue type. This enables quick searches for expression profiles of specific genes over a wide range of tissues. The web-based R2 analysis tool and database will be made publicly available at the ANR.

SS2

Current and future strategies to improve outcomes in neuroblastoma: An update from the Children's Oncology Group Neuroblastoma Disease Committee

John M. Maris
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The long-term objectives of our committee rely on continued robust specimen collection and annotation to support the discovery and validation efforts required for a personalized approach to neuroblastoma diagnosis, prognostication, treatment and surveillance. Ongoing projects focused on discovery of oncogenic drivers, detection of rare residual tumor cells and identifying genomic signatures of tumor behavior will all be highlighted at ANR2010, and will be integrated into future clinical trials. For patients with high-risk disease, the recent demonstration that passive immunotherapy with ch14.18 combined cytokines (alternating GM-CSF and IL2) to improve antibody dependant cellular cytotoxicity dramatically improves survival rates when administered shortly after myeloablative therapy (*Yu et al., NEJM in press*) will provide the new baseline for which future studies will be compared. Ongoing clinical research is focused on further defining the toxicities associated with this regimen, and future studies will seek to further improve efficacy (e.g. hu14.18-IL2) and/or reduce toxicity. A parallel major goal is to improve the quality of induction/ consolidation response to cytotoxic therapy. Major efforts include plans test the efficacy of ^{131}I-MIBG in frontline therapy in a randomized controlled trial, and to integrate molecularly targeted agents into the current chemotherapy backbone of induction therapy. Inhibitors of ALK, IGF1R and AURKA are lead candidates at this time, but it is clear that a major obstacle is a continued paucity of validated targets. Prospective identification of cases harboring mutated ALK receptors may provide for the opportunity for individualized therapy in the next generation of studies. Finally, the overall strategy for patients with non-high-risk disease will be to continue to utilize biomarkers allowing for reduction of cytotoxic therapy (*Baker et al., NEJM in press*). However, special emphasis on patient subsets with continued suboptimal outcomes, such as very young infants with INRG Stage MS disease, older children with Stage L2 disease and unfavorable genomic features, and the adolescents and young adults with any stage of disease, will require international cooperation and harmonization of approaches.

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SS3

Strategies to improve outcome and quality of life in patients with neuroblastoma: Activities of the SIOP European Neuroblastoma Group

Ladenstein, Ruth
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No abstract available

SS4

Recent achievements and future strategies of GPOH to improve outcome for children with neuroblastoma

Simon, Thorsten
Germany

No abstract available

Plenary sessions
PL1–PL36

PL1
The role of eicosanoids in health and disease

Samuelsson, Bengt

Sweden

See page 12 for biography

PL2
Fatty acids as positive and negative regulators in neuroblastoma development

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See page 12 for biography

Fatty acids are precursors to a wide range of different lipid mediators that are suggested to play fundamental different roles in cancer development. Metabolic conversion of omega-6 fatty acids, represented by arachidonic acid, mostly gives rise to the pro-inflammatory eicosanoids, prostaglandins and leukotrienes, which are potent promoters of tumour growth. Omega-3 fatty acids, on the other hand, can give rise to anti-inflammatory and pro-resolving lipid mediators that have the capacity to inhibit tumour growth.

In humans, the omega-3 fatty acid, docosahexaenoic acid (DHA), is the most abundant fatty acid found in neural cells and the active lipid mediators of DHA shield normal neural cells against cellular insults through inhibition of oxidative stress and apoptotic processes. Conversely, neuroblastoma tumour cells are profoundly deficient in DHA but contain elevated levels of the omega-6 fatty acid arachidonic acid as well as enzymes involved in the conversion of arachidonic acid to prostaglandins and leukotrienes. Unlike normal neural cells, neuroblastoma cells are not able to convert DHA to protective lipid mediators. Instead, exogenous DHA is converted to lipid intermediates that are highly toxic resulting in apoptosis. Also, inhibition of prostaglandin or leukotriene synthesis induces mitochondrial-dependent apoptosis of neuroblastoma cells, whereas exogenous addition of these pro-inflammatory eicosanoids stimulates neuroblastoma cell proliferation. Neuroblastoma expresses both prostanoid and leukotriene receptors and binding of these eicosanoids to their cognate G-protein coupled receptors results in the activation of PI3K/Akt and Erk-mediated signal transduction. These findings suggest that both prostaglandins and leukotrienes are constituents of an autocrine survival loop in neuroblastoma.

In preclinical *in vivo* models, both DHA and agents that inhibit the production of prostaglandins, leukotrienes or their receptors have profound effects both on neuroblastoma development as well as growth and progression of established neuroblastoma tumours. Moreover, DHA and nonsteroidal anti-inflammatory drugs (NSAIDs) augment the toxic effects of several established cytostatic drugs in clinical use against neuroblastoma, suggesting these novel agents as potential new elements in clinical treatment protocols. Besides the direct inhibition of prostaglandin synthesis, NSAIDs have several side-effects that increase the anti-tumourigenic potential. In neuroblastoma NSAIDs significantly inhibit angiogenesis and directly act as an inhibitor of PI3K/Akt signalling resulting in reduced expression of Mycn. NSAIDs also enhance the chemosensitivity in neuroblastoma via downregulating HDM2 and augmenting p53 stability or differentially modulate p73 isoforms leading to enhanced apoptosis. *In vivo*, IL-6 induce the expression of cyclooxygenase-2 in neuroblastoma cells resulting in increased release of prostaglandin E2 and enhanced expression of IL-6 by bone marrow stromal cells making the bone marrow microenviroment favourable to the progression of metastatic neuroblastoma.

Taken together, these results from our group and others suggest that fatty acids and inhibition of their active lipid mediators have profound regulating effects on neuroblastoma initiation, progression and metastatic potential. Since, several compounds that inhibit these lipid mediators are available and tested for other purposes these drugs should be considered to be included in clinical testing as adjuvant to current therapeutic modalities.

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PL 3

Telomeres and telomerase: their implications for human health and disease

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See page 16 for biography

PL4

Neuroblastoma genetics: From the beginning to the end(s)

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See page 16 for biography

There is a rich history of seeking to understand the genetic basis of neuroblastoma, beginning with the original description of aberrancies at the karyotypic level several decades ago. The Advances in Neuroblastoma Research Meeting has been the major venue to preview the seminal genetic discoveries in neuroblastoma, and these will be reviewed. Our laboratory has sought to build on this broad foundation to both understand how the host genome impacts susceptibility to develop neuroblastoma and how these events lead to the somatically acquired mutational events that lead to the diverse clinical phenotypes seen in the clinic. Here we will highlight some recent discoveries in neuroblastoma genetics from our laboratory, and discuss future plans designed to discover all of the major driver mutations in neuroblastoma.

Heritable genetics. We have discovered *ALK* as the major neuroblastoma predisposition gene. Activating mutations occur in the germline, but also somatically in 10-15% of primary tumors. Targeted inhibition strategies have proven effective in preclinical models, and a clinical trial is ongoing. *PHOX2B* loss of function mutations explain hereditary and sporadic neuroblastoma cases with associated Hirschsprung disease and/or congenital central hypoventilation syndrome. A third putative familial neuroblastoma locus has been tentatively mapped. In addition, by studying over 3,500 neuroblastoma cases compared to 10,000 controls, we have discovered multiple single nucleotide polymorphisms (SNPs) and copy number variations (CNVs) highly associated with neuroblastoma. The associations are phenotype specific, suggesting that neuroblastoma represents distinct diseases at the level of genetic initiation. Three proven associations result in somatic gain of function effects (*BARD1, NME7* and *LMO1*), and we now have evidence that these genes do indeed contribute to the malignant phenotype in high-risk neuroblastoma, thus providing possibilities for targeted therapeutics.

Somatic genetics. As part of the NCI-funded Therapeutically Applicable Research to Generate Effective Treatments (TARGET) project, we have generated high density SNP and Affymetrix HuEX array data on over 250 cases and used these data to further refine our genomic classification of tumors with an emphasis of refining risk classification. These date provide refined maps of copy number alterations in neuroblastoma, extend the prognostic impact of DNA and RNA signatures for predicting outcome, and have defined the frequency of mutation in over 100 regional candidate genes. The combined data further emphasize the central role of the myc family of proteins in neuroblastoma, and have identified several candidate therapeutic targets that are related to MYCN amplification and/or basal myc overexpression, such as *ALK*, *AURKA* and *CHK1*. We are currently performing a thorough resequencing of the neuroblastoma genome: 10 full genomes and 200 exomes, each with matched germline DNA; and tumor RNA sequencing in a subset. These data should provide a comprehensive catalogue of the majority (if not all) clinically relevant mutations. Ongoing epigenomic profiling will eventually be layered onto this dataset that will be freely available to all investigators.

Taken together, the data generated from these projects should provide tractable therapeutic targets for rapid translation to the clinic. Moving past discovery efforts, to mechanistic understanding of DNA sequence variation, and defining the structure-function relationship of newly identified mutations, will be essential for these translational efforts.

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PL5

Copy number variations (CNVs) in neuroblastoma

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Background: The genetic etiology of familial neuroblastoma (NB) has recently come into focus, but the genetic and environmental factors that cause sporadic NB remain largely unknown.
Methods: We are comparing germline genome-wide single nucleotide polymorphism (SNP) genotypes from 5,000 NB patients to 10,000 controls in order to discover SNP and copy number variation (CNV) associations. CNV associations are replicated in two independent cohorts using alternative methods. Correlative and mechanistic studies are performed in lymphoblastoid cell lines (LCLs) as well as primary NB tumor tissues and NB cell line models.
Results: To date, we have genotyped over 3,500 NB cases and have reported two loci containing common SNPs (*NEJM* 2008, *Nat Genet* 2008) and a common CNV (*Nature* 2009) each highly associated with NB. We have discovered an additional CNV association within *NME7* at 1q24.2 (see Maris, et al. ANR 2010 for additional SNP associations). This CNV is highly correlated with *NME7* mRNA expression in LCLs ($p < 0.0001$). To investigate whether somatic alterations of *NME7* also influence tumorigenesis, we analyzed tumor DNA copy number in 591 primary tumors and matched mRNA expression in a subset of 100 samples. We observed somatic gain of the *NME7* locus in 24% of NBs ($p = 3.3 \times 10^{-9}$). Tumor acquired somatic gain was also highly correlated with *NME7* mRNA expression ($p = 0.007$), and Western blot confirmed a strict correspondence between mRNA and protein levels. Targeted knockdown of *NME7* in NB cell lines resulted in decreased cell proliferation, restoration of contact inhibition, and decreased cell migration. No phenotype was observed after *NME7* knockdown in control RPE1 cells.
Conclusions: The NB GWAS has identified multiple susceptibility variants, and we now demonstrate functional relevance in both germline and tumor tissues. These data illustrate the utility of combining germline and somatic data in assessing GWAS signals in cancer, and identify *NME7* as both a predisposition locus and candidate oncogene in NB. Other NB-associated CNVs, including both common and rare variants, have been identified and validation efforts are ongoing.

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PL6

A potential role for the pluripotency factor LIN28B and Let-7 signalling in neuroblastoma

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Background: Multiple members of the Let-7 miRNA family are repressed in human cancers. This was shown to result in malignant transformation through stabilisation of mRNAs coding for various oncogenes like MYC and RAS. The LIN28 and LIN28B RNA binding proteins inhibit processing of Let7 pre-miRNAs to mature miRNAs. This suggests that LIN28 and LIN28B could be involved in malignant transformation of cells but no genomic aberrations of these genes have been identified.
Methods and results: We report a neuroblastoma tumor with high level DNA amplification of the LIN28B gene located on chromosome 6q21. In addition, the mRNA expression levels of LIN28B were strongly increased in a panel of 88 neuroblastoma tumors compared to normal tissues and many other human malignancies. Over-expression of LIN28B was highly correlated with a poor prognosis in neuroblastoma patients. We therefore investigated the functional role of LIN28B in neuroblastoma.
Lentiviral mediated silencing of LIN28B by various shRNA's resulted in strong neuronal differentiation in a panel of neuroblastoma cell lines, including several neuroblastoma TIC (Tumor Initiating Cells) lines.
Inducible over-expression of LIN28B in neuroblastoma cell lines with low LIN28B mRNA expression levels was well accepted and induced a slight growth advantage. Over-expression of LIN28B was able to immortalize non malignant mouse neuroblasts.
Finally, to study a functional role of LIN28B in vivo we performed correlation analysis using mRNA expression data (Affymetrix U133-plus2) and MIR expression data in 88 neuroblastoma samples using the R2 bio-informatic tool. Several miRNAs showed an inverse correlation with LIN28B mRNA expression. The 8 most significant inversely correlating miRNAs all belonged to the Let7 cluster. This suggests that the LIN28B gene is functional and inhibits Let7 miRNAs processing in vivo.
Conclusion: We conclude that LIN28B is a potential tumor driving gene in neuroblastoma and we are currently unravelling the downstream signalling cascade.

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PL7

CAMTA1, a 1p36 tumor suppressor candidate, activates differentiation programmes in neuroblastoma cells

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Background: Deletion within distal 1p characterizes about 30% of neuroblastomas (NBs) and it is widely assumed that this region harbours genetic information mediating tumor suppression. The combination of recent fine mapping studies defined a 1p36 smallest region of consistent deletion pinpointing the *CAMTA1* locus. Multivariate survival analysis identified low *CAMTA1* expression as an independent predictor of poor survival and *CAMTA1* is included in most of the recent prognostic NB expression classifier gene sets.
Aim: To analyze the potential effect of CAMTA1 on NB biology using inducible cell models.
Methods: Expression of CAMTA1 is low in NB cell lines compared to favourable tumors. We established stable NB cell models allowing Tet-inducible re-expression of CAMTA1. A specific antibody was generated to monitor CAMTA1 levels upon induction. The CAMTA1-induced phenotype was assessed using flow cytometry, colony formation, viability and soft agar assays. CAMTA1 regulation was assessed in neuroblastoma differentiation models. Trancriptome analysis upon CAMTA1 induction was done using whole genome microarrays.
Results: CAMTA1 induction in NB cells significantly decreased colony formation ability and growth rate. Growth inhibition was associated with induction of the cell cycle inhibitor p21 and an increase of the proportion of cells in G1/0 phase. In neuroblastoma cells growing in soft agar, CAMTA1 induction inhibited the capacity of anchorage-independent growth. Further, CAMTA1 induction induced morphological changes and markers characteristic of neuronal differentiation. *CAMTA1* expression was upregulated upon differentiation of NB cells induced by external stimuli. Time-resolved transcriptome analysis revealed 683 genes regulated upon CAMTA1 induction. Among CAMTA1 induced genes, GO terms related to neuronal function and differentiation were significantly enriched. Among CAMTA1 repressed genes, the majority of enriched GO terms related to cell cycle progression.
Conclusion: Together, our data imply that CAMTA1 is a 1p36 tumor suppressor candidate that inhibits features of malignant cells and is involved in neuronal differentiation.

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PL8

Whole genome and transcriptome sequencing of ten stage IV primary neuroblastoma tumors: a TARGET project report

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Studies from the NCI's Therapeutically Applicable Research to Generate Effective Targets (TARGET) Initiative use genome-wide profiling of expression levels and DNA copy number alterations as well as candidate gene re-sequencing to identify novel biomarkers and drug targets for neuroblastoma (NB). As a complementary approach to identify novel genomic alterations in NB, we used massively parallel paired-end Illumina sequencing to profile tumor transcriptomes as well as tumor and normal genomes of ten NB TARGET cases. We generated more than 5 Gb of aligned sequence per sample from polyA-enriched RNA of ten stage IV primary NB tumors. The same technology was used to sequence the corresponding tumor and matched constitutional DNA to 30-fold aligned coverage. The reads were mapped to a reference human genome and, in the case of RNA data, to a custom database of known exon junctions using MAQ and BWA software. The transcriptome alignments were used to detect more than 25,000 annotated Ensembl transcripts per sample while profiling over 12,000 protein-coding genes at 5-fold average coverage or higher across their whole length. We used a binomial mixture model, SNVmix to identify between 601 and 869 candidate protein-coding mutations in expressed genes per sample, including 310 that recurred in at least two samples. The SNVmix model was also used to identify protein-coding changes in tumor and normal genome sequencing data. Comparison of SNVs detected in the transcriptome, tumor, and normal genomes revealed somatic mutations and germline polymorphisms, as well as candidate RNA editing events. Genes harbouring novel candidate somatic mutations (protein-coding changes observed in the tumor genome and transcriptome, but not in the corresponding normal genome) identified to date include FAM21C, JAG1, HDAC6, GPC1, MRO, THADA, PDE4D and ZNF800. These results suggest that Illumina sequencing is a powerful method to identify novel genetic alternations in neuroblastoma, and may be used to reveal novel targets for therapeutic intervention.

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PL9

Role of a novel inducible dependence receptor UNC5D in spontaneous regression of neuroblastoma: its functional cooperation with p53 and E2f1 for inducing programmed cell death

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Background: The signals through NGF receptors (TrkA and p75NTR) may regulate induction of spontaneous regression in neuroblastomas (NBs). However, the molecular mechanism remains elusive. We have cloned a novel dependence receptor UNC5D, a member of netrin-1 receptors (DCC and UNC5 family), from the cDNA libraries generated from primary neuroblastoma tissues. Here we show that UNC5D is a novel inducible gene after NGF deletion and other stresses, and that it dramatically enhances the NGF-depletion-induced neuronal programmed cell death by cooperating with E2F1 and p53.
Methods: DNA and mRNA were extracted from 108 primary NBs. The mRNA expression and the protein/protein interactions were examined by a quantitative RT-PCR and immunoprecipitation experiments, respectively. Transcriptional activation was investigated by luciferase reporter assays. We generated *Unc5h4* knockout mice and prepared MEF cells and sympathetic neurons.
Results: The significantly high expression of UNC5D, but not UNC5A-C, was observed in favorable NBs and associated with good prognosis (p=0.003). The ligand, netrin-1, was only weakly detected in stromal cells by immunohistochemistry. Like E2F1 and p53, the NGF withdrawal strongly upregulated UNC5D, but not UNC5A-C, in both favorable NB cells in primary culture and PC12 cells, suggesting that UNC5D is an inducible gene. In addition, UNC5D was a direct transcriptional target of p53. The NGF depletion as well as DNA damage induced cleavage of intracellular domain of UNC5D by caspases 2 and 3, and the cleaved fragment translocated into nucleus to form a transcriptional complex with E2F1, which in turn selectively transactivated pro-apoptotic genes including caspases, Bid and E2F1 itself. This positive feedback loop dramatically enhanced apoptotic cell death through UNC5D. The analyses using MEF cells and sympathetic neurons obtained from the *Unc5h4* knockout mice we generated supported the above observations.
Conclusion: Our results demonstrate that UNC5D contributes to NGF depletion-mediated programmed cell death in neuroblastoma via nuclear translocation of its intracellular fragment (UnICD) which acts as a co-activator of E2F1.

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PL10

Metastatic neuroblastoma cancer stem cells display a mixed phenotype of tumor and niche origin required for survival

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Neuroblastoma (NB) is a pediatric tumor of neural crest origin, and is the most common cancer of infancy. 50% of patients have metastases at diagnosis, of which 85% will die after multiple relapses from metastatic disease. We identified tumor-initiating cells (TICs) from bone marrow (BM) metastases of high-risk patients that are propagated as spheres and that have many properties of cancer stem cells, and form NB in mice with as few as 1 cell (see Yan et al abstract). To understand patient relapse and disease progression, we compared NB-TICs from BM with those from tumor and brain metastases and SKPs, a normal pediatric stem cell counterpart, by cDNA microarray, flow cytometry, and whole genome shotgun sequencing transcriptome analysis. BM-derived NB-TICs expressed primitive neural crest and neuronal markers as well as hematopoietic markers from primitive, myeloid, and B-cell lineages and contained VDJ gene rearrangements, which are normally associated with B-cell leukemias. Hematopoietic genes were not expressed or expressed at very low levels in tumor-derived sphere lines and a line from an NB brain metastasis, however brain metastasis-derived TICs expressed CD133, while BM-derived NB-TICs did not. Furthermore, we found that shRNA to CD74, which is upregulated in B-cell lymphoma and multiple myeloma and is a therapeutic target for those cancers, induced the rapid death of NB-TICs but not normal SKPs. Interestingly cells co-staining for the hematopoietic markers CD45 or CD74 and the neural neural progenitor marker nestin were found in BM smears of patients with relapsed NB in the BM.
We suggest that metastatic TICs from some tumors adopt resident niche-specific gene expression signatures, which may aid diagnosis and the development of novel treatments. We hypothesize that drugs used for leukemia may be efficacious therapeutics for metastatic NB, and are currently testing this hypothesis in mouse models.

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PL11

Cell-cell communication via ion fluxes in control of neuroblastoma cell cycle

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Background: Ion channels control proliferation and self-renewal in stem cells and may act as tumor suppressors in cancer cells. We recently found an unexpected signaling pathway that controls proliferation via GABA gated ion channel activity and the DNA damage response (DDR) pathway (Nature 451, 2008). Our new data suggest yet another example of such a cell-cell signaling modality via the sodium pump, Na,K-ATPase. Intracellular ion homeostasis is driven by the Na,K-ATPase, which requires ATP for its activity. Na,K-ATPase has been explored as a target in cancer therapy e.g. using ouabain; one of the cardiac glycosides (digitalis) and a ligand of Na,K-ATPase.
Method/approach: We investigated primary human neuroblastomas, cultured neuroblastoma cell lines and TICs with immunostaining, electrophysiology, and qRT-PCR, and randomized nude mice with SH-SY5Y xenografts for targeted therapy or as controls.
Results: We found that low levels of ouabain (<10% pump inhibition) controlled Na,K-ATPase activity in neuroblastoma cells and mediated a fully reversible cell cycle block via the DDR pathway without causing DNA damage. A DDR pathway inhibitor reduced the cell cycle block, confirming a causal mechanistic link. Oral oubaine treatment of mice with xenografts significantly reduced tumor volume, inhibited proliferation and increased apoptosis, suggesting that the mechanism may be reversible also in vivo. Our continued expression analysis of primary neuroblastoma tumors, showed that all tested non-MYCN amplified tumors express the critical Na,K-ATPase subunit for ouabain sensitivity, whereas MYCN amplified tumors did not.
Conclusion: Ouabain is synthesized by the adrenal glands, an environment where most neuroblastoma tumors reside. Therefore we expect that ouabain sensitivity may have a physiological relevance for tumor growth. These data suggest novel options for targeted therapy.

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PL12

***PHOX2B* is essential for peripheral sympathetic neuronal differentiation in the zebrafish**

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Background: Heterozygous mutations in *PHOX2B* have been identified in neuroblastoma, although the effects of these variants (gain- or loss-of-function) remain unclear. To determine the consequences of phox2b deficiency during embryogenesis, we studied the effects of *phox2b* knockdown on sympathetic nervous system development in the zebrafish model.
Method: One-cell embryos were injected with anti-sense morpholinos (MO) that block translation or mRNA splicing of *zphox2B*. Genes involved in sympathetic superior cervical ganglion (SCG) neurogenesis were monitored throughout development by RNA in-situ hybridization.
Results: Knockdown of *zphox2B* caused a significant decrease in *th*- and *dbh*- expressing neurons in the SCG that was rescued by overexpression of wild type (WT) human *PHOX2B*. Lack of apoptosis in the SCG of phox2b-deficient embryos was demonstrated by acridine orange staining. However, analysis of *th*- and *phox2b* expression in phox2b-deficient embryos revealed an increase in the numbers of *phox2b*-expressing cells in the SCG, with a concomitant decrease in the numbers of *th*-expressing cells compared to WT controls. Moreover, the SCG cells in phox2b-defcient embryos could not be induced to differentiate by retinoic acid (RA). In addition to increased *phox2b* expression itself, abrogation of *zphox2B* expression also led to increased expression of another pro-neurogenic marker *zash1a*, while the expression of markers indicative of more differentiated neurons *zdhand*, *tfap2*α, and *gata3* were markedly decreased. To determine if *phox2b* and *zash1a* act redundantly with respect to differentiation, we injected embryos with *zash1a* MO and found only slight decrease in *th*- expression in the SCG. However simultaneous knockdown of both *zash1a*and *phox2b* expression resulted in complete loss of *th*- and *dbh*- expression.
Conclusion: Our data show that loss of zphox2b function during development causes an increase in the number of neuroblasts at the expense of more differentiated cell types. We suggest that *PHOX2B* loss of function variants give rise to an undifferentiated phenotype that is more vulnerable to second hits that induce malignant transformation.

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PL13

Infections linked to human cancers

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See page 54 for biography

PL14

The Yin and Yang functions of the Myc oncoprotein in cancer development and as targets for therapy

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See page 54 for biography

The *MYC* gene was originally identified in avian retroviruses as the oncogene responsible for inducing myelocytomatosis in birds. The cellular homologue, c-*MYC*, was found to be evolutionarily conserved. Later, *MYCN* and *MYCL* were found amplified in neuroblastoma and in small cell lung cancer, respectively. The *MYC* genes encode short-lived nuclear phosphoproteins with a half-life of 20–30 min that are subsequently ubiquitinated for proteasomal degradation. MYC is a basic Helix–Loop–Helix Leucine Zipper (bHLHZip) protein that heterodimerizes with the small bHLHZip protein Max resulting in dimers with DNA-binding ability at CACGTG and similar E-box sequences. The MYC proto-oncoproteins coordinate a number of normal physiological processes necessary for growth and expansion of somatic cells by controlling the expression of numerous target genes. Deregulation of *MYC* as a consequence of carcinogenic events enforces cells to undergo a transition to a hyperproliferative state. This increases the risk of additional oncogenic mutations that in turn can result in further tumor progression. However, MYC activation also provokes intrinsic tumor suppressor mechanisms including apoptosis, cellular senescence and DNA damage responses that act as barriers for tumor development, and therefore needs to be overcome during tumorigenesis. MYC thus possesses two seemingly contradictory “faces” here referred to as “Yin and Yang”. Observations that many tumor suppressor pathways remain intact but are latent in tumor cells opens the possibility that pharmacological inhibition of the Yin or activation of the Yang functions can prevail and offer new attractive approaches for treating diverse types of cancer including neuroblastoma.

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PL15

Identification of selective inhibitors of neuroblastoma stem cells - targeting the kinome

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Background: We previously isolated sphere-forming cells from neuroblastoma (NB) tumors and metastases with many of the properties of cancer stem cells, including the expression of stem cell markers, and the ability to self-renew in culture and form orthotopic metastatic tumors in immunodeficient animals with as few as a single cell (L. Hansford; S. Yan abstracts, ANR 2010).
Methods: To identify signalling pathways important for survival and self-renewal of NB tumor-initiating cells (TICs) as well as potential therapeutic targets we undertook a comprehensive high-throughput screening program using diverse chemical libraries including: (1) kinome library of 160 protein kinase inhibitors, including 33 used clinically, (2) 4400 bioactive compounds and neuroactive drugs of the Prestwick, Lopac, and Spectrum collections that we previously used to identify dequalinium-14, primaquine, and quinicrine as drugs that selectively kill TICs but not normal pediatric neural crest stem cells, and (3) 700,000 drug-like compounds of the Genomics Institute of the Novartis Research Foundation, which we are using to identify agents that promote NB TIC differentiation as detected by a MAP2 neuronal promoter-driven luciferase reporter.
Results: Here we report on our findings screening early passage NB TIC lines derived from bone marrow metastases from relapsed patients with the kinome library using a 96 hour growth/survival assay (Alamar Blue). In secondary sphere formation assays, we confirmed in vitro cytostatic or cytotoxic activity of kinase inhibitors that target PI3K/Akt, PKC, Aurora, mTor, ERBB2, Trk, Src, and polo-like kinase 1 (PLK1). Treatment with PLK1 siRNA or low nanomolar concentrations of PLK1 inhibitor BI 2536 that is currently in a phase II clinical trial for solid tumors, was selectively cytotoxic to TICs but not normal pediatric stem cells. Preliminary data indicate that BI 2536 significantly inhibits TIC tumor growth in a therapeutic xenograft model.
Conclusion: Our findings have identified candidate kinases that regulate primary NB TIC growth and survival, and suggest that PLK1 inhibitors may be an effective therapy for metastatic NB.

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PL16

A new Aurora kinase inhibitor (CCT241736) regulates Mycn protein expression and prevents neuroblastoma growth *in vitro* and *in vivo*

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Background: Amplification of the MYCN oncogene occurs in 25% of neuroblastoma (NB) correlating with poor clinical outcome. We have examined indirect methods of destabilising Mycn by targeting key components of upstream signalling pathways responsible for maintenance of Mycn protein levels. Aurora kinases regulate cell cycle transit from G2 through to cytokinesis, are amplified in a variety of cancers. Recently, Otto *et al*also showed that Aurora stabilizes the Mycn protein by specifically interfering ubiquitination/degradation of Mycn in the proteasome. We have a significant chemistry effort targeted at developing refined Aurora A kinase inhibitors with enhanced ability to destabilize Mycn.
Method/Results: CCT241736 is a potent inhibitor of Aurora A with IC50 values in the nanomolar (nM) range. We show that CCT241736 inhibits phosphorylation of Histone H3 (Ser10) in vitro in a panel of isogenically derived wild-type (wt) N-myc and N-myc phosphomutant cell lines indicating Aurora Kinase inhibition. N-myc phosphomutants are resistant to destabilisation by broad spectrum PI3K blockade (Chesler, 2006). CCT241736 targeted wt N-myc expressing cells at nanomolar concentrations in SRB and MTS assays. In comparison, N-myc phosphomutant cells treated with CCT241736 show no decrease in the steady state levels of the N-myc protein on western blots and showed less decrease in cell viability. CCT241736 was significantly effective against tumour progression concomitant with destabilization of Mycn protein in a therapeutic intervention trial in the TH-*MYCN* model.
Conclusions: We conclude that targeting of Aurora A kinase destabilizes Mycn protein *in vitro* and *in vivo* and may have therapeutic efficacy in MYCN-amplified neuroblastoma. CCT241736 displayed promising *in vivo* and *in vitro* activity and refined CCT compounds targeted more specifically against Mycn are under development.

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PL17

Mycn as a critical target of PI3K/mTOR inhibitors in neuroblastoma; paracrine effects on tumor vasculature

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Inhibitors of PI3K and of mTOR are now in clinical trials, with preclinical studies suggesting efficacy against tumor vasculature. We showed previously that this class of inhibitors also blocks Mycn protein, and that blockade of Mycn contributes to in vivo efficacy. Here, we extend this result, demonstrating that BEZ235 (Novartis), a dual inhibitor of PI3K and mTOR, led to decreased levels of Mycn protein and improved survival in mice transgenic for TH-MYCN. Murine tissues analyzed after BEZ235 treatment showed decreased proliferation and viability of both tumor cells, and tumor-associated endothelial and perivascular cells, with no untoward effects observed in normal retinal vasculature. To evaluate whether destabilization of Mycn contributes to the effects of BEZ235 on the tumor microenvironment, we co-cultured human endothelial and neuroblastoma cells, the latter transfected with vector, wt, or phospho-defective alleles of N-myc. Phospho-defective alleles of N-myc were unaffected by BEZ235 treatment, with cocultures showing increased levels of endothelial proliferation (angiogenesis) and increased levels of secreted Vascular Endothelial Growth Factor (VEGF) relative to tumor cells carrying vector or wild-type N-myc. Control co-culture experiments using TET21 cells (doxycycline regulated expression of Mycn), and using siRNA against MYCN confirmed a role for Mycn in driving paracrine VEGF signaling and promoting tumor angiogenesis. These results support: 1). Mycn as a critical target of PI3K/mTOR inhibitors, 2). A role for Mycn in sustaining tumor vasculature, in tumors driven by MYCN, and 3). PI3K/mTOR inhibitors including BEZ235 as a promising new class of compounds for therapy of MYCN-amplified neuroblastoma.

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PL18

Exploitation of ALK as a therapeutic target in neuroblastoma

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Background: The recent discovery of germline and somatic gain of function mutations in the receptor tyrosine kinase ALK provides a tractable therapeutic target for new drug development in neuroblastoma.
Methods: We report a comprehensive survey of ALK genomic status in all neuroblastoma phenotypic subsets. We determine the sensitivity of neuroblastoma in vitro and in vivo models to PF-02341066, an ATP-competitive, orally bioavailable small molecule inhibitor of ALK and MET, evaluate dose-dependent inhibition of phosphoprotein signaling, and begin to predict for resistance in the clinic.
Results: To date, we have sequenced and genotyped of 594 primary neuroblastomas and have identified non-synonymous sequence variations in 7.2% of samples (43/594), which grouped into four hotspots within the kinase domain. In the extracellular domain, we discovered and validated seven nonsynonymous sequence variations. We detected high-level amplification of ALK in 2.4% of tumors and show that ALK amplification and regional gain of the ALK locus are associated with increased ALK expression. We engineered human ALK cDNAs harboring the three most common germline mutations and the F1174L mutation, stably overexpressed these in retinal pigment epithelial cells, and show that these are gain-of-function mutations that induce differential constitutive kinase activation. We show that cytotoxicity to pharmacological ALK inhibition both in vitro and in vivo is dependent upon ALK genomic status, and correlates with abrogation of phospho-ALK and differential inhibition of downstream signaling pathways. We use homology modeling to predict a structural basis for differential activity against R1275Q and F1174L mutations.
Conclusions: Our data demonstrate that cytotoxicity to PF-02341066 is highly associated with ALK genomic status and evidence for constitutive activation, and provides the pre-clinical rationale for an ongoing phase 1/2 clinical trial in the Children's Oncology Group. Establishing the molecular mechanisms underlying the emergence of resistance, and understanding the structural basis of ALK inhibitory activity will be crucial for the development of ALK inhibition strategies.

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PL19

An RNAi screen of the protein kinome identifies CHK1 as a therapeutic target in neuroblastoma

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Background: Despite intensification of therapy for neuroblastoma, survival is poor. Therefore, patients will benefit from treatment strategies that rationally exploit signaling pathways for which a tumor cell is selectively dependent. In an effort to identify novel therapeutic targets, we performed a comprehensive loss of function screen of the protein kinome in neuroblastoma cell models.
Method: Using a validated siRNA library targeting the human protein kinome, 529 individual kinase siRNAs were transfected into four neuroblastoma cell lines and substrate adherent growth was measured.
Results: Thirty kinase targets had broad activity in the RNAi screen, but the cell cycle checkpoint kinase CHK1 was the most potent. CHK1 mRNA expression was significantly higher in MYCN amplified ($p < 0.0001$) and high risk tumors ($p < 0.03$). Western blotting revealed that CHK1 is constitutively phosphorylated in 9 of 10 neuroblastoma cell lines and a panel of high risk primary tumors, but not in control cell lines or low risk primary tumors. As the next step in translation of our genetic screen, we tested two CHK1 tool compounds. Pharmacologic inhibition by SB21807 and TCS2312 showed cytotoxicity in 7 of 9 neuroblastoma cell lines with median IC50s of 564 nM (62-695 nM) and 548 nM (159-973 nM), respectively. In contrast, the control lines were resistant with high micromolar IC50s. There was a near perfect correlation of CHK1 phosphorylation and CHK1 inhibitor sensitivity. The mechanism of selective inhibition in neuroblastoma is unclear as we did not identify mutations in the coding exons of CHK1. However, cell cycle analysis suggests that CHK1 inhibition in neuroblastoma causes apoptosis during S-phase, consistent with its role in replication fork progression.
Conclusion: We have identified CHK1 as a potential therapeutic target in neuroblastoma and are currently extending our work to understand the mechanism of this tumor's apparent selective sensitivity to CHK1 inhibition. As CHK1 inhibitors are currently in phase I/II clinical trials as chemosensitizers, we are also focused on determining the in vivo efficacy of combination CHK1 inhibition with chemotherapy in neuroblastoma.

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PL20

Recruitment of histone deacetylase 2 by N-Myc and c-Myc to a transrepressor complex is a general therapeutic target in Myc-driven cancer

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Increased histone deacetylase (HDAC) activity is a major factor in human carcinogenesis, in part by repression of tumour suppressor gene transcription, highlighting the potential for HDAC inhibitor therapy in cancer. We have shown that the N-Myc oncoprotein blocks terminal differentiation of neuroblastoma cells by recruiting the HDAC1 protein to a transrepressor complex at the transglutaminase-2 gene promoter, thus blocking it's expression. It is unclear whether this is a widespread feature of the transcriptome during Myc oncogenesis, or whether other HDAC proteins may be involved.
Here we show that N-Myc and c-Myc induced HDAC2, but not HDAC1, transcription by binding an E Box at the HDAC2 gene promoter in neuroblastoma and pancreatic cancer cells, respectively. Comparative cDNA microarray revealed that a small subset of genes, including cyclin G2 (CCNG2), were repressed by N-Myc and HDAC2 in neuroblastoma cells. We found that CCNG2 transcription was commonly repressed by N-Myc and HDAC2 in neuroblastoma cells, and, by c-Myc and HDAC2 in pancreatic cancer cells. Transcriptional repression of CCNG2 was, in part, responsible for N-Myc- and c-Myc-induced cell proliferation, and HDAC inhibitors reactivated CCNG2 expression. Dual-step, cross-linking, chromatin immunoprecipitation and protein co-immunoprecipitation showed that N-Myc recruited the HDAC2 protein to a transrepressor complex at an Sp1-binding site in the CCNG2 core promoter, in a manner distinct from its action as a transactivator. TH-MYCN transgenic mice develop neuroblast hyperplasia as a first step in neuroblastoma tumorigenesis. HDAC2 was up-regulated, and CCNG2 downregulated, in this precancerous hyperplasia, indicating a role for the N-Myc-HDAC2 transrepressor complex in neuroblastoma tumor initiation. N-Myc and c-Myc expression levels correlated with HDAC2 levels in human neuroblastoma and pancreatic cancer tissues, respectively. Taken together, our data indicate the critical roles of HDACs in Myc oncogenesis, and have significant implications for the use of HDAC inhibitors in the Myc-driven cancer.

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PL21

Genome-wide mapping of MYCN binding sites in neuroblastoma reveals e-box motif frequencies and associations with regions of DNA hypermethylation

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Background: Genomic amplification of *MYCN*, a member of the *MYC* family of oncogenic transcription factors, is a powerful prognostic indicator of poor clinical outcome in neuroblastoma (NB). In this study we have characterised MYCN genome-wide promoter occupancy in various NB cell lines and correlate these patterns with regions of DNA hypermethylation.
Methods: MYCN chromatin immunoprecipitates from SK-N-AS, Kelly and SHEP-21 (containing a repressible *MYCN* transgene) was applied to microarrays (NimbleGen) representing all annotated promoters in the genome. Only sites identified by two independent MYCN antibodies that recognize different epitopes were used in our analyses. In order to evaluate MYCN binding with respect to other genomic features, we determined the methylation status of all annotated CpG islands and promoter sequences using methylated DNA immunoprecipitation (MeDIP).
Results: Assessment of E-box usage within consistently positive MYCN binding sites revealed a predominance for the CATGTG motif ($p = 0.0016$),with significant enrichment of additional motifs CATTTG, CATCTG, CAACTG in the MYCN amplified state only. Gene ontology analysis revealed enrichment for the binding of MYCN at promoter regions of numerous molecular functional groups including DNA helicases and mRNA transcriptional regulation in cell lines over-expressing MYCN. A highly significant positive correlation between MYCN binding and DNA hypermethylation was identified upon integration of MYCN ChIP-chip and MeDIP data. This association was also detected in regions of hemizygous loss, indicating that the observed association occurs on the same homologue.
Conclusion: These findings suggest that MYCN binding occurs more commonly at CATGTG as opposed to the classic CACGTG E-box motif, and that disease associated overexpression of MYCN leads to aberrant binding to additional weaker affinity E-box motifs in neuroblastoma. The co-localization of MYCN binding and DNA hypermethylation further supports the dual role of MYCN, namely that of a classical transcription factor affecting the activity of individual genes, and that of a mediator of global chromatin stucture.

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PL22

Accurate prediction of neuroblastoma outcome based on miRNA expression profiles

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Background: Identification of new biomarkers and therapeutic targets is mandatory to improve risk stratification and survival rates of neuroblastoma (NB). MicroRNA (miRNA) expression is deregulated in most cancers, including NB. The purpose of this study was to evaluate miRNAs as NB biomarkers and identify miRNAs involved in NB tumor biology and prognosis.
Method/approach: MiRNA expression was analyzed in 69 NB patients using stem-loop RT-qPCR. Patient outcome was predicted based on miRNA expression patterns using support vector machines (SVM). Survival times were analyzed with Cox regression-based models (CASPAR).
Results: Of the 430 miRNAs analyzed, 307 were readily detectable. Prediction of event-free survival (EFS) with SVM and CASPAR were highly accurate, and reached 88.7% for SVM on a training set. Five-year EFS was 19% for patients predicted by CASPAR to have a poor outcome versus 78% for patients predicted to have long-term survival. Validation in an independent test set yielded accuracies of 94.7%(SVM) and 5y-EFS probabilities (CASPAR) of 25% for predicted poor outcome versus 100% for predicted long-term survival. Kaplan-Meier analysis revealed that both classifiers effectively separated patients with adverse clinical course ($p<0.001$). MYCN-amplification was highly correlated with deregulated miRNA expression, including miRNAs of the miR-17-92 cluster, the miR-181 family and miR-34a. Interestingly, 37 miRNAs correlated with expression of the TrkA neurotrophin receptor ($p<0.05$). Overexpression of TrkA in vitro regulated 6 of 11 miRNAs further analyzed, suggesting a functional relationship. Among the miRNAs most significantly correlated with TrkA expression in vivo was miR-542-5p. This miRNA was also induced upon TrkA overexpression in vitro, was inversely correlated with MYCN amplification in NB tumors and was a marker of EFS in the corresponding NB patients ($p<0.001$).
Conclusion: NB patient outcome prediction using miRNA expression is feasible and effective. Specific miRNAs such as miR-542-5p are likely to be important in NB tumor biology, and may qualify as potential therapeutic targets.

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PL23

Individual survival time prediction from gene-expression and/or global genomic data of neuroblastoma patients using CASPAR

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Background: Both array-CGH (aCGH) and gene-expression (GE) data have been used to predict outcome of neuroblastoma patients. However, integration of information from both platforms is not established. Here, we report on an extension of our CASPAR algorithm that allows using data from either platform alone or in combination to predict individual survival time (as a continuous variable) of neuroblastoma patients.
Methods/approach: GE and aCGH data were generated from 128 neuroblastoma patients. Then, CASPAR was applied to GE data alone, aCGH data alone and to combined GE+aCGH data (Comb) using a leave-one-out crossvalidation. In addition, a simple genomic predictor based on intrachromosomal variation (VAR) was built. Subsequently, CASPAR predicted OS times for the total cohort and for patients with 11q-deletion (n=37), 17q-gain (n=71) and MYCN-amplified disease (n=27). Prediction accuracy was assessed by Kaplan-Meier analyses, and ROC curve analyses.
Results: For the total cohort, CASPAR separated patients with distinct outcome with high accuracy from all data sets (GE: 5y-OS 0.81 (patients with predicted long survival) vs. 0.28 (predicted short survival); aCGH 0.77 vs 0.46; Comb 0.82 vs. 0.23; VAR 0.78 vs. 0.37; all $p<0.0001$). Predictions from GE data alone and combined GE+aCGH data achieved highest accuracies as detemined by area under the ROC curve (AUC) calculation (GE: 0.86±0.07, Comb: 0.87±0.03; aCGH alone 0.73±0.06). Remarkably, predictions based on VAR were similar to predictions on full aCGH data (AUC 0.75±0.06), indicating that global genomic information can be condensed without loosing predictive capability. Similar results were observed for subgroups with del11q and gain17q, while outcome of MYCN-amplified patients could not be predicted accurately with neither data set.
Conclusion: CASPAR is the first algorithm able to predict patients' individual OS time from either GE or aCGH data or a combination of both. In case of neuroblastoma, expression information alone appears to reflect tumor behavior more accurately than global genomic information, and combined information from aCGH and GE data does not significantly improve prediction accuracy.

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PL24

Identification of multiple protein disrupting mutations in stage 4 neuroblastoma using next generation sequencing transcriptome analysis

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Background: Neuroblastoma is a small round blue cell tumor of childhood. Fifty percent of patients present with high risk disease and despite aggressive multimodal therapy approximately 60% percent of these patients die from their disease. Currently, only a handful of molecular alterations are known to influence prognosis, but no clear mechanism of pathogenesis has been demonstrated.
Method/approach: We sequenced the transcriptomes of 20 stage 4 tumors, including ten MYCN amplified and ten MYCN non-amplified samples, using massively parallel sequencing technology. In our analysis pipeline, 50 nucleotide filtered reads are aligned to the reference human genome (hg 18). Reads that align were analyzed for: 1) base coverage, 2) transcript expression levels, 3) calling SNVs and 4) determination of damaging SNVs by Sorting Intolerant From Tolerant (SIFT) analysis.
Results: Initial analysis of the first six samples, yielded an average of 86.6 million uniquely mapped reads per sample. On average we detected the expression of 6,000 genes to a depth of 10x. The RNA seq expression profile correlated well with expression array data from the same sample (r=0.62), while the sequencing data identified an additional 3,000 genes, not detected by microarray. Using the SAMtool, an average of 1,255 nonsynonymous SNVs were detected per sample. Of these nonsynonymous SNVs, 69-160 per sample were predicted by the SIFT algorithm to be damaging. Interestingly, 10 different genes had damaging nonsynonymous SNVs in at least 20% of the samples.
Conclusion: Next generation sequencing of transcriptomes is a powerful and more sensitive method than microarrays for expression profiling and allows for the identification of novel transcripts including non-coding RNAs. Here we report the most extensive profiling of the neuroblastoma transcriptome to date. We identified several hundred protein disrupting SNVs, and of these 10 were commonly altered. Ongoing analysis is underway to validate our results. The identification of recurrent genetic alterations will assist in developing a better understanding of the mechanisms of pathogenesis of neuroblastoma and lead to new therapeutic targets

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PL25

Modeling neuropsychiatric disorders in the mouse

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PL26

Genetic and developmental therapeutic studies in a transgenic mouse model for high-risk neuroblastoma

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See page 71 for biography

Neuroblastoma is the third most common tumor of childhood. Amplification of MYCN is the best-characterized genetic marker for neuroblastoma, and generally marks high-risk disease. We targeted expression of MYCN to the peripheral neural crest of transgenic mice to generate a mouse model for this disease. We and others have characterized this model, and have demonstrated significant genetic and biological parallels with high-risk neuroblastoma. The talk will focus on our use of this model to dissect basic biology and genetics in neuroblastoma and as a platform for developmental therapeutics.

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PL27

Widespread dysregulation of miRNAs by MYCN amplification and chromosomal imbalances in neuroblastoma: Association of miRNA expression with survival

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Background: MiRNAs regulate gene expression at a post-transcriptional level and their dysregulation can play major roles in the pathogenesis of different forms of cancer, including neuroblastoma. The purpose of this study was to identify patterns of differential miRNA expression predictive of outcome in neuroblastoma.

Methods: We analyzed a set of neuroblastoma (n = 145) that is broadly representative of the genetic subtypes of this disease for miRNA expression (430 loci by stem-loop RT qPCR) and for DNA copy number alterations (array CGH). The tumors were stratified and then randomly split into a training set (n = 96) and a validation set (n = 49) for data analysis.

Results: Thirty-seven miRNAs were significantly differentially expressed in MYCN amplified relative to MYCN single copy tumors, indicating a potential role for MYCN in either the direct or indirect dysregulation of these loci. We also identified a significant correlation between miRNA expression levels and DNA copy number, indicating a role for large-scale genomic imbalances in the dysregulation of miRNAs. To directly assess if miRNA expression was predictive of clinical outcome, we used the Random Forest classifier to identify miRNAs most significantly associated with poor overall survival (OS). A 15 miRNA signature predictive of OS with 72.7% sensitivity and 86.5% specificity in the validation set was identified. Furthermore, 11q- tumors could be split into two subgroups which differed significantly in both clinical outcome and the overall frequency of other large scale genomic imbalances using the 15-miRNA survival signature. Interestingly, the two subgroups differed significantly in frequency of genomic imbalances that were independently associated with poor survival in the overall tumor cohort.

Conclusion: There is widespread dysregulation of miRNA expression in neuroblastoma tumors caused by over-expression of MYCN and by large-scale chromosomal imbalances. We show a miRNA signature predicative of clinical outcome, and capable of subdividing 11q- cases into two distinct clinical groups. This work highlights the potential for miRNA mediated diagnostics and therapeutics.

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PL28

Evaluation of *PHOX2B*, tyrosine hydroxylase (*TH*), *GD2* and *ELAVL4* expression for minimal residual disease (MRD) detection in neuroblastoma patients

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Aim: To assess applicability of PHOX2B, TH, GD2 and ELAVL4 expression for MRD detection in NB patients(pts).

Methods: Expression of molecular markers was evaluated by real-time PCR(RQ-PCR) in 84 bone marrow(BM) samples from 27 NB pts on different stages of disease, in 16 BM samples from 16 pts without malignancies and in 3 NB cell lines(IMR-32, SK-N-MC, Kelly). Values of the tumor genes expression were normalized to ABL. Analytical sensitivity(AS) of PQ-PCR was assessed by 10-fold dilution series of IMR-32 RNA in RNA of healthy volunteers. All NB pts' samples were also tested by cytology. Samples were considered as true positive(TP) in case of detectable PHOX2B expression or tumor cells presence in BM smears. For each MRD marker threshold level(TL) was calculated by ROC curve analysis. Diagnostic sensitivity(DS), specificity(Sp), positive and negative predictive values(PPV, NPV), overall correct prediction(OCP) were calculated for every marker.

Results: AS of PHOX2B, TH and ELAVL4 expression detection in IMR-32 cell line achieved $1 \bullet 10^{-6}$, while AS of GD2 assessment was $1 \bullet 10^{-5}$. All tested markers were highly expressed in NB cell lines. PHOX2B and TH expression was not detected in normal BM. GD2 and ELAVL4 expression was revealed in 10 of 16 normal BM. 47 of 84 (56.0%) NB samples were TP. Results of diagnostic performance tests are shown in table.

	Diagnostic sensitivity (DS)	Specificity (Sp)	Positive predictive value (PPV)	Negative predictive value (NPV)	Overall correct prediction (OCP)
PHOX2B+	0.915	1.000	1.000	0.902	0.952
TH+	0.872	0.947	0.953	0.857	0.917
ELAVL4+	0.447	1.000	1.000	0.594	0.702
GD2+	0.383	0.974	0.947	0.561	0.655
PHOX2B+ or TH+	0.957	0.946	0.957	0.946	0.952

Despite relatively high Sp of GD2 and ELAVL4 expression evaluation, these markers had low DS. Due to relatively low OCP GD2 and ELAVL4 were excluded from further analysis. In contrast, TH and PHOX2B showed both high DS and Sp. As OCP for both TH and PHOX2B was high, we estimated applicability of MRD monitoring approach where samples were defined positive in case of either PHOX2B or TH expression higher than TL. In comparison with PHOX2B, TH addition led to higher DS, but lower Sp, while OCP remained stable.

Conclusions: In our series PHOX2B and TH were the most sensitive MRD markers in NB pts. TH addition did not bring significant benefits in comparison with PHOX2B only. GD2 and ELAVL4 assessment did not show any relevance for MRD monitoring.

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PL29

QRT-PCR for TH and Phox2B mRNA in peripheral blood and bone marrow from children with high risk neuroblastoma predicts overall survival; a SIOPEN molecular monitoring group study

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Aim: To determine the clinical significance of detecting Phox2B and/or tyrosine hydroxylase (TH) mRNA by QRT-PCR in bone marrow (BM) and peripheral blood (PB) from children entered into the HR-NBL-1/SIOPEN trial.

Methods: BM and PB samples were collected and processed according to SOPs (Viprey et al, 2007, EJC, 43, 341-350; Viprey et al, 2008, J. Pathol, 216, 245-252). QRT-PCR was performed on BM samples from 230 children at diagnosis (Dx) and 175 pre-myeloablative therapy (preMAT) for TH mRNA, and from 199 children at Dx and 159 preMAT for Phox2B mRNA. TH mRNA was also measured in 224 and 156 PB samples at Dx and preMAT respectively, Phox2B was measured in 212 and 144 PB samples at Dx and preMAT respectively.

Results: The frequency of detecting TH and Phox2B mRNA in BM at Dx was 93% (214/230) and 88% (176/199) respectively, and preMAT 82% (144/175) and 58% (92/159). TH and Phox2B were highly correlated at Dx ($r=0.92$, $p<0.001$) and preMAT ($r=0.77$, $p<0.001$). The BM-Dx values for TH and Phox2B predicted strongly for survival and relapse, showing independent predictive power in this high risk group of children. These factors predominated in multivariate Cox model analyses, with an additional $\beta 2$ of 13.4 ($p=0.0003$) for analysis using TH and allowing for the prior inclusion of stage in the model, and $\beta 2$ of 10.3 ($p=0.001$) with prior inclusion of positive histology in BM at diagnosis. Log values of TH and Phox2B mRNA levels showed a clear threshold effect in predicting survival. The threshold log value differed depending on time point (Dx or preMAT) and sample type (BM or PB). Hazard ratios (HRs) for survival were 3.14 (95% CI 2.02-4.89) and 3.28 (95% CI 1.99-5.39) respectively for TH and Phox2B in BM at Dx using the threshold cut-off. Similar effects were observed for values in the PB at Dx (HR=2.98 [95% CI 1.61-5.50] for TH and HR=3.30 [95% CI 1.96-5.57] for Phox2B) and for values in the BM preMAT (HR=2.39 [95% CI 1.44-3.96] for TH and HR=2.89 [95% CI 1.65-5.09] for Phox2B).

Conclusion: Phox2B and TH mRNA detected by QRT-PCR in BM and PB from children with high risk neuroblastoma at diagnosis and preMAT predicts overall survival.

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PL30

Analyses of mIBG scoring as a prognostic indicator in patients with stage 4 neuroblastoma. A Children's Oncology Group (A3973) report

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Background: Over the past 2 decades, radiolabeled metaiodobenzylguanidine (mIBG) has proven to be a highly sensitive marker for the detection of neuroblastoma (NBL). Recently, a semiquantitative mIBG scoring method (Curie score) has been developed. The aim of this study was to correlate mIBG scores with outcome in a group of uniformly treated patients (pts).

Methods: Newly diagnosed pts with stage 4 NBL enrolled on COG A3973 were examined. MIBG scans were evaluated at the time of diagnosis (n=280), post-induction (n=274), post-transplant (n=203), and upon completion of biotherapy (n=99). Pts with non-mIBG avid disease at diagnosis (Dx) were excluded (n=29). For each time point, mIBG scans were evaluated at 10 anatomic sites. Scans were read by 2 observers, using a semiquantitative scoring method. Individual sites were scored 0-3, based upon extent of disease at each site. Absolute scores (cumulative score at each time point) and relative scores (absolute to initial score ratios) were correlated with event free (EFS) and overall survival (logrank test).

Results: The median Curie score at Dx was 12 (range 1-30). There was no correlation between Curie score at Dx and EFS. In contrast, pts with a Curie score > 5 following induction therapy had a significantly worse EFS when compared to those with a score < 5 (3-yr EFS: 8.3±4.6% vs 41.5±3.5%, $p<0.0001$). The presence of mIBG avid disease in either the T or L-spine, chest, pelvis or distal lower extremities post-induction was associated with a 3-yr EFS <15%. Curie scores (0 vs >0) post-induction had a greater impact in pts with MYCN amplified tumors (3 yr EFS: 45.2±7.0% vs 15.0±8.0%, $p=0.014$) than those with MYCN non-amplified disease (3 yr EFS: 45.4±5.8% vs 35.9±6.8%, $p=0.088$). Pts with Curie scores > 0 post-transplant had a lower EFS than those with a Curie score of 0 (3-yr EFS: 28.6±6.5% vs 45.2 ±4.6%, $p=0.023$). Relative scores (≤ 0.5, ≤ 0.25) were highly significant, but did not change results using absolute scores at any time point.

Conclusion: Pts with Curie scores >5 after induction have a 3 yr EFS <10% and should be considered for alternative therapy regimen.

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PL31

Characterization of neuroblastoma imaging studies using F-18-DOPA PET/CT

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Objectives: Tumors of ganglion cell origin including ganglioneuroma, neuroblastoma and ganglioneuroblastoma are common tumors in children. Iodinated MIBG and FDG PET-CT are the choice for functional imaging studies. In the recent year, F-18 DOPA has emerged as a new diagnostic tool for neuroendocrine tumors. We try to apply and characterize the functional status of the neuroblastoma in limited number of cases of our institution.
Methods: After 100 mg of carbidopa was given orally for 60 mins, the patients was injected with 200 MBq (5.4 mCi) of F-18-DOPA and wait for 90 mins for imaging. Whole body imaging was performed using PET/CT. The patients also received the standard I123-MIBG, FDG PET-CT imaging studies.
Results: Nineteen patients with neuroblastoma were enrolled in this study. Their ages ranged from 0.5-12.8 years old. All patients had F-18-DOPA PET scan. Three patients were primary diagnosis/staging of disease and 16 cases were restaging of disease. Three primary diagnosis patients showed positive uptake of F-18-DOPA in primary and metastasis lesions. In restaging patients, five patients without uptake of F-18-DOPA showed negative standard imaging studies. Eleven patients with uptake of F-18-DOPA showed only 6 positive I123-MIBG and 7 positive FDG PET/CT. In organ-region-specific analysis, there were different uptake pattern in 3 imaging studies.
Conclusion: No study on the possible role of F-18-DOPA in neuroblastoma has been published yet. In our study, we found a major drawback of FDG PET/CT was lack of visualization of lesions in the liver and cranium because of high physiologic activity. Another disadvantage of FDG PET/CT was not disease-specific. F-18-DOPA is a better substrate for the cell membrane norepinephrine transporter than MIBG and a more specific substrate for neuroblastoma cells than FDG. F-18-DOPA might provide more additional information than FDG PET/CT in this area. F-18-DOPA positivity indicates the ability of tumor cells to accumulate and the ability to decarboxylate F-18-DOPA by AADC in a well differentiated tumor or tumor component. This might indicate better prognosis. The clinical significance needs further follow up.

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PL32

Clinical and biological features predictive of survival after relapse of neuroblastoma: A study from the International Neuroblastoma (NB) Risk Group (INRG) Database

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Background: In NB, most patients (pts) who relapse eventually die. Prognostic factors are used to stratify treatment at diagnosis, but typically not at the time of relapse. Our goals were to determine a) which factors were predictive of time to death post-relapse; b) if time from diagnosis until relapse/progression has a predictive role.
Methods: Retrospective analysis included INRG pts with first event of relapse, progressive disease, or secondary malignancy (excluding pts whose first event was death). Time from diagnosis until event ("time-to-first-event") was calculated and analyzed as <1 year (yr) vs ≥1 yr. 5-yr estimates of overall survival (OS ± standard error), time from first event until death or last contact, are presented (lifetable methods). Time-to-first-event was tested in a multivariable Cox model (adjusting for nonproportional hazards) with clinical and biologic factors; hazard ratios (HR) for increased risk of death post-relapse were calculated.
Results: From 8,800 INRG pts, 2,266 experienced a non-death first event. Median time to relapse was 13.2 months (mo) (range: 1 day to 11.4 yrs). The 5-yr OS after first event was 20%±1%. Time-to-first-event (HR=1.8), age >18 mo (HR=2.3), INSS stage 4 (HR=3.4), MYCN amplified (HR=2.8), diploidy (HR=1.6), high MKI (HR=2.0), undifferentiated grade (HR=1.6), and 1p aberration (HR=1.7) were significantly predictive of death after relapse (p<0.0001), but not 11q aberration. Compared to pts whose first event occurred <6 mo from diagnosis, pts who relapsed 6-<18 mo from diagnosis had increased risk of death, while relapses ≥18 mo from diagnosis had decreased risk of death. Shorter time-to-first-event was not independently predictive of death after adjustment for undifferentiated grade, high MKI, MYCN amplification, or diploidy. We found the same results, when we analyzed relapses in stage 4> than 18m.
Conclusions: Time to first relapse is a significant predictor of time to death after relapse; the risk of death is higher for pts who relapse within 6-<18 mo, but lower for pts who relapse ≥18 mo from diagnosis. Stratification of relapsed NB pts according to the timing of first relapse, age, stage, *MYCN*, and MKI, and diploidy is important in retrieval study designs.

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PL33

Topotecan-vincristine-doxorubicin in metastatic neuroblastoma failing to respond to rapid COJEC. Preliminary results of a SIOPEN Group Study

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Background: This study has evaluated activity and toxicity of the Topotecan-Vincristine-Doxorubicin (TVD) combination administered to patients (pts) with stage 4 neuroblastoma failing to achieve remission after induction therapy (rapid COJEC) according to the HR-NBL-1 SIOPEN protocol.
Methods: Pts above 1 year of age with stage 4 neuroblastoma, who failed to achieve metastatic remission with rapid COJEC were eligible. Topotecan was administered at 1.5 mg/m2/day for 5 days, followed by 48-hour infusion of vincristine, 2 mg/m2, and doxorubicin, 45 mg/m2. Tumor response was assessed after 2 TVD courses, according to the INSS criteria. Pts achieving CR or VGPR (metastatic CR) underwent myeloablative therapy (MAT) made of BU-MEL or CEM, followed by PBSC rescue. Pts who achieved PR received 2 further TVD courses and then were re-assessed. In case of CR or VGPR, treatment was continued according to HR-NBL-1 (BU-MEL) standard arm. Pts who failed to achieve PR after 2-4 TVD courses, or developed PD were withdrawn from the study and were treated at physicians' discretion.
Results: Sixty-six pts who did not achieved CR or VGPR after rapid COJEC were enrolled in the study. After 2 TVD courses, responses of 51 assessable pts included CR in 3, VGPR in 11, PR in 17, MR in 6, NR in 12, PD in 2 (overall response rate 60 %). Twenty-three pts who achieved CR or VGPR or PR (metastatic CR) received MAT (random BU-MEL vs CEM) according to protocol. Twenty-six/66 patients are presently alive. Toxicity was mostly hematopoietic. 49 pts experienced grade 4 neutropenia, 44 grade 4 thrombocytopenia and 11 grade 4 anemia after the first course. 43 pts developed grade 4 neutropenia, 35 grade 4 thrombocytopenia and 12 grade 4 anemia after the second course. Systemic antibiotic therapy required hospitalization for 19 pts after the first course, and for 15 pts after the second. Results were recorded in the SIOPEN-R-NET database.
Conclusion: TVD combination was active and tolerable in pts with metastatic neuroblastoma after treatment with rapid COJEC.

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PL34

Suppression of human anti-mouse antibody response by rituximab plus cyclophosphamide permits continuation of anti-GD2 immunotherapy

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Background: Anti-GD2 monoclonal antibodies (MoAb) are effective against high risk NB. Human anti-mouse antibodies (HAMA) arising after mouse or chimeric MoAb treatment can interfere with MoAb binding to GD2 and neutralize the benefit of further immunotherapy. We hypothesized that destruction of B and T cells by the combination of rituximab and cyclophosphamide may suppress preformed HAMA and allow MoAb therapy to continue.
Method/approach: Ultra-high risk NB patients receiving anti-GD2 immunotherapy with mouse anti-GD2 MoAb 3F8 plus GMCSF (NCT00072358) who developed HAMA (>1000U/ml) were treated with intravenous (IV) rituximab (375mg/m2/dose on days 1 and 15) plus IV cyclophosphamide (750mg/m2 on day 16) (R-C). Patients resumed immunotherapy when HAMA levels subsided.
Results: 41 patients with elevated HAMA titers received R-C. Patients had previously received a median of 4 (range 1-10) cycles of 3F8/GMCSF with median HAMA titer of 2521 (range 1034-21385) U/ml. 6 patients developed allergic reactions to rituximab: 4 completed R-C; 1 received a single dose of rituximab while 1 could not complete the first dose and was inevaluable for response. Febrile neutropenia or unexpected infections were not observed in any patient. HAMA titers were abrogated in 30/40 (75%) evaluable patients at a median of 60 (range 9-245) days but remained >1000U/ml in 10(25%) patients at a median of 127 (range 13-511) days after R-C. 28/30 R-C-responsive patients went on to receive further 3F8/GMCSF. 17/26 evaluable patients (65%) continued to be HAMA-negative after rechallenge receiving a median of 3 (range 1-9) further 3F8/GMCSF cycles. 9/26 (35%) redeveloped HAMA that persisted at a median 141 (range 17-395) days after 3F8 rechallenge.
Conclusion: R-C therapy was safe and well tolerated in heavily pretreated patients with NB. It suppressed preformed HAMA response and permitted continuation of MoAb therapy in most patients. This is the first report of an effective strategy to suppress ongoing host immune response against IgG proteins. It may have general application in suppressing or delaying anti-MoAb responses, thereby rendering MoAb immunotherapy more effective.

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PL35

Long term outcome: the price of treatment for surviving high-risk neuroblastoma

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Background: Megatherapy has been proven to be superior in randomized trials and is currently the backbone of most treatment regimen for high risk neuroblastoma. However, long term outcome and the risk of late effects have not been addressed in these patients so far.
Methods: We analysed our previously published cohort of 295 randomized high risk neuroblastoma patients (stage 4 or MYCN amplified) with special focus on late recurrences and therapy related late effects. After induction chemotherapy, patients were randomized to megatherapy with stem cell rescue or to maintenance therapy (4 cycles of oral cyclophosphamide).
Results: The cohort is currently followed for up to 12 years (median observation time: 8.4 years). The long-term results confirmed the better outcome after megatherapy in the “as-randomized”, “as-treated” and “treated-as-randomized” analysis. Megatherapy (n=143) was early complicated by pneumonia in ten, other severe infectious complications in eight, veno-occlussive disease in eight and renal failure in three patients. Five patients died due to megatherapy-related complications. Only one out of 119 patients treated with maintenance therapy developed pneumonia. No patient died to maintenance therapy related complications. Relapses occurred later in patients treated with megatherapy (74/143 pts., 8-85 months, median 21 months) than in patients with maintenance therapy (83/119 pts., 7-60 months, median 16 months, p<0.001). Patients died up to nine years after diagnosis (megatherapy: median 30 months; maintenance therapy: 20 months, p<0.001). One patient of each arm developed secondary leukaemia. Major late effects were analysed in 109 patients surviving five years or longer (megatherapy n=68, maintenance n=41), and were more often found in patients of the megatherapy arm: hearing loss 72% vs. 51% (p=0.04), tubular damage 18% vs. 12% (n.s.), hypothyroidism 19% vs. 2% (p=0.02), focal nodular hyperplasia of the liver 10% vs. 0% (p=0.04), impaired growth 10% vs. 0% (p=0.04).
Conclusion: Megatherapy proofed effective with respect to long term outcome, but is complicated by acute toxicity and by an increased risk of long term effects.

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PL36

Long-term toxicity in survivors of ENSG5 trial for children with high-risk neuroblastoma

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Background: Due to the use of intensive therapies survivors of high-risk neuroblastoma potentially face many complications. Our aim is to provide a long-term follow-up of metastatic neuroblastoma survivors included in the ENSG5 protocol from 1990 to 1999.
Methods: Patients were randomised to receive the same induction drug doses but in one arm the dose intensity was 1.8 times greater (OPEC/OJEC vs. COJEC), surgical removal of primary tumour and high-dose melphalan with stem cell rescue. 262 children were randomized, 62 survived more than 5 years and 57 of them were analyzed. Information from ENSG5 yearly updated database was gathered and questionnaires were sent to participating centres (73.7% responses).
Results: Median follow-up was 12.87 (6.88-16.49) years. Overall, 44 children (77.2%) developed at least one complication and these were severe in 11 cases (19.3%). Twenty-eight children (49.1%) developed hearing loss. This was severe (Brock grade 3 and 4) in 5 (8.8%). 9 patients (15.8%) developed decreased GFR, but no cases of renal failure or tubulopathy were documented. Endocrine complications (28.1% of children) included mainly hypogonadism, delayed growth and delayed puberty. Neurocognitive issues (behavioral problems, speech or learning difficulties) were reported in 12 cases (21%). Three children developed second malignancies 5 years after diagnosis: one localized osteosarcoma, one carcinoma of the parotid gland and one anaplastic ependymoma. No haematologic malignancies/myelodysplasia were documented. There were no deaths in remission during follow-up. There were no differences between the two induction treatment arms.
Discussion: This study presents a homogeneous cohort of high-risk neuroblastoma survivors from a multi-institutional randomized trial with a low profile of long-term toxicity compared to previous studies. However, 3 cases of secondary malignant solid neoplasms have developed so far. Long-term toxicity was not increased in patients receiving more intensive COJEC chemotherapy compared to standard arm. It is likely that with current more intensive treatment regimens using radiotherapy for local control, the burden on survivors could increase.

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Parallel session 1 – Targeting kinases OR1–OR07

OR1

The KidsCancerKinome: Validation of Aurora kinases as potential drug targets in neuroblastoma and other pediatric tumors

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KidsCancerKinome (KCK) is a translational research effort connecting 9 European labs with 2 European SME's aimed at the systematic investigation of the human protein kinase family to validate novel drug targets and develop targeted therapies. Many novel kinase inhibitors are under development for adult oncology and KCK will test their in vitro activity against the tumor-driving kinases identified in this program. Successful small molecule inhibitors will be taken further to in vivo validation in established xenograft models of the six childhood tumor types.
The experimental approach encompasses:
1. target presence analyses (mRNA and protein expression of the human kinome)
2. molecular validation of kinase tumor dependency (RNAi)
3. kinase mutation analysis
4. in vitro drug efficacy testing
5. in vivo proof-of-principle of drug efficacy
We have validated Aurora kinase A and B as potential drug targets in six highly malignant pediatric tumor types (i.e Ewing sarcoma, osteosarcoma, rhabdomyosarcoma, neuroblastoma, medulloblastoma and ALL). The stepwise procedure started with the extensive analyses of expression of human kinases using Affymetrix mRNA profiles of over 500 tumors and cell lines. Clustering analyses on the combined data of all tumor types revealed a cluster containing many G2M kinases that showed significantly higher expression patterns than in the reference tissues. Prominently present in the G2M cluster were Aurora kinase A and B, which expression could be correlated to poor prognosis in the individual tumor types in further analyses.
Subsequently, lentiviral shRNA-mediated knockdown of AURKA and AURKB protein expression has been performed in cell line panels for each tumor type to evaluate the kinases for their potential as drug targets. Inhibition of the Aurora kinases resulted in significant phenotypes in several pediatric tumor cell lines ranging from growth inhibition to extensive cell death. The knockdown of AURKA or AURKB often leads to induction of apoptosis, although preceded by a different type of cell arrest. These findings were promising for further evaluation of Aurora kinase inhibitors in the core panel of sensitive pediatric tumor cell lines.

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OR2

Inhibition of Aurora-A as an approach to control N-Myc levels in neuroblastoma

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The oncogene MYCN belongs to the MYC family of transcription factors with the basic region/helix-loop-helix/leucine zipper domain (bHLHZip). Amplification of MYCN in neuroblastoma is one of the strongest predictors of aggressive disease, resistance to therapy and poor prognosis. To explain the aggressive phenotype of MYCN-amplified neuroblastoma and in order to identify potential molecular targets for the therapy of these tumours, we used a RNA-interference screen to identify a small group of genes that are required for the growth of MYCN-amplified neuroblastoma cells, but largely dispensable in cells without MYCN-amplification. One of the identified genes encodes Aurora-A. We have previously shown that Aurora-A has a critical function in stabilizing the N-Myc protein (Otto et al., 2009 Cancer Cell).
We have now found that several small molecule inhibitors of the Aurora-A kinase reduce N-Myc protein levels in neuroblastoma cells, demonstrating that catalytically active Aurora-A is required to maintain N-Myc protein levels. Our results show that Aurora-A uses two distinct mechanisms to stabilize N-Myc: First, Aurora-A interacts in a kinase-independent manner with both N-Myc and the ubiquitin ligase SCFFbxw7 and promotes the synthesis of non-K48-linked ubiquitin chains that do not support degradation. We found that Aurora-A recruits the ubiquitin-conjugating enzyme UbcH5 that can conjugate to K11, K63 in addition to K48 ubiquitin chains, providing a mechanistic basis for these observations.
Second, Aurora-A inhibitors decrease phosphorylation of GSK-3beta at Ser 9, demonstrating that Aurora-A directly or indirectly controls phosphorylation at this site. As a result, inhibition of Aurora-A activates Gsk3, promoting degradation of N-Myc. We are currently testing whether Aurora-A is a direct Gsk3 kinase and whether inhibition of Aurora-A and of Akt, a key kinase for this residue, synergize in regulating Gsk3ser9 phosphorylation and N-Myc levels.
Taken together, our data indicate that small molecule inhibitors of Aurora-A may be a tool to inhibit MYCN activity for neuroblastoma tumor therapy.

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OR3

Molecular analysis and therapeutic targeting of the PI3K/AKT/mTOR pathway in paediatric neuroblastoma

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Background: The PI3K/AKT/mTOR cell-signalling pathway plays a key role in major cellular functions including cell growth, survival and angiogenesis. Deregulation of this pathway is observed in MYCN amplified neuroblastoma (NBL), making it an attractive target for inhibitors of this pathway. Novel imaging modalities such as small animal ultrasound (US) and fluoro-deoxy glucose-positron emission tomography (FDG-PET) are emerging as useful tools for evaluating interventions in murine models.
Method/approach: Homozygous TH-MYCN transgenic mice underwent serial abdominal US from four weeks of age until neuroblastomas greater than 75mm^3 were detected. Tumour volumes were calculated and baseline FDG-PET scans were performed followed by a 7-day intervention with PF04691502, a combined PI3K/mTORC1 inhibitor. Repeat US and FDG-PET were performed at 48 hours. Tumour:background (T:B) ratios for the radio-labelled FDG tracer were used as a measure of avidity. Tumours were harvested for western blot (WB) and immunohistochemistry (IHC) analysis of key proteins in the PI3K/AKT/mTOR as well as markers of senescence, apoptosis and angiogenesis.
Results: A significant decrease in the FDG uptake was observed following treatment with PF04691502 (T:B ratios 4.6±1.8 reduced to 1.7±0.3) when compared with vehicle (T:B ratios 3.5±0.7 compared to 5.0± 0.8 post treatment, $p<0.001$). Treatment with PF04691502 also improved survival at 7 days (100%) compared with vehicle (33.3%), $p<0.01$. WB analysis demonstrated decreased levels of MYCN. WB and IHC showed reduced tumour vascularity as evidenced by a significant decrease in CD31 staining and the induction of apoptosis.
Conclusion: Both small animal FDG-PET and US have been validated as robust tools for use in the TH-MYCN transgenic mouse model. PF04691502 significantly decreased uptake of FDG, suggesting inhibition of metabolism and/or tumour viability. Treatment with PF04691502 induced apoptosis, reduced expression of MYCN and also reduced tumour vascularity. These data indicate the PI3K/AKT/mTOR pathway is a promising therapeutic strategy in MYCN amplified neuroblastoma, targeting multiple cellular mechanisms.

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OR4

PI3K inhibitors prime neuroblastoma cells for chemotherapy *in vitro* and *in vivo* by shifting the balance towards pro-apoptotic Bcl-2 proteins and increased mitochondrial apoptosis

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Aberrant activation of the PI3K/Akt/mTOR cascade is a characteristic feature of human cancers and has been associated with poor prognosis. We recently identified Akt activation as a novel predictor of poor outcome in neuroblastoma. Therefore, we investigated whether inhibition of PI3K/Akt signaling presents a novel approach for chemosensitization of neuroblastoma. Here, we provide first evidence that the PI3K inhibitor PI103 synergistically induces apoptosis in combination with various anticancer drugs including Doxorubicin, Etoposide, Topotecan, Cisplatin, Vincristine and Taxol. Mechanistic studies reveal that PI103 cooperates with Doxorubicin to downregulate Mcl-1, to upregulate Noxa and Bim levels and to inhibit Bim phosphorylation. This shifted ratio of pro- and anti-apoptotic Bcl-2 proteins results in increased Bax conformational change, loss of mitochondrial membrane potential, cytochrome c release, caspase activation and apoptosis upon combined treatment with PI103 and Doxorubicin. Knockdown of Mcl-1 enhances Doxorubicin-induced apoptosis, while silencing of Noxa, Bax/Bak or p53 reduces Doxorubicin-mediated cell death. The central role of the mitochondrial pathway for chemosensitization is underscored by Bcl-2 overexpression, which inhibits Bax activation, mitochondrial perturbations, cleavage of caspases and apoptosis. Interestingly, the caspase inhibitor zVAD.fmk blocks caspase activation and apoptosis without interfering with Bax activation or mitochondrial outer membrane permeabilization. This places mitochondrial events upstream of caspase activation. PI103 and Doxorubicin cooperate to induce apoptosis in patients' derived primary neuroblastoma cells, supporting the clinical relevance of the results. Most importantly, combined treatment with PI103 and Doxorubicin is superior to either agent alone to suppress tumor growth in an in vivo model of neuroblastoma. By demonstrating that PI3K inhibitors such as PI103 prime neuroblastoma cells for chemotherapy-induced apoptosis in vitro and in vivo, these findings have important clinical implications for the development of targeted therapies to increase chemosensitivity of neuroblastoma.

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OR5

PLK1 is a novel target for high-risk neuroblastoma therapy

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Background: High-risk neuroblastoma remains a therapeutic challenge for pediatric oncologists. The Polo-like kinase 1 (*PLK1*) is a key regulator of eukaryotic cell division and serves as a negative prognostic marker in many human cancers. This enzyme is a target of the novel small-molecule inhibitor BI 2536, which has already shown promising anti-cancer activity in adult malignancies. In this study, we investigated the effect of BI 2536 on neuroblastoma cells *in vitro* and *in vivo* to explore PLK1 as a potential target in high-risk neuroblastoma therapy.
Methods: Oligonucleotide-microarray profiles of 476 neuroblastoma specimens were analyzed and mRNA levels of *PLK1* were correlated with prognostic markers and outcome. To explore the effect of *PLK1* inhibition on growth properties of neuroblastoma cells, seven cell lines were treated with various concentrations of BI 2536 and changes in cell proliferation and cell cycle distribution were determined. Furthermore, nude mice with IMR-32 neuroblastoma xenografts were treated with BI 2536.
Results: Up-regulation *PLK1* transcript levels is associated with markers of unfavorable prognosis like disseminated stage 4, age >18 months and *MYCN* amplification ($p<0.001$ each). Moreover, high *PLK1* expression is significantly correlated with unfavorable gene expression-based classification and adverse patient outcome ($p<0.001$ each). Analysis of *PLK1* protein levels revealed high expression in neuroblastoma cell lines and in samples from patients with poor outcome. On treatment with nanomolar doses of BI 2536, all seven neuroblastoma cell lines showed reduced proliferation, cell cycle arrest and cell death with LC50 values ranging from 2.2 nmol/L to 31.2 nmol/L. BI 2536 treatment of nude mice bearing IMR-32 neuroblastoma xenografts resulted in significant inhibition of tumor growth ($p<0.001$).
Conclusions: High expression of *PLK1* is significantly associated with high-risk neuroblastoma and unfavorable patient outcome. Inhibiting its function with BI 2536 has strong anti-tumor activity on human neuroblastoma cells *in vitro* and *in vivo*, opening encouraging perspectives for the treatment of high-risk neuroblastoma.

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OR6

MicroRNA-184 inhibits neuroblastoma cell proliferation and promotes apoptosis by targeting the serine/threonine kinase *AKT2*

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Background: Prior studies indicate that miR-184 is significantly under-expressed in MYCN amplified neuroblastoma (NB) tumors and that ectopic up-regulation of this miRNA results in the induction of a caspase mediated apoptotic pathway in NB cell lines (*Cancer Res*. 2007;67:976). Here, we elucidate the target responsible for the molecular mechanism of miR-184 action.
Method/Results: MiR-184 is computationally predicted to target the 3'UTR of *AKT2*. Given that *AKT2* is a member of the PI3K pathway, one of the most potent pro-survival pathways in cancer, the interaction between miR-184 and *AKT2* was further explored. Analysis of primary NB tumors indicated a significant inverse correlation between miR-184 and *AKT2* expression, while transfection of miR-184 into Kelly or SK-N-AS cell lines resulted in a highly significant decrease in both *AKT2* mRNA and protein levels. Conversely, transfection of the miR-184 antagomir into these cell lines led to increased AKT2 protein and a statistically significant increase in the rate of cell proliferation. siRNA mediated knock-down of *AKT2* resulted in decreased cell proliferation through the induction of significant apoptosis, while ectopic up-regulation of AKT2 using an expression plasmid led to increased cell proliferation, mimicking the effect of miR-184 knockdown. Co-transfection of miR-184 with an *AKT2* expression plasmid lacking the miR-184 target site rescued cell proliferation, indicating that miR-184 exerts pro-apoptotic effects primarily through the targeting of *AKT2*. Finally, the inverse correlation between MYCN and miR-184 levels was experimentally confirmed using the SHEP-TET cell line containing a repressible *MYCN* transgene. Suppression of *MYCN* resulted in an 8 fold increase in miR-184 levels. Using chromatin immunoprecipitation based methods we also demonstrate that MYCN binds weakly to an up-stream region of miR-184 containing ebox motifs previously demonstrated to bind MYCN.
Conclusion: MYCN induces a tumorigenic effect in part through down regulating miR-184, leading to increased AKT2 protein. MiR-184, as an inhibitor of *AKT2*, could be of potential benefit in microRNA mediated therapeutics.

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OR7

Exploring a new therapy for neuroblastoma: silencing of doublecortin-like kinase using RNA-interference

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Neuroblastoma is one of the most common childhood cancers. Microtubule-destabilizing agents are used in the treatment of these tumors. However, resistance to chemotherapeutic agents and systemic toxicity make neuroblastoma a difficult drug target. In our previous work, we found that doublecortin-like kinase (DCLK) gene transcripts are crucial microtubule-associated proteins for correct proliferation and differentiation of neuroprogenitor cells. Gene expression profiling revealed a high expression of these transcripts in neuroblastoma patients. Furthermore, these transcripts are endogenously expressed specifically in neuroblasts but are not found in other cell types. Suppression of DCLK by short interfering RNA (siRNA) disrupted the mitotic spindles in neuroblastoma cells and gene expression profiling revealed numerous differentially expressed genes indicating apoptosis. Apoptotic cell death of neuroblastoma cells by DCLK knockdown was further confirmed by several assays. Interestingly, mitochondria were the most affected cell components after DCLK-long knockdown. We also found in human neuroblastomas a significant correlation between DCLK expression and genes related with mitochondria activity. Furthermore, we showed a successful delivery of siRNA targeting DCLK to neuroblastoma cells by using specific peptide-siRNA conjugates. In conclusion, silencing of the DCLK gene by siRNA interference is a novel potential therapeutic approach for neuroblastoma with the promise of combining high specificity with fewer side effects. Peptide-siRNA conjugates might be the tool needed for specific neuroblastoma delivery.

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Parallel session 2 – Tumour initiating stem cells OR8–OR14

OR8

HIF-2α maintains an undifferentiated state in neural crest-like human neuroblastoma tumor-initiating cells

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Background: Cancers are phenotypically heterogeneous and hypoxia, being one cause of heterogeneity, dedifferentiates neuroblastoma cells towards a neural crest-like phenotype. Low stages of tumor cell differentiation are frequently coupled to advanced disease and high hypoxia-inducible factor 2a (HIF-2a) protein levels predict poor outcome in neuroblastoma.
Method/approach: We aimed to phenotypically characterize neuroblastoma tumor-initiating/stem cells (TICs) isolated from the bone marrow of patients with high-risk neuroblastoma with a focus on HIF expression and expression and regulation of stem cell/neural crest marker genes as well as markers of SNS differentiation.
Results: Here, we identify HIF-2a as a marker of normoxic neural crest-like neuroblastoma TICs isolated from bone marrow of patients with high-risk neuroblastoma. Knockdown of HIF-2a reduced VEGF expression and induced expression of sympathetic neuronal differentiation markers while expression of neural crest-associated genes diminished. Xenograft tumors of HIF-2a-silenced cells were widely necrotic, poorly vascularized and resembled the bulk of tumor cells in clinical neuroblastomas by expressing sympathetic neuronal markers including tyrosine hydroxylase, while control tumors were immature, well-vascularized and stroma-rich. HIF-2a-silenced xenograft tumors were high in HIF-1a, a feature that unlike HIF-2a was not associated with adverse clinical outcome and correlated negatively with advanced clinical stage and thus tumor spread in human neuroblastoma.
Conclusion: We conclude that HIF-2a is required for maintaining an aggressive undifferentiated phenotype of neuroblastoma TICs. As low expression of differentiation markers predict poor outcome in neuroblastoma and angiogenesis is crucial for macroscopic tumor growth, HIF-2a is an attractive target for neuroblastoma therapy.

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OR9

Identification of signaling pathways and drug candidates using primary neuroblastoma cancer stem cells by phosphoproteomics and transcriptome sequencing

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A major cause of fatality in neuroblastoma (NB) is relapse in the bone marrow (BM). Tumor-initiating cells (TICs) isolated from the BM of relapsed patients have many properties of cancer stem cells and form metastatic NB in vivo with 1 cell (L. Hansford; S. Yan abstracts, ANR). These cells express both neural-specific and hematopoietic genes that we hypothesize are required for survival in the BM niche. To identify the signaling pathways required for the survival of TICs, as well as drugs that will be cytotoxic on a patient-specific basis, we performed two types of complementary analyses: (1) phosphoproteomics by mass spectroscopy (MS) to identify kinases of constitutively active signaling pathways, and (2) next-generation RNA sequencing to reveal genes that are highly expressed in TICs compared to normal pediatric neural crest stem cells and a panel of cancer tissues. The presence of TIC-enriched transcripts at the protein level was confirmed using MS. Three constitutively activated signaling pathways were identified, FGF2 and insulin receptor, beta1-integrin, and B-cell receptor (BCR) and their activated effectors including Lyn, LCK, BTK, Syk and FAK. Treatment of TICs with Src and Syk inhibitors, including dasatinib, bosutinib, and R406, which are used clinically for hematopoietic malignancies, or shRNA knockdown of Src family members was rapidly cytotoxic. Transcriptome profiling also identified genes of the BRCA1 DNA damage response, CHK checkpoint control, and G2/M DNA damage checkpoint regulation as highly upregulated. One gene of the BRCA1 pathway, Aurora B kinase, which has not been considered as a drug target in NB, was analyzed further. Aurora B knockdown or treatment with AZD1152, a selective Aurora B inhibitor in phase I clinical trials for AML, was cytotoxic to TICs. This work is the first high-resolution analysis of the transcriptome, proteome, and phosphoproteome of NB TICs and identifies candidate TIC-enriched proteins and transcripts for development as therapeutic targets. Furthermore, we suggest that targeting hematopoietic survival pathways, which have thus far not been predicted to play a role in NB, may provide new drug therapies.

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OR10

Induced stable neuroblastoma cancer stem cells

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Background/Aim: Cancer stem cells (CSC) are known for their phenotypic drift toward non-CSC. An established tumor would therefore contain a mixture of CSC and non-CSC. To understand the biology of NB CSC and to identify effective therapeutic agents against them, one needs to isolate and maintain phenotypically stable CSC. Our goal is to establish and characterize stable NB stem cells induced by small molecules (i.e., induced CSC or iCSC).
Methods: Sphere forming culture conditions without growth factors were used with epigenetic modifier treatments to establish stable iCSC from NB cell lines SKNAS and SKNBE(2)C. TaqMan real-time PCR, gene expression profiling, and Western blot assay examined the expression of genes and proteins (stemness factors, stem cell markers). MTS assay examined the effect of chemotherapeutics on iCSC.
Results: Short-term treatments of NB cells with epigenetic modifiers significantly enhanced the expression of stemness factors (SOX2, OCT4, KLF4, LIN28, NANOG) and stem cell markers (ABCG2, CD133, CD44, CXCR4) in the iCSC as compared to monolayer and sphere cultures that were not treated with epigenetic modifiers, resulting in expression levels equivalent to those in NT2 teratocarcinoma cells. The established iCSCs also retained high-level expression of MYC or MYCN. Even with the short treatment of epigenetic modifiers, the stemness phenotype of the iCSC has been stable over 150 days. The iCSC conferred clonal expansion, a fundamental characteristic of CSC, and clonal iCSC populations retained the characteristics of the bulk iCSC. Gene expression profile analysis of SKNAS iCSC revealed that they expressed elevated levels of genes involved in NOTCH/DELTA, WNT/FZD pathways and genes expressed highly in other CSC types (BIN1, ENPP2, PDPN, THY1). Genes for some isoforms of the stem cell marker ALDH were also up-regulated in the iCSC. MYC-destabilizing agents were >100-fold more effective against SKNAS iCSC over the monolayer counterpart in MTS growth assay. Tumor seeding ability of SKNAS iCSC in vivo is currently being evaluated.
Conclusion: This study opens doors to a better understanding of the true nature of NB CSC as well as CSC in general.

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OR11

Inhibition of global DNA methylation induces differentiation of human neuroblastoma tumor-initiating cells

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Background: In neuroblastoma, low stage of differentiation correlates with aggressive disease and poor outcome. Human bone marrow-derived neuroblastoma tumor-initiating cells (TICs) are characterized by an immature stem cell- and neural crest-like phenotype with expression of typical markers such as Nestin and ID2. We have recently shown that HIF-2a maintains an undifferentiated state of neuroblastoma TICs and knockdown of this protein results in induced differentiation towards a sympathetic neuronal lineage. Here, we have studied the effects of epigenetic modification and growth factor treatment on neuroblastoma TIC lineage specification and differentiation.
Method/approach: Neuroblastoma TICs were treated with DNA methylation inhibitor 5-Aza-2'-deoxycytidine (DAC) in combination with growth factors involved in normal sympathetic neuronal and glial development, respectively, and effects were analyzed by quantitative RT-PCR and immunoflourescence.
Results: Inhibition of global DNA methylation by 5-Aza-2'-deoxycytidine induces differentiation of neuroblastoma TICs, giving rise to a mixed population of cells with both neuronal lineage features with induced expression of SNS markers, such as βIII-tubulin and neurofilaments, as well as Schwannian/glial lineage features with expression of typical glial markers GFAP and S100β. Additional growth factor treatment induces lineage-restricted differentiation, with nerve growth factor (NGF) and neurotrophin-3 (NT-3) driving TIC differentiation into a sympathetic neuronal lineage whereas ciliary neurotrophic factor (CNTF) and glial cell derived neurotrophic factor (GDNF) drive differentiation into a distinct glial lineage. Further, response to DNA methylation inhibition and growth factor treatment seems to be cell-differentiation stage-specific as differentiation is induced only in cells devoid of SNS marker expression, as tested in classical neuroblastoma cell lines.
Conclusion: Neuroblastoma TICs are immature and neural crest-like and resemble normal neural crest cells in that they have the capacity to differentiate into distinct ganglionic and glial/Schwann cell populations.

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OR12

Exploiting the embryonic environment to reprogram cancer stem cells in neuroblastoma

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Background: Neuroblastoma (NB) tumours can undergo spontaneous regression. Coupled with the knowledge that a cell's microenvironment exerts control over its eventual fate, this has lead to the hypothesis that an embryonic environment capable of differentiating a particular cell lineage should be able to reprogram cancers derived from that lineage. For example, malignant melanoma (MM) cells express markers of differentiation following neural crest transplantation [1]. We are investigating whether NB cells migrate, differentiate and become non-tumorigenic following injection into the neural crest (their site of origin, therefore an environment with the "correct" developmental cues), and in contrast, proliferate to form a tumour when transplanted into an inappropriate embryonic environment. We ultimately hope to determine precisely which factors tame the aggressive cancer cells.
Methods: GFP- or DiI-labelled NB cells were injected into the chick embryo eyecup and venous circulation at E3, and following 4-7 days incubation, body tissues sectioned and stained for the presence of markers of cell proliferation and apoptosis.
Results: NB cells from the SK-N-BE(2)C and Kelly cell lines were found within the sympathetic ganglia, heart, meninges and gut following intravenous administration. In some locations, typically the meninges, cells formed small aggregates. In others, typically sympathetic ganglia and gut, cells integrated and adopted a migratory morphology. These cells, like those injected into the eyecup, have so far failed to stain for markers of proliferation or apoptosis, suggesting that following implantation the majority of cells integrate into host tissue or form aggregates and do not divide.
Conclusions: Results so far suggest that the embryonic environment as a whole, regardless of location, may tame NB cells. We plan to test whether cells in the various locations express stem cell or differentiation markers, and compare these results with those of cells injected into the neural crest at E2, and those injected intravenously at a later stage (E6) when early developmental cues will no longer be present.

1. Hendrix, M.J., et al., Nat Rev Cancer, 2007. 7(4): p. 246-5

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OR13

Endocrine-gland vascular endothelial growth factor (EG-VEGF) in neuroblastoma tumor initiating cells

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Background: Endocrine-gland vascular endothelial growth factor (EG-VEGF) is a potent angiogenic and neurotrophic factor. We have previously demonstrated that it is implicated in the neuroblastoma (NB) progression by promoting growth and migration of NB cells. Here, we further delineated its roles in mediating NB tumor initiating cells (TICs).
Methods: Expression profiles of EG-VEGF receptors (PK-R1 and PK-R2) in different NB subclones, bone marrow metastasized TICs as well as TICs isolated from primary tumor were analyzed using RT-PCR. Implications of EG-VEGF in the various NB subpopulations were directly demonstrated using flow cytometric analysis. Clonegenicity, migration capability and proliferation of the EG-VEGF responsive subpopulations and TICs were also examined in this study.
Results: PK-R1 and PK-R2 were differentially expressed in various malignant NB stem cell and neuronal subclones. In particular, these receptors were expressed at higher level in a malignant neuronal subclone (SH-SY-5Y) than its parental clone (SK-N-SH), suggesting the implication of EG-VEGF in the growth of the malignant neuroblastic subpopulation. Subsequent flow cytometric analysis directly showed that EG-VEGF/ profoundly increases the c-kit$^+$/GD2$^+$/p75^{NTR+}/CD133$^+$ population of SH-SY-5Y cells. This subpopulation of cells consistently expressed both PK-R1 and PK-R2 and was highly responsive to EG-VEGF treatment. More importantly, these cells were highly proliferative, clonegenic and migratory, may contribute to the malignant phenotype of the NB. Concordant results were independently obtained from NB TICs. EG-VEGF could consistently enhance the colony formation capacity of both the bone marrow metastasized TICs and TICs isolated from primary tumor. Interestingly, only TICs from primary tumor expressed both PK-R1 and PK-R2, while only PK-R1 was expressed in the bone marrow metastasized TICs. This data may imply that PK-R1 would be curial for tumor dissemination.
Conclusions: Taken together, our data suggests that EG-VEGF could be a niche factor for NB TICs to promote tumor growth and dissemination.

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OR14

Novel cardiac glycoside analogues selectively target neuroblastoma tumor initiating cells (TICs)

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Background: Drug screens to identify agents that specifically target neuroblastoma (NB) tumor initiating cells (TIC) isolated from bone marrow metastases identified compounds that selectively inhibit TIC growth, including many cardiac glycosides (CGs). Since CGs are ionotropic agents with inhibitory effects on Na,K-ATPase pump, and at high doses induce cardiotoxicity, we designed novel CG analogues in an attempt to separate the NB growth inhibitory effects from the cardiotoxicity.
Method/approach: *In vitro drug* efficacy of known CGs (digoxin, digitoxin, ouabain) and synthesized analogues (RIDKs) was tested in adherent NB lines (IMR5, SKNBE2) and in NB TICs (NB12, NB88R, NB122R2), as well as skin-derived precursor SKPs (non-transformed neural crest stem cells) by MTT, alamar blue, sphere assays and cleaved-PARP IB. CG effects on Na,K-ATPases were assessed via cellular rubidium uptake. Expression of Na,K-ATPase a and β isoforms in TICs was determined by qRT-PCR. a1 isoform-specific Na,K-ATPase pump shRNA was performed.
Results: Parental and novel CGs(RIDKs) induce apoptosis and are moderately selective toward NB TICs in comparison to SKPs. TICs are more sensitive to CGs than adherent NB cells. In TICs the IC50 for RIDK 34 of 1-2nM is lower than the IC50 for digitoxin (300 nM). The doses of both RIDK 34 (1.042 uM) and digitoxin (1.038 uM) required to inhibit the Na,K pump activity were equivalent for both drugs, and higher than the IC50 required for TIC growth inhibition. NB TICs predominately express isozyme combinations of a1 with either β1 or β2. a1 isoform -specific shRNA does not significantly affect CG mediated-NB TIC growth inhibition.
Conclusion: RIDK34 actively inhibits TIC growth at a concentration that is significantly lower than the dose required to inhibit the Na,K-ATPase. This data, together with preliminary results in TICs in which shRNA knockdown of the pump does not affect CG sensitivity, suggest that CG activity is not dependent on the pump and thus, may be predicted to have diminished cardiotoxicity. Studies to determine in vivo sensitivity and toxicities are in progress

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Parallel session 3 – Immunotherapy OR15–OR23

OR15

Anti-GD2 murine monoclonal antibody (MoAb) 3F8/Granulocyte-Macrophage colony stimulating factor (GM-CSF) plus 13-Cis-Retinoic acid (13-cis-RA) for consolidation of >2nd complete remission/very good partial remission (CR/VGPR) of neuroblastoma

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Purpose: To report results of a prospective study using 3F8/GM-CSF plus 13-cis-RA to consolidate >2nd CR/VGPR of NB.
Patients and Methods: 68 patients (pts) with NB in >2nd CR/VGPR, but at high risk for another relapse, were enrolled on Memorial Sloan-Kettering Cancer Center protocol 03-077 [ClinicalTrials.gov NCT00072358], which opened in 2003. Pts received 3F8/GM-CSF cycles every 1-3 months for 24 months in the absence of human anti-mouse antibody (HAMA), plus 6 courses of 13-cis-RA. In 2006, the protocol was amended to all pts who developed early HAMA (i.e., before cycle 3 of 3F8/GM-CSF) to receive low-dose maintenance chemotherapy (e.g., temozolomide) until the immunotherapy could resume.
Results: The 68 pts included 39 (57%) who were post-stem-cell transplantation (SCT) and 53 (78%) previously treated with 13-cis-RA. Event-free survival at 36 months was 35% +7% for all 68 pts; 31% +8% for 41 pts not previously treated with 3F8; and 45% +7% for 27 pts who had previously received 3F8. 15 pts relapsed early (<3 months from start of 3F8). Among the 12 pts who remained in CR/VGPR with long follow-up (25-72+ [median 46+] months), 11 had prior relapse post-SCT, 9 had MYCN-amplified NB, and 6 never received maintenance chemotherapy after starting 3F8/GM-CSF on this protocol. Common toxicities were as expected: pain and urticaria with 3F8/GM-CSF, dry skin and cheilitis with 13-cis-RA. Treatment was outpatient.
Conclusions: One-third of pts who achieved 2nd CR/VGPR of high-risk NB became long-term event-free survivors using consolidation that included anti-GD2 MoAb immunotherapy plus 13-cis-RA, despite having previously failed both modalities. These results offered an alternate approach to relapsed NB, which may no longer be uniformly lethal. If CR/VGPR could be established again, reapplication of anti-GD2 immunotherapy and 13-cis-RA should be considered.

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OR16

Combined administration of *in vitro* expanded Vγ9Vδ2+ T cells and bisphosphonate zoledronic acid as preclinical immunotherapeutic approach for neuroblastoma

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Background and aims: Human TCR Vγ9Vδ2+ γδ cells are unconventional T cells that mediate HLA-unrestricted killing against most human tumor cell types. They display an unique reactivity towards posphoantigens and respond to cells exposed to aminobisphosphonates. These compounds are well established in the clinic as inhibitor of osteoclastic activity and several data support their antiangiogenic and proapoptotic properties for different human tumors. Our study investigated the cytotoxic properties of human Vγ9Vδ2+ T cells in vitro expanded by Zoledronate (Zol) against Zol-sensitized human neuroblastoma (NB) cells in NB for γδ T cell-based NB immunotherapy.
Methods: Human V9Vδ2+ T cell lines were generated by in vitro stimulation of PBMC from normal donors with 5μM Zol plus IL-2 and tested for cytotoxicity against Zol-sensitized human NB cells. SH-SY-5Y human NB cells, transfected with a luciferase reporter gene, were injected orthotopically in the adrenal gland of athymic Nude mice. After seven days, mice were randomized in four group: i) untreated; ii) treated with Zol (3 μg/mouse); iii) treated with γδ T cells (5x106/mouse); iv) treated with Zol (3 μg/mouse) and γδ T cells (5x106/mouse) 16 hr after Zol injection. All treatments were performed by intravenous administration once a week for 4 weeks.
Results: Overnight pretreatment of SH-SY-5Y and HTLA 230 NB cells with Zol at 10μM and 50 μM respectively, significantly enhanced their in vitro killing by Vγ9Vδ2+ T cells generated from different donors. In vivo experiments showed a statistically significant increased survival in all treated mice groups compared to untreated group. However, the most significant results were obtained with mice pretreated with Zol before γδcells injection (Zol, P 0.03; γδ T cells, P 0.01; Zol+γδ P 0.0009).
Conclusion: These preliminary results might sustain a new immunotherapeutic approach for human NB where bisphosphonate treatment sensitize tumor cells to cytotoxic effect of ex vivo expanded Vγ9Vδ2+ γδ T cells.

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OR17

Galectin-1 modulates immune response towards a state of tolerance in neuroblastoma

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Background: Galectin-1, a highly conserved glycan-binding protein, endows dendritic cells (DC) with a regulatory phenotype which contributes to sustain a tolerogenic microenvironment at sites of tumor growth. Galectin-1 is upregulated in neuroblastoma (NB) patients and its expression is associated with poor outcome. Here, we investigated the role of galectin-1 as a modulator of the anti-NB immune response in syngeneic NB mouse model.
Method/approach: Galectin-1 expression and secretion by murine (NXS-2) and human (LAN-1, Kelly, SK-N-AS) NB cells was evaluated by westernblot and immunohistochemistry. The effect of NB supernatant on I-A/I-K expression on bone marrow derived DC (BMDC) as an indicator for DC maturation was analysed by flow cytometry. Next, we stably transfected NXS-2 cells with galectin-1 antisense DNA (LAG-1) to suppress galectin-1 expression (LAG-1-NXS-2). LAG-1-NXS-2 was injected into A/J mice s.c. and its growth was compared to that of NXS-2 (galectin+). Cytotoxic Cr51 assays were performed to evaluate NB cell lysis.
Results: Galectin-1 is overexpressed in all tested human and murine NB cell lines in contrast to the very weak expression in A/J mice organs. We found galectin-1 in NXS-2 cell supernatant, which suppressed DC maturation as indicated by decreased I-A/I-K expression in the CD11c population (32%) in contrast to DC matured in dendritic cell medium (64%). LAG-1-NXS-2 s.c. injection prevented the tumorgrowth in contrast to s.c. NXS-2. We could not find metastases in the livers of the LAG-1-NXS-2 group. In contrast, on average of 6 metastases were detected in the livers of NXS-2 group. Splenocytes from mice receiving s.c. injections of LAG-1-NXS-2 showed 20-45% higher NXS-2 target cells lysis (82%; E:T 100:1) compared to splenocytes from mice injected with NXS-2 (37%; E:T 100:1).
Conclusion: Galectin-1 is secreted by NB cells which may inhibit DC maturation thus leading to increased tumor growth and dissemination. This effect may explain the state of tolerance observed in NB patients. Galectin-1 is therefore an interesting target to develop novel anti-NB immunotherapeutic approaches.

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OR18

Lenalidomide activates anti-tumor functions of NK cells and overcomes immune suppression by IL-6 and TGFβ1

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Background: Tumor progression occurs from residual disease in 40% of high-risk neuroblastoma patients. Effective natural killer (NK) cell-based immunotherapy may improve outcome. The milieu of neuroblastoma cells and mononuclear phagocytes, which includes IL-6 and TGFβ1, can suppress NK functions and promote tumor growth. We determined the ability of lenalidomide, an immune modulator, to activate NK cell anti-tumor functions (direct cytotoxicity, ADCC, secretion of cytokines) and to overcome NK suppression by IL-6 and TGFβ1.
Methods: Purified NK cells from normal adults were activated with IL-2 ± lenalidomide ± CD16 stimulation (anti-CD16 mAb or anti-GD2 mAb ch14.18 with neuroblastoma cells) for 24-72 hrs. Direct cytotoxicity and ADCC with ch14.18 were then determined by co-culturing NK cells with calcein-AM labeled neuroblastoma cells for 6 hrs and then quantifying loss of calcein from target cells. Cytokine release was quantified with a BD Cytometric Bead Array (CBA) assay or with individual ELISAs.
Results: Activation of NK cells for direct cytotoxicity and for ADCC with ch14.18 against multi-drug sensitive and resistant neuroblastoma cell lines was increased by adding lenalidomide to IL-2. Lenalidomide increased NK secretion of IL-2, GM-CSF, IFNγ, TNFa, MIP1a, and MIG, release of granzyme A and B, and synthesis of perforin but decreased secretion of IL-6, IL-10 and TGF-β1. IL-6 + sIL-6R and TGF-β1 suppressed IL-2 + CD16 activation of NK ADCC and secretion of IFNγ, but lenalidomide reversed this suppression. Lenalidomide suppressed IL-6 and TGF-β1 mediated phosphorylation of STAT3 and SMAD2/3 respectively in NK cells. Lenalidomide significantly improved the frequency of no tumor growth in NOD/SCID mice co-injected with PBMC and luciferase-labeled neuroblastoma cells as quantified by bioluminescence imaging.
Conclusion: Lenalidomide enhanced anti-tumor cytotoxicity and cytokine secretion by NK cells and overcame suppression by IL-6 and TGFβ1. These data support clinical testing of lenalidomide with anti-tumor cell mAbs in patients with recurrent, high-risk neuroblastoma.

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OR19

Treatment of high risk neuroblastoma with autologous T lymphocytes engineered to recognize GD2

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Background: Adoptive transfer of tumor directed T cells may offer an alternative therapy for patients with advance stage solid tumors as they can expand in vivo, actively migrate through tissue planes, and use direct and indirect cytotoxic mechanisms to kill tumor cells. Two major limitations have been the ability of tumors to downregulate MHC expression and decrease susceptibility to antigen-specific T cell killing, and the lack of co-stimulatory molecules leading to incomplete T cell activation and poor survival. We attempted to overcome these limitations by generating an MHC-independent chimeric antigen receptor (CAR) targeting GD2, a tumor antigen expressed on almost all neuroblastoma cells. We transduced activated T cells (ATC), and Epstein Barr virus-specific cytotoxic T lymphocytes (EBV-CTL) with a distinguishable GD2-CAR, infused them into patients with high-risk neuroblastoma, and evaluated the safety, persistence and clinical response after infusion.
Method/approach: This was a Phase I, dose-escalating, safety trial administering GD2 ATC and EBV-CTL in high-risk neuroblastoma patients.
Results: Nineteen patients received autologous ATC and EBV-CTL transduced with GD2-CARs. No dose limiting toxicities were identified. Twelve of 18 patients had detectable GD2 T cells/CTLs within the peripheral blood 6 weeks post-infusion. 4 of 6 followed >1 year continue to have detectable GD2 T cells/CTL populations. Clinically, 8 subjects had no evidence of disease at the time of infusion: 6 remain disease free 2-32 months and 1 is alive with disease (AWD) 29 months after infusion. Of 11 with relapsed/resistant disease: 7 had bulky disease, 3 had solitary bone lesions, and 1 had bone marrow disease. 2 patients with bulky disease had evidence of tumor necrosis, and a 3rd is AWD 25 months post-infusion. The subject with bone marrow disease cleared within 6 weeks. Lastly, 2 complete responses were seen in those with bone lesions: >4 years and >12 months post-infusion.
Conclusion: Treatment of high-risk neuroblastoma with adoptively transferred T cells expressing GD2 CARs appears safe and can be associated with both long term persistence and anti-tumor activity.

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OR20

A novel lentiviral-transduced dendritic cell vaccine targeting the survivin antigen is effective against neuroblastoma

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Background: The inhibitor of apoptosis protein survivin emerges as a promising target for anti-neuroblastoma (NB) immunotherapy. Our recent generated survivin minigene DNA vaccine was able to induce a protective CD8 T cell-mediated immune response using attenuated Salmonella typhimurium (aSL) as carrier in a NB model. However, the potential application of aSL as a vaccine carrier might be hazardous for immunocompromised stage 4 NB patients. For this reason, we now focus on the development of an alternative survivin-based DNA vaccination approach using ex vivo lentiviral-transduced dendritic cells (DC).
Method/approach: Lentiviral vector (LV) particles carrying the survivin minigen S-high were generated in HEK293T cells by CaCl2 precipitation (10^8-10^9 TU/ml). Empty LVs were used as mock controls. Generation of murine DC was accomplished by cultivation of isolated murine bone marrow cells (BMDCs) in the presence of GM-CSF. DC transduction was performed on day 2 of BMDC culture. DCs were stimulated for 24 hours by LPS-(100ng/ml)/TNF-alpha (250U/ml) on day 7 of BMDC culture. Maturation (I-A/I-E, CD40, CD80, CD86) of CD11c+ DC was analysed by flow cytometry. Vaccination was carried out on days 3, 10 after s.c./i.p. injection of 2x10^6 NXS-2 cells into A/J mice. Tumor growth was calculated by calliper measurement and direct NXS-2 target cell lysis was analyzed by Cr51 assay.
Results::We achieved a 60% transduction efficiency in the CD11c+ BMDC population at MOI 2. In a therapeutic setting, the resulting S-high encoding survivin DC vaccine (DC-Shigh) was able to reduce primary tumor growth by 75% in contrast to application of a mock control DC vaccine (DC-mock). This was demonstrated in a syngeneic NB mouse model for subcutaneous as well as intraperitoneal DC injection. Isolated splenocytes from the DC-Shigh vaccinated group showed 8-12% higher target cell lysis at various E:T ratios than splenocytes from DC-mock mice.
Conclusion::Vaccination with DCs transduced with survivin-minigene carrying LV is able to induce effective anti-neuroblastoma immune reponse and is the baseline for the development of a novel NB immunotherapy.

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OR21

Immunotherapy for neuroblastoma by GD2 specific chimeric antigen receptor

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Background: Chimeric antigen receptors (CARs) are single molecules comprising the antigen-binding moiety of a monoclonal antibody with activation motifs from endogenous T cell receptors and/or costimulatory receptors. T-cells transduced with CAR recognize and kill target cells expressing the cognate antigen. This technology allows the generation of large numbers of T-cells specific to any cancer antigen without requiring T-cell selection/expansion. We have previously tested GD2 specific CAR transduced autologous T-cells in a clinical trial in refractory neuroblastoma (Pule et al, Nat Med, 2008) with promising clinical responses.
Methods: We have made a series of refinements to the CAR and the adoptive transfer methodology used in the initial clinical study, with a view to a follow-on study.
Results: We have performed the following modifications to the anti-GD2 CAR:
(1) We have humanised the original murine antibody (muk666) component of the CAR and added co-stimulatory domains. When expressed in T-cells, the humanised CAR mediates specific lysis of GD2-bearing neuroblastoma cells to comparable levels as the murine derivative, and undergoes two fold greater antigen specific proliferation as well as greater specific IL2 and IFNγ secretion.
(2) We codon-optimised the entire CAR sequences for improved expression.
(3) To manage possible toxicity, we have incorporated an iCasp9 suicide gene within the CAR vector. Co-expression of iCasp9 induced by an inert small molecule inhibitor allows >95% killing of transduced T-cell after a single exposure.
(4) We improved the vector cassette to include a Genomic Scaffold Attachment Sequence to create more homogenous bright expression, improving expression and suicide gene activity.
(5) We have generated syngeneic models of luciferase and GD2 expressing tumours in which bioluminescence imaging successfully demonstrates safety, efficacy and kinetics of tumour kill within an immunoreplete host.
Conclusion: Our highly optimised retroviral cassette incorporating additional safety features for the treatment of neuroblastoma with a CAR, alongside lymphodepleting conditioning is likely to improve responses in a follow up clinical study.

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OR22

NKT cells co-localize with tumor-associated macrophages in neuroblastoma in an innate response to tumor-induced hypoxia

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The potential importance of CD1d-restricted Va24-invariant Natural Killer T cells (iNKTs) for antitumor immunity and immunotherapy has been demonstrated in multiple models of cancer as well as in cancer patients. However, the mechanism by which iNKTs mediate antitumor responses against solid tumors, most of which are CD1d-negative, has remained enigmatic. We recently reported that instead of attacking tumor cells directly, iNKTs target CD1d-positive tumor-associated macrophages (TAMs) that play essential roles in tumor progression (JCI 2009). To explain the observed co-localization of iNKTs with TAMs in primary human neuroblastoma (NB) we hypothesized that TAMs actively chemoattract iNKTs. To test this hypothesis, we used a multiplex quantitative RT-PCR to analyze changes in the expression of CC and CXC chemokine genes in primary human monocytes upon co-culture with human NB cells in normoxic (20% O2) and hypoxic (1% O2) conditions. We found that hypoxia alone selectively up-regulated CCL20 gene expression that was confirmed at the protein level by ELISA. Of interest, co-culture with NB cells even in normoxia resulted in a cell contact-dependent up-regulation of CCL20 in monocytes and the effect was further amplified up to 70 fold in hypoxia (N=8, P<0.001, t-test). All primary human iNKTs expressed high levels of CCR6, the only receptor for CCL20 and the expression was not affected by hypoxia for at least 24 h. In the functional experiments we found that co-culture of NB cells with monocytes was significantly more chemoattractive for iNKT cells than either NB cells or monocytes alone and this effect of the co-culture was further enhanced in hypoxia. Anti-CCL20 neutralizing mAb strongly inhibited iNKT-cell in vitro migration towards tumor-conditioned hypoxic monocytes. Furthermore, in vivo neutralization of human CCL20 in NOD/SCID mice prevented co-localization of adoptively transferred human iNKT cells with TAMs within human neuroblastoma/monocyte xenografts. Thus, TAMs via CCL20 production attract iNKTs inside tumor tissues that reveals a novel mechanism of an innate response to hypoxia and should be exploited for cancer immunotherapy.

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OR23

Bone marrow response evaluation with a quantitative device identifies prognostic groups in patients over 18 months

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Background: Bone marrow (BM) based response criteria are still lacking or controversial in stage 4 neuroblastomas. We hypothesized that the dynamics of BM clearing mirrors the response to cytotoxic treatment and is thus able to identify subgroups of stage 4 patients with unfavourable prognosis.
Methods: BM samples from 81 stage 4 patients registered in two neuroblastoma Trials were tested with a fully automatic fluorescence based device which combines GD2 based immunocytology and subsequent molecular-cytogenetic analyses of identical cells (automatic immunofluorescence plus FISH, AIPF). Inclusion criteria of the study were: BM specimens at diagnosis and given time points during treatment and genomic information on the primary tumour. After exclusion of 37 patients (tumor cell free BMs at diagnosis, lack of data or material), 44 patients (age 0 to 239 months, 219 BM specimens, median observation time 8.2 years) remained for whom a complete data set was available.
Results: BM clearing after 2 to 4 cycles of chemotherapy was achieved by 28 patients (63.6%) and was significantly associated with overall survival (OS) in patients above 18 months of age at diagnosis ($p<0.0001$, Logrank test) but not in the younger age group. Stage 4 patients below 18 months of age had a good prognosis irrespective of BM clearing and tumour genetics. None of the genetic markers, like MYCN amplification (MNA), 1p and 11q loss and 17q gain showed a correlation with OS in this patient cohort. For MNA, a tendency with BM clearing was observed ($p=0.059$, Fisher's Exact Test).
Conclusion: The determination of BM clearance reaches the so far highest prognostic impact in stage 4 neuroblastoma patients over 18 months of age making accurate BM monitoring an important tool for risk assessment in this patient group.

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Parallel session 4 – p53 and molecular targets OR24–OR32

OR24

Cooperative induction of apoptosis through p53 signaling and mTOR inhibition in neuroblastoma

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Background: The use of MDM2 inhibitors to induce apoptosis is an attractive approach for neuroblastoma which is predominantly p53 wild-type at diagnosis and relapse. We sought to identify additional pathways to potentiate the p53 response. Mammalian target of rapamycin (mTOR) signaling pathway is active in neuroblastoma and it was recently demonstrated that two p53 target genes, Sestrin1 and Sestrin2, inhibit mTOR activity, linking genotoxic stress, p53 and the mTOR signaling pathway.
Aim: We investigated the role of Sestrin1 and Sestrin2 in p53-mediated apoptosis in neuroblastoma and the interaction of mTOR and p53 pathways after their simultaneous blockade using the mTOR inhibitor, Temsirolimus and the MDM2 inhibitor, Nutlin 3a.
Methods: We used microarray expression profiling and quantitative real-time PCR to define the transcriptional response to MDM2 inhibition in primary neuroblastoma lines. We studied growth and apoptosis of neuroblastoma lines in the setting of concurrent therapy with Temsirolimus and Nutlin 3a. Flow cytometric analysis was used to assess the status of phospho-S6 ribosomal protein. We used an orthotopic xenograft model of neuroblastoma to test the in vivo response to mTOR inhibition.
Results: We show that the global transcriptional response to Nutlin is p53-dependent and that Sestrin1 and Sestrin2 are significantly upregulated in response to Nutlin in neuroblastoma cells. With MTT and Tunnel assays we demonstrate a p53-dependent synergistic effect of combined Nutlin 3a and Temsirolimus treatment on cell growth and apoptosis. Flow cytometric analysis of the phospho-S6 ribosomal protein demonstrates a profound dephosphorylation of S6 in vitro when low dose Nutlin 3a is combined with Temsirolimus. Additional in vivo studies suggest that mTOR inhibition reduces tumor burden and phospho-S6 of neuroblastoma xenografts in nude mice. Conclusions. We conclude that MDM2 inhibition and p53 driven Sestrin1 and Sestrin2 activation may enhance the apoptotic response to mTOR inhibition. Further in vitro and in vivo studies will support the novel therapeutic strategy of combined MDM2 and mTOR inhibition for relapsed and de novo neuroblastoma.

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OR25

Repressed p53 stress responses in normal perinatal cells provides a susceptibility to N-Myc oncogenesis as an initiating event in embryonal malignancy

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Embryonal cells which are not required for organogenesis must be deleted by mechanisms such as trophic factor withdrawal, otherwise embryonal rests will form postnatally and may later become cancerous. We have previously shown that N-Myc expression caused neuroblast or granule neurone precursor (GNP) cell rests as tumor-initiating events in murine models of neuroblastoma (TH-MYCN transgenic) and medulloblastoma (Ptch1+/- hemizygous knockout). However, prolonged Myc expression in normal cells should trigger protective apoptosis or senescence barriers via stress response signals involving p53 and p19ARF. Here we show that exogenous N-Myc expression instead caused resistance to trophic factor withdrawal-induced death in perinatal precursor cells for neuroblastoma (neuroblasts), medulloblastoma (granule-neurone precursors or GNPs), and acute lymphoblastic leukaemia (pre-B lymphocytes). N-Myc-induced death resistance was transient, since exogenous N-Myc expression increased death of mature normal ganglia and neuroblastoma tumor cells. This transient resistance to death stimuli was due to an inherent inactivation of p53 responses in perinatal cells. Trophic factor withdrawal induced robust p53 responses in 6-week old normal ganglia cells, but not in normal and TH-MYCN neuroblasts, or normal and Ptch-1+/- GNPs. Exogenous p53 restored death sensitivity to normal and N-Myc-expressing neuroblasts. Mdm2 promotes ubiquitin-mediated degradation of p53 protein. The Mdm2 inhibitor, Nutlin 3a, restored death sensitivity of N-Myc-expressing neuroblasts, GNPs and pre-B cells. Moreover, we showed that Mdm2 expression was required in vivo for N-Myc-induced rest formation. N-Myc increased transcription of the polycomb group protein, Bmi-1, a known repressor of p19ARF transcription in rest cells. TH-MYCN mice crossed with Bmi-1+/- knockout mice exhibited reduced rest formation and tumorigenicity, indicating Bmi-1 was necessary for embryonal tumor initiation. Our findings indicate a unique susceptibility of some specialized embryonal cells to oncoprotein stress, and suggest that postnatal activation of p53 responses may eradicate postnatal rests and thus prevent embryonal cancer.

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OR26

Common and distinct MYC target genes in embryonal tumors

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Background: Deregulation of the MYC proteins MYC and MYCN plays an essential role in embryonal tumors such as neuroblastoma, medulloblastoma and Ewing sarcoma family tumors. Because MYC proteins control opposing cellular processes, such as proliferation, apoptosis and differentiation, it is important to delineate which transcriptional targets of MYC proteins contribute to cancer development, progression and regression. Our main goal is to precisely decipher the transcriptional functions of MYC proteins in these embryonal tumor types.
Method/approach: To define tumor type-associated target genes of MYC proteins the strategy was as follows: model systems that allow modulation of MYC transcriptional activity either by knockdown and/or overexpression in the given disease background were used followed by global gene expression profiling. Direct regulation by MYC proteins was further analyzed by assessing the binding to target gene promoters in each tumor type by using chromatin immunoprecipitation (ChIP) -chip/sequencing. Differential regulation of the target genes of the MYC proteins in the different tumor types were defined by the status of epigenetic marks for H3K4me3 (activation), H3K36me3 (elongation), H3K27me3 and H3K9me2 (silencing) at gene promoters using ChIP. Targets of the MYC proteins were validated by global gene expression profiles from primary tumors of each tumor type.
Results: As a principle function for all tumor types, deregulated MYC proteins favor G1-S phase transition through hyperphosphorylation of pRB via CDKs and D cyclins. However, MYC proteins achieve this via distinct sets of cell cycle regulators in each tumor type. In neuroblastoma the induction of *CDK4* by MYCN or c-MYC is the predominant route. Epigenetic silencing of *CCND2* that abrogates binding of MYC to the *CCND2* promoter was observed. On the other hand, in medulloblastoma *CCND2* activation by c-MYC is the predominant route.
Conclusion: These data support the idea that the progenitor cell sets a different epigenetic program for each tumor type which is then modulated by MYC proteins.

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Addiction of MYCN amplified neuroblastomas to B-MYB underscores a reciprocal regulatory loop

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Background: Amplification of MYCN is is the most important genetic aberration in neuroblastoma. B-MYB is a transcription factor of the MYB family associated with advanced neuroblastoma stages and whose over-expression confers drug resistance to neuroblastoma cells. In this study, we investigated the relationship between B-MYB and MYCN in neuroblastoma.
Method/approach: Expression of MYCN and B-MYB in neuroblastoma patients was assessed in silico (Oncomine, Genesapiens, Oncogenomics). For transcription studies, we carried out transient transfection and luciferase assays, ChiP and gel shift analyses. Infection with lentiviral vectors carrying B-MYB and MYCN shRNAs were performed for functional studies.
Results: The expression of B-MYB is significantly associated to that of MYCN in neuroblastoma samples and strongly predicts poor patients' survival. B-MYB and MYCN are bound to the promoter of each other in living neuroblastoma cells. B-MYB is required for the expression of the MYCN amplicon and the proliferation of MYCN amplified, but not MYCN non amplified, neuroblastoma cells.
Conclusion: In this study we identify B-MYB as key MYCN downstream effector, required for expression of the MYCN amplicon and whose inhibition causes synthetic lethality in MYCN amplified cells. In the light of our study, we hypothesize that in neuroblastoma, following MYCN amplification, hundreds of copies of the gene in DM bodies or HSRs chromosomes causes accumulation of MYCN oncoprotein and activation of B-MYB expression. This, in turn, will initiate an aberrant regulatory cycle where B-MYB will enhance the expression of MYCN and vice versa. This theory should explain the apparent paradox of a ubiquitous factor -B-MYB- driving the expression of a tissue specific factor –MYCN- and clarify why MYCN is not activated in non-neuronal tumours where B-MYB is overexpressed or amplified. Our work has potential clinical implications. Given the absolute requirement for B-MYB expression, tumours with MYCN amplification should be exquisitely sensitive to B-MYB targeting, indicating that the search for small molecule inhibitors of B-MYB is warranted.

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OR28

Combined massively parallel sequencing and synthetic lethal screening identifies multiple druggable targets in neuroblastoma

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Background: Despite aggressive multimodal treatments the overall survival of patients with high risk neuroblastoma remains poor. Rational methods are needed to identify novel targets and select therapeutic strategies for patients with neuroblastoma. RNAi-based screening has become a powerful tool to identify drug targets for cancer therapy and recent advances in next generation sequencing make it possible to identify mutations in all of the protein coding genes in a massively parallel manner.

Methods: We took a combined approach of parallel sequencing and siRNA based synthetic lethal screening to identify mutated genes whose inhibition leads to growth suppression or synergizes with topotecan, a topoisomerase I inhibitor currently used to treat high-risk neuroblastoma. We screened siRNAs designed against ~7000 druggable targets of the human genome in 4 neuroblastoma cell lines representing both MYCN amplified and non-amplified tumors. In parallel we sequenced the transcriptome and whole exome of these cell lines.

Results: Many important biological processes, including cell cycle, phosphorylation, and protein modification, were significantly enriched in 173 growth suppressive siRNAs common to all 4 cell lines. By integrating the siRNA functional data with sequencing analyses at depth, we identified >40 candidate genes that are essential for cancer cell proliferation and also have non-synonymous damaging coding mutations in the 4 different neuroblastoma cell lines. Further validations are underway to establish if the same mutations are present in neuroblastoma tumor samples.

Conclusions: Taken together, our integrative approach of siRNA screening and highly parallel sequencing analyses will facilitate a rapid and rational identification of drug targets in neuroblastoma.

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OR29

Tumor regression and curability of preclinical neuroblastoma models by the novel targeted camptothecin EZN-2208

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Background/Aims: Treatment of neuroblastoma (NB) is successful in less than half of patients with high-risk disease. Here, the anti-tumor activity of a water soluble pegylated SN38 drug conjugate (EZN-2208), was evaluated in preclinical models of human NB.

Methods: The in vitro cytotoxicity of EZN-2208 was tested by counting trypan blue dye- and annexin-V-positive cells, while its therapeutic efficacy was evaluated, in terms of survival, anti-tumor and anti-angiogenic activities, in subcutaneous, pseudometastatic and orthotopic NB animal models.

Results: In vitro, EZN-2208 was about 100-fold more cytotoxic than CPT-11 in a panel of NB cell lines, by inducing apoptosis/necrosis and p53 expression and by reducing HIF-1a/ HIF-2a expression. Compared to the SN38 equivalents of CPT-11, EZN-2208 led in vivo to a significant tumor regression in subcutaneous luciferase-transfected xenografts and to 100% of disease-free mice in the pseudometastatic model. In the orthotopic model, EZN-2208-treated mice showed a dramatic arrest and regression of primary tumor growth. Long term survival was seen in 100% of EZN-2208-treated animals with tumors almost disappeared, as assessed by staining histological sections of tumors with antibodies recognizing NB cells and cell proliferation. At MTD, while CPT-partially prolonged mice survival, all immunocompetent and immunodeficient, EZN-2208-treated NB-bearing mice were cured. Compared to CPT-11, EZN-2208 significantly reduced the number of radiating vessels invading the tumor implanted onto the chorioallantoic membranes. Mechanistic experiments showed statistically significant enhanced TUNEL and Histone H2ax staining and decreased VEGF, CD31, MMP-2 and MMP-9 expression in tumors removed from EZN-2208-treated mice. In orthotopic NB model resistant to Doxorubicin (D), Cisplatin, Vincristine (V), Fenretinide and Topotecan (T), EZN-2208 induced 100% curability. EZN-2208 blocked tumor relapsed after TVD-combined treatment.

Conclusion: EZN-2208 could be considered the most promising novel anti-NB agent, to be administered in different clinical settings.

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OR30

Selective targeting of neuroblastoma tumor inititating cells by a telomerase inhibitor IMETELSTAT

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The ability of neuroblastoma (NB) to recur after maximal therapy could be related to limitless replicative potential of tumor intiating cells (TIC) and telomerase activation. The role of telomerase activation and its inhibition role in neuroblastoma TIC is still unkown.

Aims: To demonstrate that telomerase-dependent telomere maintenance is critical to the survival of the self-renewing neuroblastoma TICs , targeting telomerase activity may selectively block tumorigenicity.

Methods: Telomere maintenance was assessed for normal stem cells (SKPs, NSC, MSC) and TICs NB12, NB88R2 and NB122R isolated from NB bone marrows. NSC and NB TICs were treated with Imetelstat 5 uM (Geron). Telomerase activity was assessed by Telomeric Repeat Amplification Protocol (TRAP) assays. Telomere length was assessed by TRF assay and stem cell renewal was assessed by sphere forming assays.

TICs NB12 were Xenografted in NOD/SCID mice then treated with IP Imetelstat (30mg/kg twice a week) tumor volumes and survival were monitored. TIC harvested from these animals were subjected to sphere forming ability.

Results: NB TIC lines exhibited very short telomeres and high telomerase activity. Strikingly, normal stem cells revealed undetectable telomerase and very long telomers. Inhibition of telomerase by Imetelstat of 3 different neuroblastoma TICs lines resulted in a dramatic loss of replicative potential after 3-5 weeks of treatment, accompanied by rapid telomere attrition and loss of the sphere forming ability. In contrast, normal neural stem cell lines were insensitive to telomerase inhibition even after 12 weeks of therapy.

Animals xenografted with NB12 TIC which were pre-treated with Imetelstat failed to form tumors (p=0.009). In established tumors, early treatment with imetelstat resulted in late tumor growth arrest and a significant increase in survival when compared to controls (p=0.03). TIC harvested from treated animals had significantly reduced in sphere forming ability .($p<0.05$)

Conclusion: Telomerase inhibition causes tumor growth delay and irreversible loss of TIC self-renewal capacity. Targeting TIC with telomerase inhibitors may represent a new therapeutic approach in NB

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OR31

ABCC transporters influence multiple aspects of neuroblastoma biology, as well as clinical outcome, independent of cytotoxic drug efflux

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Background/Aims: We have previously shown that high levels of the multidrug transporters ABCC1/MRP1 (J Clin Oncol 24:1546, 2006) and ABCC4/MRP4 (Mol Cancer Ther 4:547, 2005), are strongly predictive of poor outcome in neuroblastoma. Although the prognostic significance of ABCC1 may be explained in terms of cytotoxic drug resistance, none of the drugs used to treat children in these studies were ABCC4 substrates. This suggests that multidrug transporters can contribute to the malignant phenotype, independent of cytotoxic drug efflux, as we have recently outlined (Nat Rev Cancer, 10:147, 2010).
Methods: A MYCN-driven transgenic mouse neuroblastoma model was crossed with an Abcc1-deficient mouse strain or alternatively, treated with an ABCC1 inhibitor. ABCC genes were suppressed using siRNA or overexpressed by stable transfection. Quantitative PCR was used to examine the clinical significance of ABCC family gene expression in a large prospectively accrued cohort (n=209) of primary neuroblastomas. Survival curves were compared with a logrank test.
Results: Pharmacological inhibition or genetic depletion of ABCC1 significantly inhibited neuroblastoma development in MYCN transgenic mice, while knockdown of ABCC1 or ABCC4 resulted in reduced proliferation and migration, and enhanced morphological differentiation of cultured cells. Analysis of a large neuroblastoma cohort confirmed the predictive power of ABCC4 expression and revealed that low ABCC3 expression was highly predictive of reduced survival (p<.0001). These results were confirmed by analysis of a large publicly available neuroblastoma gene expression database. No other ABCC transporter genes were predictive of clinical outcome. Expression levels of ABCC1, ABCC3 and ABCC4 were independently prognostic for outcome and their combined expression pattern defined a subgroup of patients with particularly poor survival. Over-expression of ABCC3 reduced neuroblastoma cell migration and proliferation.
Conclusions: Transporters of the ABCC subfamily can influence important biological characteristics of neuroblastoma independently of their role in drug efflux and represent attractive targets for therapeutic intervention.

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OR32

EZH2 mediates epigenetic silencing of candidate neuroblastoma tumor suppressor gene Casz1

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Casz1 localizes to Chr1p36, which is a region of LOH found in 20-40% of NB tumors. Low CASZ1 expression is detected in NB tumors from patients who have a poor prognosis (58 patients, P= 2.07x10-6). Functional studies showing CASZ1 inhibits NB growth, soft agar cloning and tumorigenicity in vivo indicate CASZ1 is a candidate NB tumor suppressor gene. Casz1 expression is restored upon treatment of NB cell lines with HDAC inhibitors, such as Depsipeptide (Depsi) (2-6 fold in AS, SY5Y and NGP, 7-20 fold in KCNR) while components of the polycomb repressive complex 2 (PRC2) decrease. We hypothesize that epigenetic silencing via PRC2 complex contributes to low CASZ1 expression in NB tumors. Enhancer of Zeste Homologue 2 (EZH2) is a methyltransferase, which interacts with EED, SUZ12 and YY1 to form PRC2, which represses gene expression through trimethylation of Lysine 27 on Histone 3 (H3K27me3). Increased levels of EZH2 are found in prostate and breast cancer, Ewing's sarcoma and glioblastoma. We find high expression of EZH2 and EED associated with poor prognosis in NB tumors (58 patients, median value, EZH2 (P=5.87x10-4), EED (P=2.6x10-3). Chromatin Immunoprecipitation (ChIP) assays show increased H3K27me3 at the Transcriptional Start Site of Casz1, which decreased after Depsi treatment. ChIP analysis also reveals binding of PRC2 complex subunits (EZH2, EED and SUZ12) to the CASZ1 transcription start site in an area co-incident with increased H3K27me3 binding. PRC2 complex binding also decreased after Depsi treatment. Targeting of EZH2 using siRNA to EZH2 led to increased Casz1 expression (2 fold) in NB cells. Also Casz1mRNA is 3-fold higher in EZH2-/- mouse embryonic fibroblasts (MEFs) compared to EZH2+/+ MEFs. Treatment of NB cells with a small molecule inhibitor of EZH2, DZNep, caused a 2-fold increase in CASZ1 expression. Our data shows CASZ1 expression is epigenetically silenced by PRC2 complex and the decrease of PRC2 complex expression and binding leads to increased CASZ1 expression. This is consistent with a model in which aberrant EZH2 mediated epigenetic gene silencing of CASZ1 contributes to NB tumorigenesis.

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Parallel session 5 – Genomics, clinical correlation OR33–OR39

OR33

Discovering epistatic genetic interactions associated with high-risk neuroblastoma

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Background: We have recently demonstrated that polymorphisms in the BRCA1 interacting gene BARD1 are associated with high-risk neuroblastoma (NBL) using a genome-wide association approach (Capasso, Nat Genet 2009). The mechanism by which BARD1 impacts susceptibility to NBL remains undefined. Here, using a two-locus analytic method, we sought to identify genes that might increase susceptibility to NBL development by their interaction with BARD1.
Methods: We performed an interaction analysis based on a genome-wide SNP array dataset comprising 1433 cases and 3221 controls. Six databases (BIND, BIOGRID, MINT, HPRD, STRING, IntAct) were queried to identify the proteins known or predicted to interact with BARD1. A regression analysis method implemented in PLINK was utilized to test for pairwise interactions between BARD1 SNPs and SNPs of candidate interacting genes.
Results: A total of 109 proteins were identified that were known or predicted to interact with BARD1. Twenty-two of these were identified in three or more databases surveyed, and were the focus of subsequent interaction analyses. The most significant interactions were found between the intronic SNPs rs919581 and rs1880791 of the PTN gene (pleitropin) and rs17487792 of BARD1 (OR=1.45, $P=9x10^{-5}$; OR=1.39, $P=7x10^{-4}$). The PTN SNPs showed no effect in the single SNP analysis (OR=0.97, P=0.50; OR=0.96, P=0.41) while the BARD1 SNP showed highly significant association with NBL (OR=1.36, $P=1.7x10^{-16}$). The OR of the highest-risk genotype (homozygote at the minor alleles for rs17487792 and rs919581) relative to the most common genotype at the two loci combined was 9.69. SNP rs17487792 is in LD (r2=0.96) with rs2070096, which is predicted to regulate BARD1 splicing. Ongoing experiments are testing if the BARD1 isoforms related to this SNP affect physical interaction with PTN.
Conclusions: PTN and BARD1 variants may act in an epistatic fashion to promote NBL tumorigenesis. Future work will focus on identifying the mechanisms for this interaction, and discovering if somatically acquired alterations in these genes may also contribute to a high-risk NBL phenotype.
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Accumulation of segmental alterations determines progression in neuroblastoma

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Background: Neuroblastoma is characterized by two distinct types of genetic profiles, consisting of either numerical or segmental chromosome alterations. The latter are associated with a higher risk of relapse, even when occurring together with numerical alterations. We explored the role of segmental alterations in tumor progression and the possibility of evolution from indolent to aggressive genomic types.
Methods: Array-based comparative genomic hybridization data of 394 neuroblastoma samples was analyzed and linked to clinical data.
Results: Integration of ploidy and genomic data indicated that pseudo-triploid tumors with mixed numerical and segmental profiles may derive from pseudo-triploid tumors with numerical alterations only. This was confirmed by the analysis of paired samples, at diagnosis and at relapse, as tumors with a purely numerical profile at diagnosis frequently acquired segmental alterations at relapse. New segmental alterations were also usually observed at relapse in cases with segmental alterations at diagnosis. Such an evolution was not linked to secondary effects of cytotoxic treatments since it was observed even in cases treated with surgery alone. A higher number of chromosome breakpoints was correlated with higher age at diagnosis, higher stage of disease, and a higher risk of relapse and a poorer outcome.
Conclusion: These data provide further evidence of the role of segmental alterations, suggesting that tumor progression is directly linked to the accumulation of segmental alterations in neuroblastoma. This possibility of genomic evolution should be taken into account in treatment strategies of low- and intermediate risk neuroblastoma and should warrant biological reinvestigation at the time of relapse. -(GS and IJL contributed equally to this work.)

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OR35

Improved outcome prediction of children with neuroblastoma using a miRNA signature

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Background: More accurate assessment of prognosis is important to further improve the choice of risk-related therapy in neuroblastoma (NB) patients.
Method/approach: 430 human mature microRNAs (miRNAs) were profiled on two patient subgroups with maximally divergent clinical courses. Univariate logistic regression analysis was used to select miRNAs that correlated with NB patient survival. Subsequently, a 25-miRNA gene signature was built using 54 training samples, tested on 197 test samples, and validated on an independent set of 278 tumors.
Results: The 25-miRNA signature significantly discriminates the test patients with respect to progression-free survival (PFS) and overall survival (OS) ($p<0.0001$). Multivariate analysis indicates that the miRNA signature is a significant independent predictor of PFS and OS after controlling for currently used risk factors: patients at high molecular risk have a 5-fold higher risk for relapse/progression and a 6-fold higher risk to die from disease compared to patients at low molecular risk. Patients with increased risk for both a shorter PFS and OS can also be identified in the cohort of high risk patients based on currently used risk factors, showing the potential of this signature for improved clinical management in this subgroup. These results were confirmed in an external validation set, in which the signature is also independently statistically significant for PFS and OS. A separate logistic model for PFS and OS for the 25-miRNA signature and a previously published 59-mRNA signature was performed on a subgroup of 236 samples and shows that the 25-miRNA signature is an independent significant predictor for PFS, as is the 59-mRNA signature for OS. Currently, we are evaluating the possibility of combining the power of both mRNA and miRNA signatures in order to establish an integrated and even more improved prognostic classification.
Conclusion: Based on miRNA expression data for an unprecedentedly large number of more than 500 NB patients, we established and validated a robust miRNA classifier, able to identify a cohort of high risk NB patients at greater risk for adverse outcome. *shared 1st aut

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OR36

Gene expression-based classification improves risk estimation of neuroblastoma patients

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Background: The broad range of neuroblastoma clinical phenotypes and of corresponding therapeutic regimens make accurate risk estimation at the time of diagnosis an essential prerequisite for treatment decisions. This study aimed at evaluating the potential clinical impact of a pre-defined gene expression-based classifier in neuroblastoma patients.
Method/approach: Gene-expression profiles of 440 internationally collected neuroblastoma specimens (Germany, n=325; other countries, n=115) were investigated by microarray analysis. Of the German samples, 125 were examined prospectively as part of the trial NB2004. Patients were classified as either favorable or unfavorable by a 144-gene PAM classifier established previously on a separate set of 77 patients. PAM classification results were compared with those of current prognostic markers and risk estimation strategies.
Results: Patients with divergent outcome were reliably distinguished by the PAM classifier (favorable, n=249, and unfavorable, n=191; 5-year EFS 0.84±0.03 vs. 0.38±0.04, 5-year OS 0.98±0.01 vs. 0.56±0.05, respectively; both p<0.001). Different clinical courses were robustly discriminated in both the German and international cohorts, as well as in the prospectively analyzed samples (p≤0.001 for both EFS and OS each). In subgroups with clinical low, intermediate and high risk, the PAM predictor significantly separated patients with divergent outcome (low risk, 5-year OS 1.0 vs. 0.75±0.10, p<0.001; intermediate risk, 1.0 vs. 0.82±0.08, p=0.042; and high risk, 0.81±0.08 vs. 0.43±0.05, p=0.001). In multivariate Cox regression models based on both EFS and OS, PAM was a significant independent prognostic marker (EFS, p<0.001, HR 3.38, CI 2.08-5.49; OS, p<0.001, HR 11.12, CI 2.49-49.70). The highest potential clinical impact of the classifier was observed in patients currently considered as non-high risk (n=289; 5-year EFS 0.87±0.02 vs. 0.44±0.07; 5-year OS 1.0 vs. 0.80±0.06; both p<0.001).
Conclusion: Gene expression-based classification using the 144-gene PAM predictor can contribute to improved therapy stratification of neuroblastoma patients.

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OR37

Genomic portrait of tumor progression using next-generation sequencing

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Background: Genetic alterations are thought to enable cancers to proliferate and survive more effectively, or to resist cytotoxic therapies. However, the molecular basis of tumorigenesis and progression is not fully understood.
Method/approach: In order to understand the genetic aberrations underlying tumor progression in refractory neuroblastoma, we performed the next-generation sequencing on the whole transcriptomes and exomes for three sequential samples taken from a high-risk stage 4 neuroblastoma patient at diagnosis, after cytotoxic therapies, and at death. In parallel, we performed whole genome sequencing on patient's constitutional genomic DNA at 16X coverage to establish the genetic background.
Results: Whole transcriptome sequencing of three samples yielded total >500 million mappable reads representing >14,000 genes (>20,000 transcripts). The average coverage for each transcript is >14X, while >5297 genes have an average per-base-coverage of >10. Single nucleotide variant (SNV) analysis of transcriptome sequencing data identified approximately 10,000 exonic SNVs of which ~4000 are in the coding regions and about half of them are nonsynonymous. Using SIFT analysis, we predicted approximately 400 SNVs to be damaging. Of them, >150 SNVs are shared among two or more samples. In addition, 188 SNVs were present at diagnosis, but not in the second sample after cytotoxic therapies. These deleterious mutations that arise during therapies are currently being validated by whole exome sequencing, and investigated for pathway disruption that may lead to refractory disease.
Conclusion: In summary, we present the most comprehensive genomics analysis of a neuroblastoma genome to date in a single patient during the course of the disease. Our data show that multiple passenger and driver mutations are found in the presentation and sequential tumor biopsies indicating the presence of significant genomic instability of these tumors. This approach will allow us to develop tumor progression models and potential targeted therapies against mutated gene products in neuroblastoma.

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OR38

A multi-locus technique for risk evaluation of patients with neuroblastoma

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Background: Precise and comprehensive analysis of tumour genetics is essential for the most accurate risk evaluation and only pangenomic/multi-locus approaches fulfil the present-day requirements. Here, we present the establishment of the multiplex ligation-dependent probe amplification technique (MLPA) for neuroblastoma.
Methods: A neuroblastoma specific MLPA kit was designed by the SIOPEN (SIOP Europe Neuroblastoma) Biology Committee in co-operation with MRC Holland. The kit used in this study contained target sequences for 106 genetic loci corresponding to 19 chromosomal arms and reference loci. The validation was performed by fluorescence in situ hybridization (FISH, n=125), BAC array (aCGH, n=39) and by SNP array (n=10). Dilution experiments for determination of minimal tumour cell percentage were performed as well as testing of reproducibility which was checked by inter-laboratory testing involving nine laboratories.
Results: Inter-technique validation showed a high concordance rate (99.5%) as well as the inter-laboratory MLPA testing (kappa 0,95, p<0.01) with seven discrepant out of 1490 results (0.5%). Validation of MLPA results by SNP and aCGH showed a single discordance out of 190 consensus results (0.5%). The test results led to the formulation of interpretation standards and to a revision of the kit. The minimal amount of tumour cell content was fixed at 60% to detect segmental aberration, for detection of amplification, it can be lower.
Conclusions: The recently designed neuroblastoma specific MLPA kit not only covers the chromosomal regions demanded by the International Neuroblastoma Risk Group (INRG) for therapy stratification but also includes all hitherto described genetic loci of possible prognostic interest for future studies. Moreover, the technique turned out to be cost effective, reliable and robust with a high inter-laboratory and inter-technique concordance.

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OR39

Detecting the cutting edges. Highly sensitive and absolute specific detection of *MYCN* amplified neuroblastoma cells by amplicon-fusion-site (AFS) PCR

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Background: To detect tumor cells as sensitively and specifically as possible is one major goal of cancer diagnostics. It is indispensable to develop new, feasible tools to reliably classify tumor stage at time of initial diagnosis and to monitor the response to therapy as well as recurrence of disease as early as possible.
Method/approach: We mapped the amplified genomic regions (ampGR) around the proto-oncogene *MYCN* from about 40 primary human neuroblastomas and 3 neuroblastoma cell lines, using a high resolution Tiling Array (HR-TA). Based on the HR-TA data we were able to precisely describe the telomeric and centromeric borders of the ampGR and to deduce virtual fusion sites of the connected ampGRs (amplicon-fusion-sites (AFS)). These AFS served as blueprints for a first AFS-PCR primer design. The specific AFS-PCR fragments were then sequenced. Based on the exact AFS sequences we were able to establish high sensitive, quantitative real time PCR assays (final AFS-PCR).
Results: All ampGR and thus, all AFS identified were absolute tumor cell specific and unique for each patient. AFS-PCR was highly sensitive and uncovered one tumor cell out of 10^6 - 10^7 control cells. We successfully proved the *"in-vivo"* practicability of AFS-PCR by detecting and quantifying the specific AFS-DNA of *MYCN* amplified neuroblastomas in peripheral blood (PB) and bone marrow (BM) samples of the corresponding patients.
Conclusion: AFS-PCR promises an important contribution to an exact definition of the tumor stage of *MYCN* amplified neuroblastoma at time of initial diagnosis, monitoring the response to therapy by quantifying circulating tumor cells in the PB or infiltration of the BM and for detecting smallest amounts of minimal residual disease (MRD) or recurrent disease over time. Furthermore, AFS-PCR is not limited to a specific tumor type but is rather transferable to every entity of malignancy, provided that the individual tumor cells harbour ampGR. Once established, AFS-PCR represents a powerful but nevertheless feasible, personalized diagnostic tool for a large number of cancer patients including children with *MYCN* amplified neuroblatomas.

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Parallel session 6 – Differentiation and epigenetics OR40–OR46

OR40

The homeobox transcription factor HoxC9, a key regulator of development, suppresses tumourigenicity of neuroblastoma

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Background: The embryonic nature of neuroblastoma suggests the involvement of key developmental regulator genes in its pathogenesis. Homeobox genes constitute an important family of developmental regulators which play a fundamental role in morphogenesis and cell differentiation during embryogenesis. This study aimed at elucidating the role of HoxC9 in neuroblastoma spontaneous regression and differentiation.
Methods: Gene expression profiles from 476 patients were generated using microarray technology and *HOXC9* expression levels were calculated. The methylation pattern of 26 *HOXC9* promoter CpG sites was determined in 46 neuroblastic tumours and 2 cell lines. Genomic aberrations on the *HOXC9* locus were determined by aCGH in 216 primary tumours. A tetracycline-inducible system of *HOXC9* expression was established in 3 neuroblastoma cell lines to investigate the effect of HoxC9 on cell differentiation, proliferation, viability, cell cycle distribution, migration and anchorage-independent growth. The effect of HoxC9 on *in vivo* tumourigenicity was investigated in neuroblastoma xenograft models.
Results: *HOXC9* down-regulation is significantly associated with unfavourable prognostic markers (stage 4, age >18 months at diagnosis, *MYCN* amplification), unfavourable gene expression-based classification and adverse patient outcome ($p<0.001$ each). Bisulfite sequencing and aCGH analysis suggested that neither hypermethylation nor copy number alterations are regularly involved in *HOXC9* gene silencing in neuroblastoma. Re-expression of HoxC9 resulted in significant reduction of cell viability and clonogenic growth as well as in increased apoptosis. In SK-N-AS cells, restored expression of HoxC9 reduced cell migration. In IMR-32 cells, neuronal differentiation was observed upon HoxC9 upregulation. In neuroblastoma xenograft models (IMR-32 and SK-N-AS cell lines), tumour growth was impeded almost completely after HoxC9 re-expression.
Conclusions: Our data suggest that HoxC9 is a critical factor for spontaneous regression and differentiation in neuroblastoma.

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OR41

Identification of miRNAs implicated in neuronal development and neuroblastoma oncogenesis using fetal neuroblast miRNA profiles

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Background: MiRNAs have been shown to be involved in both normal development and oncogenesis. Data are emerging that deregulation of miRNAs also plays an important role in neuroblastoma. In order to identify miRNAs implicated in neuroblastoma pathogenesis, we compared the miRNA profiles of fetal adrenal neuroblasts with a well defined cohort of neuroblastoma tumors.
Method/approach: Using a high-throughput stem-loop reverse transcription qPCR assay, we profiled the expression of 430 miRNAs in 100 primary untreated neuroblastoma tumors and 7 neuroblast samples, isolated from fetal adrenal glands using laser capture microdissection.
Results: Differential expression analysis of neuroblast and neuroblastoma miRNA profiles resulted in a list of 60 candidate miRNAs (32 lower and 28 higher expressed in neuroblast compared to neuroblastoma). A critical selection for potential oncogenic or tumor suppressive miRNAs was based on literature data, correlation with patient survival, genomic localization, correlation with genomic aberrations and putative targets. Furthermore, we compared the predicted targets with our previously described list of differentially expressed mRNAs between neuroblast and neuroblastoma. This allowed the selection of 10 miRNAs, including the well known oncomiRs miR-21 and miR-25, for further functional validation. We also selected miRNAs putatively targeting genes known to be involved in neuroblastoma such as *MYCN*, *ALK* or *PHOX2B*. Functional analysis supported a role in neuronal differentiation for 3 miRNAs. Further analyses are focused on the miRNA regulation of *PHOX2B*, a known master regulator of peripheral neuronal differentiation and implicated in a subset of familial neuroblastoma.
Conclusion: Comparison of miRNA profiles of fetal neuroblasts versus neuroblastomas yielded a distinct subset of miRNAs with possible implication in neuroblastoma. Functional evidence was obtained for a role in neuronal differentiation for 3 miRNAs. Further in vivo studies are warranted to explore the contribution of these miRNAs to the malignant phenotype in neuroblastoma and possibly other neuronal tumors.

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OR42

Neuroblastoma Phox2b variants stimulate proliferation and de-differentiation of immature sympathetic neurons

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Background: Neuroblastoma is a pediatric tumor that is thought to arise from autonomic precursors in the neural crest. Mutations in the PHOX2B gene have been observed in familial and sporadic forms of neuroblastoma and represent the first defined genetic predisposition for neuroblastoma.
Methods/approach: We address the mechanisms that may underlie this predisposition, comparing the function of wildtype and mutant Phox2b proteins ectopically expressed in proliferating, embryonic sympathetic neurons.
Results: Phox2b displays a strong antiproliferative effect, which is lost in all Phox2b neuroblastoma variants analyzed. In contrast, an increase in sympathetic neuron proliferation is elicited by Phox2b variants with mutations in the homeodomain when endogenous Phox2b levels are lowered by siRNA-mediated knockdown to mimic the situation of heterozygous PHOX2B mutations in neuroblastoma. The increased proliferation is blocked by Hand2 knockdown and the antiproliferative Phox2b effects are rescued by Hand2 overexpression, implying Hand2 in Phox2b-mediated proliferation control. A Phox2b variant with a nonsense mutation in the homeodomain elicits, in addition, a decreased expression of characteristic marker genes.
Conclusion: These results suggest that PHOX2B mutations predispose to neuroblastoma by increasing proliferation and promoting de-differentiation of cells in the sympathoadrenergic lineage.

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OR43

Multidrug resistance-associated protein 4 regulates cAMP-dependent differentiation in neuroblastoma and represents a target for therapeutic inhibition

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Background/Aims: cAMP is a central second messenger that regulates key cellular responses such as survival and differentiation, in a range of cancers, including neuroblastoma. cAMP pathway activation induces neuronal differentiation and cell growth inhibition. The multidrug transporter, MRP4/ABCC4 actively effluxes cAMP from mammalian cells, and we have previously shown that expression of MRP4 is a powerful, independent prognostic marker in primary neuroblastoma. MRP4 may therefore contribute to aggressive poor prognosis neuroblastoma through regulation of this important signalling pathway.
Methods: Neuroblastoma cells were treated with dibutyryl-cyclic AMP (db-cAMP), forskolin (adenylate cyclase agonist) or 2'5'-dideoxiadenosine (DDA; adenylate cyclase antagonist), either alone or following siRNA-mediated downregulation of MRP4. A 30,000 diverse chemical small molecule library (Chembridge) was screened for potential inhibitors of MRP4.
Results: Analysis of 3 publicly available neuroblastoma gene expression databases, demonstrated that high levels of MRP4 and low levels of cAMP pathway genes including adenylate cyclase 1, CREB1, and cAMP-dependent protein kinase genes, were all strongly predictive of poor outcome. MRP4 siRNA downregulation in MRP4 overexpressing BE(2)-C neuroblastoma cells, led to enhanced morphological differentiation and reduced proliferation. This phenotype was augmented following treatment with the cAMP agonists forskolin or db-cAMP, or reversed using the antagonist DDA suggesting that MRP4 acts as an important endogenous regulator of intracellular cyclic nucleotide levels. In order to isolate MRP4 small molecule inhibitors, a 30,000 chemical compound library was screened using a cell-based assay. Following a range of filters, 19 MRP4-specific inhibitors were identified. Of these, two in particular were shown to mimic the effects of MRP4 siRNA on neuroblastoma cells.
Conclusion: Our results suggest that MRP4 plays a fundamental role in neuroblastoma biology specifically through regulating the efflux of cAMP. Promising small molecule inhibitors of MRP4 have been identified with potential for development as pharmacologic therapeutic agents.

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OR44

Versatile *in vivo* roles for caspase-8 in neuroblastoma tumorigenesis

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Background: Caspase-8 (C8) has roles in receptor-mediated apoptosis, cellular migration and attachment, although enzymatic activity is not required for these two later processes. C8 is silenced in a high percentage of neuroblastoma (NB) patients and methylation of C8 can serve a prognostic marker for NB. Loss of C8 expression also potentiates metastasis via the prevention of integrin-mediated cell death. Here, we utilized several mouse models to determine whether C8 has a role in vivo in NB.
Method/approach: To test the effects of loss of C8 expression on NB formation tissue-specific deletion of the C8 was achieved by mating C8 conditional knockout mice with mice expressing a neural crest specific TH-cre recombinase. These mice were then mated with NB prone transgenic mice which over-express N-myc (TH-MYCN). To test the role of C8 in attachment and migration, NB cells with and without C8 were introduced into SCID mice by tail vein injection and disseminated tumor formation was assayed.
Results: Although no tumors were observed when C8 alone was deleted, mating the C8 conditional knockout mice with TH-MYCN mice significantly increased the frequency of N-myc-induced tumors, without changing the latency. Interestingly, tumors without C8 were less differentiated. A role for C8 in metastasis was also observed in SCID mice. Mice that were injected with cells expressing physiological levels of C8, had a much higher incidence of macro tumors than mice injected with NB cells that did not express C8, although these large tumors no longer expressed C8.
Conclusion: Our work suggests both apoptotic and nonapoptotic roles for C8 in NB tumorigenesis. C8 increases tumor frequency in the TH-MYCN NB model and decreases tumor differentiation suggesting a role for C8 in neuronal differentiation. In addition, C8 is important for extravasations and migration of NB cells in the blood system. These data support the hypothesis that C8 plays important non-apoptotic roles in cellular migration and attachment during metastasis, but that the down-regulation of C8 enhances the survival of both initially developing and metastatic tumors.

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OR45

Histone deacetylase 8 in neuroblastoma tumorigenesis

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The effects of pan-histone deacetylase (HDAC) inhibitors on cancer cells have shown that HDACs are involved in fundamental tumor biological processes such as cell cycle control, differentiation and apoptosis. However, due to the unselective nature of these compounds, little is known about the contribution of individual HDAC family members to tumorigenesis and progression. The purpose of this study was to evaluate the role of individual HDACs in neuroblastoma tumorigenesis. We have investigated the mRNA expression of all classical HDAC1-11 family members in a large cohort of primary neuroblastoma samples covering the full spectrum of the disease. HDACs associated with disease stage and survival, were subsequently functionally evaluated in cell culture models. Only HDAC8 expression was significantly correlated with advanced disease and metastasis, and downregulated in stage 4S neuroblastoma associated with spontaneous regression. High HDAC8 expression was associated with poor prognostic markers, poor overall and event-free survival. Knockdown of HDAC8 resulted in inhibition of proliferation, in reduced clonogenic growth, cell cycle arrest and differentiation in cultured neuroblastoma cells. Treatment of neuroblastoma cell lines as well as short term culture neuroblastoma cells with a HDAC8 selective small molecule inhibitor inhibited cell proliferation, clone formation and induced differentiation, and thus reproduced the HDAC8 knockdown phenotype. Global histone 4 acetylation was not affected by HDAC8 knockdown or by selective inhibitor treatment. Our data point toward an important role of HDAC8 in neuroblastoma pathogenesis and identifies this HDAC family member as a specific drug target for differentiation therapy of neuroblastoma.

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OR46

Genome-wide DNA methylation profiling reveals extensive and complex epigenetic alterations in neuroblastic tumors

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Background: Although a number of studies have reported aberrant methylation and inactivation of selected genes in neuroblastoma (NB), the extent of genome-wide promoter hypermethylation is poorly understood. We have applied methylated DNA immunoprecipitation (MeDIP) to genomic microarrays representing all known promoter and/or CpG islands in the human genome to more fully characterize the epigenome of neuroblastic tumors.
Methods: MeDIP analysis was applied to NB primary tumors (n=18), cell lines (n=7), ganglioneuroblastoma (GNB) (n=4) and ganglioneuroma (GN) (n=6).
Results: The total number of hypermethylated sites per sample ranged from 1,462-5,197). Consistent differences in DNA methylation patterns were identified between cell lines and tumor subtypes, indicating that epigenetic changes play a significant role in adapting cells to in vitro proliferation. Unsupervised hierarchical clustering of methylation data revealed a distinct split between the GN/GNB and NB groups. mRNA microarray expression analyses of cell lines following treatment with 5'-aza-2-deoxycytidine allowed us to explore the functional significance of the hypermethylation. The number of genes which were consistently hypermethylated in the GN/GNB group relative to NB was far greater (199 genes) than the opposite comparison (2 genes). Gene ontology analysis carried out on genes hypermethylated in >90% of GN/GNB displayed a statistically significant enrichment for protein kinases, growth factors and mitosis. In total, 70 recurrent large-scale blocks of contiguously hypermethylated promoters/CpG islands were identified, consistent with other studies of breast and colon cancer. The size of these regions ranged from 12.5 kb to 590.5 kb, with a mean length of 96.4 kb, with nearly one-third of the blocks clustering within telomeric regions.
Conclusion: Our results indicate that genome-wide hypermethylation in neuroblastic tumors is highly complex and plays important roles in many cellular processes, including in vitro cell growth and differentiation. We also identify many candidate genes which are potentially silenced through methylation and which will form the basis of functional studies.

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Parallel session 7 – Targeting MYCN OR47–OR55

OR47

SIRT1 enhances N-Myc protein stability in a positive feedback loop which converts N-Myc expression from a low to high level

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High level Myc expression due to gene amplification in the phase of genomic instability is a common feature of the advanced malignant phenotype. However, emerging evidence suggests that different Myc dose levels are required for conversion from a pre-cancerous, to a frankly malignant, state. Our own data indicates that much higher N-Myc levels drive tumor progression, than are required at embryonal tumor initiation. Here we describe a mechanism whereby low-level N-myc expression in pre-malignant tissues can be converted to a much higher level, thus driving tumor progression.
Silent information regulator 2, or human SIRT1, is a member of the class III histone deacetylase family which regulate lifespan and enhance cancer cell proliferation, in part by blocking tumour suppressor transcription. SIRT1 inhibitors have profound anti-cancer effects in vitro and in vivo. Here we show that N-Myc up-regulated SIRT1 transcription in neuroblastoma cells, which contributed to N-Myc-induced cell proliferation. Surprisingly, SIRT1 markedly up-regulated N-Myc protein levels. N-Myc is stabilised when phosphorylated at Serine 62 (S62) by phosphorylated extracellular signal-regulated protein kinase (ERK). SIRT1 increased the level of ERK phosphorylation, and, as a consequence N-Myc S62 phosphorylation. Affymetrix microarray and real-time RT-PCR revealed mitogen-activated protein kinase phosphatase 3 (MKP3) was one of the genes most significantly repressed by SIRT1 in neuroblastoma cells, and could be reactivated by the SIRT1 inhibitor, Cambinol. Repression of MKP3 blocked the effects of SIRT1 siRNA on ERK de-phosphorylation, N-Myc S62 de-phosphorylation and degradation, and, neuroblastoma cell growth arrest caused by N-Myc siRNA and SIRT1 siRNA. Importantly, SIRT1 was up-regulated, and MKP3 down-regulated, in precancerous ganglia cells from TH-MYCN transgenic mice. Perinatal Cambinol treatment of TH-MYCN mice reactivated MKP3 gene expression, and reduced tumourigenesis. Our data indicate that SIRT1 and N-Myc operate in a unique positive feedback loop to promote tumor progression. SIRT1 inhibitors may have role in therapy and prevention of neuroblastoma.

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OR48

Identification of therapeutic targets for MYCN-amplifed neuroblastoma by functional genomics

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Background: Amplification of the MYCN oncogene is a strong marker of poor prognosis and its relevance in NB development has been demonstrated. However, because MYC family encodes transcription factors the identification of small molecule inhibitors is a challenge. Furthermore, long-term inhibition of MYC has the potential to be harmful for proliferation of normal tissues. Our study aimed at identifying survival pathways of MYC-driven cancers, such as MYCN amplified NB. The existence of non-essential genes enabling the conditional survival of MYC overexpressing cells has been previously demonstrated through a candidate approach.
Method/approach: Here, we employed an unbiased synthetic lethal siRNA screening approach to identify druggable genes in an isogenic pair of cells with or without MYC-overexpression.
Results: Among ~3,500 gene tested, ~100 were identified as synthetic lethal genes with aberrant MYC expression. 45 genes were tested in additional cell pairs, including NB cell lines with and without MYCN amplification. The majority was confirmed and validated through multiple assays, such as apoptosis, DNA damage and by long term colony inhibition assay with stable RNAi. However, only a subset of the gene "Hits" conferred selective lethality in NB with MYCN amplifications. Among these, a gene was identified with no previously recognized direct connections to MYC, casein kinase 1 epsilon (CSNK1e). Investigation of CSNK1e expression in a set of primary NB (http://pob.abcc.ncifcrf.gov/cgi-bin/JK) indicated its correlation with MYCN amplification, possibly a direct consequence of MYCN transcriptional activity. Utilizing conditional silencing as well as chemical approaches we showed that CSNK1e is required only in the context of aberrant expression of either c-MYC or MYCN. In vivo validation was also obtained utilizing xenografts of human NB cells.
Conclusion: Our experiments indicate that CSNK1e is a valid therapeutic target for NB with MYCN amplification. This study provides a paradigm for the unbiased identification of therapeutic targets for molecularly defined subtypes of NB.

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OR49

MYCN transcriptionally controls the expression of the Nijmegen Brekage Syndrome gene product p95 nibrin/NBS1

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The p95 nibrin/NBS1 is a member of the human MRE11 complex whose genetic inactivation is responsible for the Nijmegen Brekage Syndrome, an autosomal recessive hereditary disorder characterized by microcephaly, facial dismorphism, growth retardation, immunodeficiency, radiosensitivity, chromosomal instability and cancer predisposition. NBS1 is a major player of the DNA double strand break repair responses and controls genetic stability. NBS1 is strongly expressed in highly proliferating tissues and is down regulated in differentiated neuronal cells. NBS1 hypomorphic mutations cause cell cycle alterations in mouse embryonic fibroblast.
MYCN is a helix-loop-helix/leucine zipper transcription factor belonging to the MYC family, amplified and overexpressed in 20-25% of human neuroblastomas. We have shown that, similar to other oncogenes, MYCN might induce a DNA damage response, possibly via replication stress and leading to p53 accumulation. We report that neuroblastoma cell differentiation and cell growth inhibition cause NBS1 repression at the protein and transcript level. In MYCN amplified neuroblastoma cell lines this is kinetically associated with MYCN inhibition. Indeed MYCN overexpression in SK-N-SH or in the SHEP Tet21/N inducible cell model stimulates NBS1 expression at the protein and transcript level. Mutagenesis of a typical CACGTG (E-box) MYCN binding site located in the human NBS1 intron 1 and chromatin immunoprecipitation experiments demonstrate that MYCN directly binds NBS1 gene in order to transcriptionally regulate its expression. NBS1 depletion via RNA interference inhibits Tet21/N colony forming ability more efficiently in MYCN overexpressing cells, by impairing cell proliferation and inducing cell death. Therefore NBS1 induction by MYCN is required for the increased proliferation induced by the oncogene. We postulate that NBS1 might be required to appropriately process the DNA damage and eventually genetic instability induced by MYCN and that inhibiting the DNA damage response pathway might lead to increased cell death in MYCN overexpressing/amplified cells.

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OR50

Bmi1 is a MYCN target gene and regulates tumorigenesis via repression of KIF1Bβ and TSLC1 in neuroblastoma

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Background: Recent advances in neuroblastoma (NB) research addressed that epigenetic alterations such as hypermethylation of promoter sequences, with consequent silencing of tumor-suppressor genes, can play significant roles in the tumorigenesis of NB. The exact role of epigenetic alterations, except for DNA hypermethylation, however, remains to be elucidated in NB research.
Results and Discussion: In the present study, we clarified the direct binding of MYCN to Bmi1 promoter by quantitative ChIP assay and up-regulation of Bmi1 transcription by MYCN by real-time PCR analysis. Mutation introduction into a MYCN binding site (E-box) in Bmi1 promoter suggests that MYCN has more important roles in the transcription of Bmi1 than E2F-related Bmi1 regulation. A correlation between MYCN and polycomb protein Bmi1 expression was observed in primary NB tumors. Expression of Bmi1 resulted in the acceleration of proliferation and colony formation in NB cells. Bmi1-related inhibition of NB cell differentiation was confirmed by neurite extension assay and analysis of differentiation marker molecules. Intriguingly, the above-mentioned Bmi1-related regulation of the NB cell phenotype seems not to be mediated only by p14ARF/p16INK4a, well-known Bmi1 targets. Expression profiling analysis using a in-house tumor-specific cDNA microarray (CCC-NHR13000 chip) addressed the Bmi1-dependent repression of KIF1Bβ (Munirajan et al., J Biol Chem 2008) and TSLC1 (Ando et al., Int J Cancer 2008), which have important roles in predicting the prognosis of NB. Chromatin immunoprecipitation assay and semi-quantitative RT-PCR of the tumor suppressors demonstrated that KIF1Bβ and TSLC1 are direct targets of Bmi1 in NB cells. These findings suggest that MYCN induces Bmi1 expression, resulting in the repression of tumor suppressors via PcG-mediated epigenetic chromosome modification.
Conclusion: NB cell proliferation and differentiation appear to be partially dependent on the MYCN/Bmi1/tumor-suppressor pathways.

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OR51

The interplay between Mycn, microRNAs and estrogen receptor-α during differentiation of the post-migratory sympathetic nervous system

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MYCN, a proto-oncogene normally expressed in the migrating neural crest, is in its amplified state a key factor in the genesis of human neuroblastoma (NB). However, the mechanisms underlying MYCN-mediated NB progression are poorly understood. Here, we present a MYCN-induced miRNA signature in human NB involving the activation and transrepression of several miRNA genes from paralogous clusters. Several family members derived from the miR-17~92 cluster, including miR-18a and miR-19a, were among the up-regulated miRNAs. Expression analysis of these miRNAs in NB tumors confirmed increased levels in MYCN-amplified samples. Specifically, we show that miR-18a and miR-19a target and repress the expression of estrogen receptor-a (ESR1), a ligand-inducible transcription factor implicated in neuronal differentiation. Immunohistochemical staining demonstrated ESR1 expression in human fetal sympathetic ganglia, suggesting a role for ESR1 during sympathetic nervous system development. Reconstitution of ESR1 expression in NB cells resulted in marked growth arrest and neuronal differentiation while inhibition of miR-18a in NB cells led to severe growth retardation, outgrowth of varicosity-containing neurites, and induction of neuronal sympathetic differentiation markers. We propose that MYCN amplification may disrupt estrogen signaling sensitivity in primitive sympathetic cells through deregulation of ESR1, thereby preventing the normal induction of neuroblast differentiation. Collectively, our findings demonstrate the molecular consequences of abnormal miRNA transcription in a MYCN-driven tumor and offer unique insights into the pathology underlying MYCN-amplified NB.

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Targeting MYCN by modulation of the fate of its mRNA: a new potential therapeutic approach for neuroblastoma.

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MYCN amplification in neuroblastoma patients is strongly associated with advanced disease stages, rapid tumor progression and poor prognosis, making this gene an obvious therapeutic target. MYCN is however a transcription factor, and therefore not a druggable protein. We took advantage of evidence of post-transcriptional regulation of MYCN gene expression to develop a cellular model for investigating the role of MYCN 3' untranslated region (3'UTR) in the modulation of MYCN mRNA fate, and to identify compounds able to affect it. Using a panel of parental neuroblastoma cell lines we firstly reported post-transcriptional MYCN alteration of expression, related in a complex way to gene amplification. We then generated luciferase reporter constructs with full length MYCN 3'UTR, which were stably inserted in the CHP134 neuroblastoma cell line. A screening was carried out in 96-well plates in triplicates, using a 2000-compound library including all the FDA-approved drugs, with luciferase activity assessed after 24h of a 2μM treatment. Molecules affecting luciferase were counterscreened for promoter effects and checked for reproducibility, dose-responsiveness and for the phenotypic outcome in terms of cytotoxic activity. We identified more than 100 hits, which were clustered based on structural and functional similarity. Cytotoxicity was high, as expected, for chemotherapeutic drugs, as well as for another chemically homogeneous class of drugs, cardiac glycosides (remarkably, 13 molecules active out of the 13 present in the library). The relationship between MYCN downregulation and toxicity of cardiac glycosides is currently under investigation. Another class of natural compounds, flavonoids, resulted instead effective in enhancing MYCN 3'UTR-induced luciferase activity, raising interest as potential triggers of MYCN-induced apoptosis. We report other experiments aimed at the definition of the mechanism of action of the two classes of compounds, which could finally result in the development of clinically relevant therapeutics for MYCN-amplified neuroblastomas.

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Bortezomib and HDAC inhibitor PCI-24781 show synergistic activity in neuroblastoma *in vitro* and *in vivo* models, inducing ROS and depressing MYCN

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Background: Current treatment of neuroblastoma (NB) often fails due to chemo-resistance and new treatments with novel mechanisms are needed. HDAC inhibitors are a diverse class of compounds which modulate transcription and control protein degradation and have shown inhibitory effects on NB both in vitro and in vivo. Our data indicate that the potent pan-HDAC inhibitor PCI-24781 is more effective than other HDAC inhibitors and is ideal to pair with the proteasome inhibitor bortezomib in NB.
Methods: NB cell lines and patient-derived primary NB cultures were treated with bortezomib and PCI-24781 alone and in combination for 48 hours. Cells were also treated with HDAC inhibitors vorinostat, sodium butyrate, and valproic acid and viability was assessed by calcein AM assays. mRNA from treated cells was evaluated at 6 and 24 hours using U133 2+ mRNA expression arrays and Ingenuity analysis. DCF was used to measure reactive oxygen species (ROS). Cell viability assays were repeated in the presence of N-acetylcysteine (NAC). Western blot evaluated caspase-3 and PARP cleavage. Nude mice were injected with 10^7 SMS-KCNR cells subcutaneously and treated with daily doses of 0.5 mg/kg bortezomib, 12.5 mg/kg PCI-24781, or a combination. Tumors were measured and imaged twice per week.
Results: NB cell lines and patient cells showed sensitivity to bortezomib and PCI-24781 with IC50's for bortezomib <50nM and IC50's for PCI-24781 <200nM. The combination of bortezomib and PCI-24781 was synergistic. Expression analysis showed upregulation of NOTCH 2 and its ligands as well as c-jun. NFκ-B and MYCN were both significantly down-regulated. DCF analysis showed formation of ROS and viability assays showed inhibition of caspase-mediated apoptosis in the presence of NAC. The NB xenograft mouse model showed a decrease in tumor volume in mice treated with both bortezomib and PCI-24781 when compared to the single agent treatment groups with significant survival benefit.
Conclusion: Bortezomib and PCI-24781 synergistically inhibit NB growth both in vitro and in vivo. This combination therapy is effective and well tolerated in the mouse model and would be a novel therapy for NB.

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OR54

Re-activation of CLUSTERIN by epigenetic drugs as a therapeutic approach for MYCN tumourigenesis

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Background: Epigenetic reprogramming is an important mechanism of oncogene-initiated tumourigenesis. MYCN, a neuronal specific member of the MYC family of transcription factors, is amplified and highly expressed in aggressive, metastatic neuroblastomas. In a previous study we showed that CLUSTERIN (CLU) is negatively regulated by MYCN and is a suppressor of MYCN induced tumourigenesis. Here we show that MYCN transcriptionally represses CLU via interaction with a non canonical E-BOX motif.
Methods: We used gel shift and Chromatin IP analyses to monitor the binding of MYCN to the CLU promoter in vitro and in vivo. Luciferase assays were carried out for assessment of transcription. In vitro invasion and proliferation/FACS assays were carried out for functional studies.
Results: MYCN inhibits the expression of CLU by direct interaction with the non-canonical E-box sequence CACGCG in the 5' flanking region of the CLU promoter. We found that binding of MYCN to the CLU gene causes the appearance of bivalent epigenetic marks, typically observed in the promoters of developmentally regulated genes in stem cells, such as acetylated histone H3, di-methylated H3K4 , tri-methylated H3K9-K27, and recruitment of histone deacetylases and polycomb proteins. Tricostatin A and Valproic acid, two inhibitors of histone deacetylases currently used in cancer clinical trials, re-activate the expression of CLU in MYCN amplified neuroblastoma cell lines, causing inhibition of their proliferation and invasive potential. Notably, the effects of the epigenetic drugs are completely abrogated when CLU expression is silenced by RNA interference.
Discussion This is, to our knowledge, the first study documenting that a MYC oncoprotein can impose a bivalent state and negatively modulate a tumour suppressor gene by direct interaction with an E-box motif. The observation that CLU is an essential mediator of the therapeutic effects of histone deacetylase inhibitors in MYCN-driven tumours suggests that re-activation of CLU in cancer patients could be used to predict a favourable response to epigenetic drugs.

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OR55

Identification of small molecules inhibiting Myc oncoprotein function

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Deregulated expression of *MYC* family oncogenes of *MYC*, *MYCN* and MYCL occurs in many types of human tumors, including the childhood tumors neuroblastoma and medulloblastoma, and is often associated with aggressive tumor development and poor prognosis. The MYC genes encode transcription factors that control the expression of thousands of genes of relevance for tumorigenesis. In mouse tumor models, inactivation of Myc often leads to tumor regression with well-tolerated side effects, suggesting that Myc is a potential and suitable target for anti-cancer therapy. However, targeting transcription factors with drugs is considered difficult and no anti-Myc drugs are clinically available today.
One potential strategy to target Myc is to try to interfere with the interaction between Myc proteins and cofactors that Myc is dependent on for its function. We have developed a system for screening for small molecule inhibitors of such interactions based on Bimolecular Fluorescent Complementation (BiFC). Cells expressing the Myc and its obligatory partner Max fused to complementary Yellow Fluorescent Protein (YFP) fragments were exposed to a NCBI library consisting of 2000 molecules. 15 potential candidates that reduced YFP fluorescence were further evaluated. Of these we have concentrated the work on two molecules. The first is named "Terminator of Myc" (ToM) and affects Myc:Max interactions indirectly by decreasing the steady state protein levels of both c- and N-Myc without affecting the level of Max. ToM induces apoptosis in a Myc-dependent manner in tumor cells, including MYCN-amplified neuroblastoma cells, at low μM concentrations while normal cells become G1 arrested without signs of apoptosis. The mechanism by which ToM acts is presently under investigation. The second molecule is named "Inhibitor of N-Myc/Max Interactions" (INMI) and selectively targets the N-Myc-Max interaction over the c-Myc-Max interaction, both in cells and *in vitro*. INMI represses N-Myc-driven transcription, and decreases neuroblastoma cell proliferation in an N-Myc dependent manner, both as adherent cells and in semi-solid medium, correlating with increased cellular senescence. Further, INMI inhibits N-Myc (but not c-Myc) + Ras-driven transformation of primary rat embryo fibroblasts. This is the first report of a molecule that targets the N-Myc:Max interaction, and may potentially be an interesting lead molecule for drug development for treatment of *MYCN*-amplified neuroblastoma. Since INMI does not affect c-Myc it is further likely to give fewer side effects.
Taken together ToM and INMI have the potential of becoming a useful anti-Myc drugs for treatment of cancer.

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Parallel session 8 – Novel clinical strategies OR56–OR64

OR56

The role of dietary restriction in the mechanisms of differential cellular protection: a strategy to enhance the efficacy of chemotherapy in the treatment of neuroblastoma

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Background/Aim: Strategies to treat cancer have focused primarily on the killing of tumor cells. Here, we described a differential stress resistance (DSR) method that focuses instead on protecting normal but not neuroblastoma (NB) cells from high dose chemotherapy. Specifically, we studied the mechanisms of starvation-(STS)dependent DSR in NB cell lines and we evaluated the toxicity and the therapeutic effects of STS in combination with different chemotherapeutic agents (chemo drugs) in suitable experimental NB models.
Methods: The in vitro cytotoxicity of chemo drugs in combination with STS was tested by Trypan Blue staining and MTT assay. The in vivo STS protocol allowed mice to consume water but not food for 48-60 hours prior to chemotherapy. All mice were monitored daily for weight loss. The in vivo therapeutic effects of appropriate combinations of different chemo drugs with STS were evaluated in term of toxicity, tumor burden and survival in A/J mice intravenously injected with NXS2-luciferase and Neuro-2a-luciferase NB cells. We tested the antitumor effect of STS alone by measuring the volume of subcutaneous ACN tumors developed in athymic mice. The effect of STS on the differential regulation of Akt, mTor, S6Kinase (S6K) and Erk in NB cell lines was evaluated by Western Blot experiments.
Results: The reduction in culture of serum from 10% to 1% and glucose from 1.0 to 0.5 g/L sensitized NB cell lines to different chemo drugs. Similarly, in vivo experiments demonstrated that STS in combination with high dose chemotherapy decreased the toxicity and significantly prolonged the survival of NB bearing mice. In addition, we demonstrated that STS alone could reduce tumor cell growth through downregulation of Phospho- Akt, Phospho-mTor and Phospho-S6K.
Conclusion: Taken together, these results support the hypothesis that STS can protect normal cells from and sensitize NB cells to chemotherapy-induced toxicity, and provide the foundation for a novel and powerful therapeutic strategy for the cure of NB.

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OR57

Fenretinide (4-HPR) orally formulated in Lym-X-Sorb™(LXS) lipid matrix or as an intravenous emulsion increased 4-HPR systemic exposure in patients with Recurrent or Resistant Neuroblastoma. A new approaches to neuroblastoma therapy (NANT) consortium trial

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Background: Fenretinide (4-HPR), a cytotoxic retinoid, obtained limited and variable plasma levels when tested in a corn oil-based capsule. 4-HPR/LXS® oral powder and 4-HPR intravenous emulsion (4-HPR IE) are new formulations intended to increase bioavailability, especially in small children.
Methods: We compared 4-HPR plasma levels and tumor responses obtained with capsule 4-HPR to those obtained with 4-HPR/LXS® and 4-HPR IE. Capsule 4-HPR was given three times a day for 7 days every 21 days, 4HPR/LXS® oral powder was mixed in Slim-Fast® nutritional supplement, twice a day for 7 days, every 21 days, and 4-HPR IE by continuous infusion over 120 hours every 21 days. Eligible patients had high-risk neuroblastoma with recurrent/progressive disease, or disease refractory or persistent after frontline therapy. Plasma levels were by a HPLC assay.
Results: Capsule trial data from CCG 09709 (Ph 1) and COG ANBL0321 (Ph 2) were compared to NANT 2004-04 (Ph 1, 4-HPR/LXS®) and NANT 2004-03 (Ph 1 4-HPR ILE). Both new formulations increased achievable mean peak plasma levels with an apparent increase in complete responses (CR) with 4-HPR/LXS. Toxicities to date in patients receiving 4-HPR/LXS ® or 4-HPR IE were modest and include dry skin, elevated triglycerides, reversible nyctalopia, and transient transaminase elevations.

Study	Mean Peak Plasma Levels (μM)	Evaluable Pt #	Responses	Prolonged Stable Disease
CCG 09709	8-10	30	1 complete	13
COG ANBL0321	6-9	58	1 partial	13
NANT 2004-04	15-20	29*	4 complete*	6*
NANT 2004-03	22-45**	ongoing	blinded	blinded

*All responses achieved at in Dose Levels 4 – 8 (1006 - 2210 mg 4-HPR/m^2/d), n = 18, the responses in NANT 2004-04 are pending central review.
** Data from dosing at 925 mg/m^2/d, dose escalation ongoing, MTD to be determined.

Conclusion: 4HPR/LXS® oral powder is well tolerated and obtained 2 - 5 fold higher (P < 0.01) 4HPR plasma levels than 4-HPR capsules on the same dose and schedule. The CR's noted in 4/18 patients at Dose Levels 4 - 8 of 4-HPR/LXS® warrant further study. An MTD for 4-HPR/LXS® was not identified; a recommended phase II dosing of 4-HPR/LXS® is 1500 mg/m^2/day, divided TID, x 7 days every 3 weeks. 4-HPR IE appears to further increase 4-HPR systemic exposure, but MTD determination of 4-HPR IE is ongoing and response data are currently blinded.

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OR58

Phase II trial of meta-iodobenzylguanidine (mIBG) with intensive chemotherapy and Autologous Stem Cell Transplant (ASCT) for high risk neuroblastoma. A New Approaches to Neuroblastoma Therapy (NANT) study

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Background: 131I-mIBG has been used as a single agent or combined with chemotherapy for the treatment of neuroblastoma (NBL). A phase II multicenter study of 131I-mIBG with carboplatin, etoposide and melphalan (CEM) and ASCT is now reported.
Methods: Fifty patients (pts) (median age 6.0 yrs, 1.1-22.8 yrs), 9 with partial response (PR), and 41 with stable (SD) or progressive disease (PD) to induction therapy, and no prior ASCT were enrolled. 131I-mIBG was administered on day -21, with CEM given days -7 to -4, and ASCT on day 0. The endpoint was response at day +60, stratified into standard risk (PR to induction), or poor risk (SD/PD to induction). Dosing of 131I-mIBG and CEM was stratified by glomerular filtration rate (GFR), with 8 mCi/kg 131I-mIBG for pts with low GFR<100 (13 pts), and 12 mCi/kg for pts with normal GFR>100 (37 pts). CEM doses were adjusted by GFR and, for the last 22 pts, by total body dosimetry.
Results: All 50 patients were evaluable for toxicity, and 49 for response. Six pts had non-hematologic dose limiting toxicity (DLT), 1/13 in the low GFR strata and 5/37 in the normal GFR strata. Dose limiting hepatic veno-occlusive disease (VOD) was seen in 5 pts (1 low/4 normal GFR). Two toxic deaths occurred, both related to VOD. Median time to engraft was 10 days for neutrophils and 15 days for platelets. Three pts required a 2nd stem cell infusion to engraft. Patients received a median 162.6 cGy (61-846 cGy), based on whole body dosimetry. Patients with severe VOD received a median 181 cGy (mean 280 cGy). Two of 3 pts receiving >500 cGy experienced DLT. In the poor risk group, 2 CR, 3 PR, 8 mixed responses (MR), 21 SD, and 5 PD were reported among 40 evaluable pts (13% CR+PR, 33% CR+PR+MR rate). One CR, 1 PR, 4 MR, 1 SD, and 2 pending were reported among 9 standard risk pts. For all 50 pts, the estimated event free survival (EFS) and overall survival (OS) at 1.5 yrs were 0.41±0.09 and 0.77±0.07 respectively (median follow-up 22.8 months, 2.6-50.7).
Conclusion:131I-mIBG with myeloablative CEM chemotherapy is tolerable and active therapy for patients with partially responsive or refractory disease.

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OR59

A phase IIa trial of ultratrace (no-carrier added) iobenguane I-131 (MIBG): A New Approaches to Neuroblastoma Therapy (NANT) study

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Background: I-131-MIBG is specifically taken up in neuroblastoma, with a response rate >30% in relapsed disease. The presence of non-radioactive "carrier" MIBG molecules may inhibit uptake of I-131-MIBG, resulting in less tumor radiation and increased risk of cardiovascular side effects. The primary aim of this study was to establish the maximum tolerated dose (MTD) of no-carrier added iobenguane I-131 (Ultratrace) with autologous hematopoietic stem cell (AHSC) support.
Method/approach: Eligible patients were age 1-30 years with resistant neuroblastoma, MIBG uptake, and cryopreserved AHSC. A diagnostic dose of Ultratrace (1-5 mCi) was followed by 3 dosimetry scans. AHSC were infused 14 days post therapy. Response and toxicity were evaluated day 60. The Ultratrace was escalated in 3mCi/kg increments from 12-21 mCi/kg using 3+3 design. The administered dose was adjusted based on absorbed dose estimates and Emami(1991) organ tolerance limits. Dose limiting toxicity (DLT) was failure to engraft or grade 3 or 4 non-hematologic toxicity except grade 3 pre-defined exclusions.
Results: Three patients each were entered and evaluable at levels 12, 15, 18 mCi/kg (444-666 GBq/kg) without required dose adjustment or DLT. Three additional patients entered at 21 mCi/kg have all required dose reductions of 6, 14, and 25% to meet organ limits. Median whole body radiation dose was 3 Gy (2.1-4.4); median liver dose, 11.5 Gy (9.8-28.1); median lung dose 11.6 Gy (6.1-16); median kidney dose 14.6 Gy (9.1-23). In 4 patients there were 8 target lesions with doses of 1.9- 11.0 mGy/MBq. There was no related grade 3 or 4 non-hematologic toxicity; all patents engrafted promptly. No patients at 15 or 18 mCi/kg had ANC<500. The response rate in 9 evaluable patients was 33%: 12 mCi/kg, 2 stable disease (SD) and 1 progressive disease (PD); 15 mCi/kg, 1 partial response (PR), 1 mixed response, 1 SD; 18 mCi/kg, 1 complete response (CR), 1PR, 1SD.
Conclusion: Ultratrace iobenguane-131 with AHSC support is feasible at 18 mCi/kg without significant toxicity and with promising responses that support proceeding to a pivotal Phase II study of 18 mCi/kg.

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OR60

Characteristics of relapsing localized neuroblastoma: A preliminary report of the second SIOPEN study (LNESG2, localized neuroblastoma European study group 2)

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Introduction: Based on the results of the first protocol, LNESG2 has been designed to expand information on factors associated with clinical prognosis, to maintain/improve the overall survival of >95 % for INSS stage 1 MYCN +/- and INSS stage 2 MYCN- patients (pts) treated by surgery alone using image defined preoperative risk factors (IDRF), to secure banking of material and to treat relapses in a uniform way.
Methods and patients: Prospective study of pts any age at diagnosis with resectable NB according to IDRF, treated by surgery only. Available preoperative LDH, negative MYCN if stage 2, performed 1pdel, centrally reviewed histology and secured material, were mandatory for inclusion. Results: Since March 2005, 237 pts have been included into the study: 116 girls, 121 boys with a median age at diagnosis of 16 months (0-165); 161 stage 1 (67.9%), 71 stage 2 (30%), 5 stage 3 pts (2.1%) with mainly abdominal, then thoracic and cervical primary. Over 4.5 years, 15/237 pts relapsed, including 6 stage 1, 2 of whom MYCN +, and 9 stage 2. Two other stage 1 pts MYCN+, and one with heterogeneous MYCN amplification, did not relapse. The latest relapse occurred 15 months after surgery, the 15 months overall relapse rate being 9.8%. Complete excision was possible in 11/15 relapsed pts, 2 had a macroscopic and 2 a microscopic residue, 2 of them having been operated in spite of IDRF. Histology was unfavorable in 3/15 pts. Relapse was local in 8, metastatic in 2, combined in 4, not precised in 1. Local relapses with favorable histology were reoperated only, whereas local relapses with unfavorable histology or combined/metastatic relapse went on the European high-risk protocol. All pts are alive. Currently, biological characteristics are evaluated by pan/multigenomic techniques on 26 case controls matched for country of origin and minimal follow-up of 15 months after surgery . The results will be presented adjusted for age and stage.
Conclusion: Relapses in localized NB are rare and occur more frequently in stage 2. Biological and pan-genomic analyses are currently under investigation in the relapsed pts and matched controls in order to identify risk factors for relapse.

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OR61

Outcome for stage 3 neuroblastoma: A report from the Children's Oncology Group

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Background: INSS Stage 3 neuroblastoma (NB) is a heterogeneous disease with variable outcome. The appropriate therapeutic intensity for this group remains controversial.
Methods: The study cohort included 324 patients with Stage 3 NB enrolled onto COG A3961 intermediate risk (n=270) or A3973 high risk (n=54) protocols. Clinical and biologic factors of age, MYCN status, tumor histology, somatic chromosome 1p or 11q aberrations and therapy intensity were analyzed for their impact on EFS and OS.
Results: The 3-year EFS and OS for the cohort were 89+/-2% and 96+/-1%, respectively. MYCN amplification (MYCN-A) was associated with significantly decreased EFS and OS compared to single copy MYCN (MYCN-NA) (EFS 74+/-10% vs 90+/-2%. p=0.035; OS 74+/-10% vs 98+/-1% p<0.0001). In patients with MYCN-NA NB (n=303), age >/= 547days (n=87) was associated with decreased EFS (p=0.004) and OS (p=0.034). For the cohort treated on A3961, age >/= 547 days was not predictive of lower EFS (p=0.16) or OS (p=0.62). Poorly differentiated/undifferentiated histology (UH) was not predictive of decreased EFS (p=0.36) or OS (p=0.20) for all stage 3 patients with MYCN-NA tumors, although both UH (p=0.04) and unfavorable INPC classification (p=0.02) predicted decreased OS for patients >/= 547 days of age with MYCN-NA tumors. Analyses of 1p and 11q data are ongoing. The 3-yr EFS and OS for Stage 3, MYCN-NA patients assigned to A3961 was 92+/-2% and 98+/-1%, respectively compared to 79+/-7% and 94+/-4% for those assigned to A3973. In a model for EFS in patients with MYCN-NA tumors, adjusting for intensity of treatment (A3961 vs A3973), age remains independently significant (p=0.004).
Conclusion: Patients with INSS Stage 3 MYCN-NA NB treated on A3961 intermediate risk protocol have an excellent prognosis and warrant further reduction in therapy. Those >/= 547 days of age with UH tumors or those with MYCN-A tumors at any age continue to have an inferior outcome, despite intensive therapy. Identification of improved therapeutic targets is needed for this group of patients with non-metastatic neuroblastoma.

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OR62

Do relapsed high-risk neuroblastoma patients have a second chance? Results of the German neuroblastoma trials

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Background: The prognosis of high-risk neuroblastoma patients has improved over the last decades. However, many patients experience relapse after successful initial treatment. This study investigates the outcome after second-line therapy.
Method/approach: We analyzed outcome and risk profile of high-risk neuroblastoma patients, i.e. patients with stage 4 disease or MYCN amplification. Patients 1 year or older diagnosed between 01.01.90 and 31.12.07 with relapse after successful first-line autologous stem cell transplantation (ASCT) were included.
Results: A total of 458 high risk neuroblastoma patients over 1 year underwent ASCT during first-line treatment, 256 experienced recurrence of disease, 181 received any salvage chemotherapy, and 24 of them finally underwent second ASCT. Multivariate analysis demonstrated that MYCN amplification, early recurrence within 18 as well as 24 months after first diagnosis, bone marrow involvement at recurrence, and lung/pleura involvement at recurrence independently predict poor survival after recurrence. The 24 patients with second ASCT had a better outcome (median survival 2.08 years, 3-year-survival from recurrence 41.7±10.6 %) compared to 75 patients who had no second chemotherapy (median survival 0.24 years, 3-year-survival rate 3.9±2.5 %) and 135 patients who underwent second line chemotherapy but did not undergo second ASCT (median survival of 0.89 years, 3-year survival 9.6±2.8 %, p<0.001). By February 2010, 3/24 patients were in complete remission, 3/24 in very good partial remission, 1/24 in partial remission. The survival time of these 7 patients was between 1.19 and 6.31 years. 15/24 patients have died of disease after successful second ASCT. Two patients have died of complications of the second ASCT.
Conclusion: Intensive second-line therapy is feasible. A small subgroup of relapsed high-risk neuroblastoma patients may benefit from intensive relapse chemotherapy and second ASCT. The potential of long term survival justifies clinical trials on intensive second-line treatment.

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OR63

Anti-GD2 murine monoclonal antibody (MoAb) 3F8 for consolidation of first complete/very good partial remission of high risk stage 4 neuroblastoma

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Background: 3F8 is active against chemoresistant NB in the bone marrow (BM). We used 3F8 to consolidate first complete/very good partial remission (CR/VGPR) patients (pts), combining it with granulocyte-macrophage colony-stimulating factor (GM-CSF) ± 13-cis-retinoic acid (CRA).
Methods/approach: 187 pts with high risk stage 4 NB (diagnosed after 18 months of age [171 pts] or with *MYCN* amplification [73 pts]; 92% with BM and/or bone metastases), were enrolled after achieving first CR/VGPR following (1) standard MSKCC or COG induction treatment [156 pts] or (2) additional high dose cyclophosphamide, plus topotecan or irinotecan, plus vincristine (CTV/CCV) for refractory disease [28 pts]. For consolidation, patients received: (Group 1) 3F8 alone (± targeted radiotherapy with ^{131}I-3F8 [NCT00002634]), or (Group 2) 3F8 + intravenous (iv) GM-CSF + CRA [NCT00002560] following myeloablative chemotherapy with stem cell transplant (SCT); or (Group 3) 3F8 + subcutaneous (sc) GM-CSF + CRA [NCT00072358] with or without SCT. Progression-free survival (PFS) and overall survival (OS) from first day of 3F8 treatment were evaluated by Kaplan-Meier analyses.
Results: PFS and OS for Group 1 (n=42) were both 41% ± 8% at 19 years (y) from first 3F8. Neither PFS nor OS changed significantly when treatment included 20 mCi/kg of ^{131}I-3F8. PFS for both Group 2 (n=58) and Group 3 (n=87) improved to 51% ± 7% at 11 y and at 7 y, respectively, while OS improved to 59% ± 7% and 80% ± 5% (p<0.01), respectively. Despite risk factors in Group 3 (36 *MYCN* amplification and 28 requiring CTV/CCV), OS was superior partly because of (1) improved second-line treatment for focal relapses and (2) a successful salvage regimen for isolated central nervous system metastases. All three groups had better PFS and OS (p<0.05) than historical control (n=28, 21% ± 8% at 24 y from diagnosis) when SCT without MoAb or CRA was the standard of care.
Conclusion: We attribute the consistent improvement in OS to a more effective use of 3F8/GM-CSF/CRA plus better salvage treatment following relapse.

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OR64

Changes over three decades in the prognostic influence of age in patients with neuroblastoma: A report from the International Neuroblastoma Risk Group Project

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Purpose: Increasing age has been an adverse risk factor in children with neuroblastoma (NB) since the 1970's, with a 12-month age cut-off for treatment stratification. Over the last 30 years, treatment intensity for children >12 months with advanced-stage disease has increased; to investigate if this reduced the prognostic influence of age, we analyzed the International Neuroblastoma Risk Group (INRG) database.
Patients and Methods: Data were analyzed for 11,037 children with NB (1974-2002) from Australia, Europe, Japan and North America. Cox modeling on event-free survival (EFS) tested if the age effect on outcome, adjusted for bone marrow metastases and MYCN status, had changed over time.
Results: Outcome improved over time: 3-year EFS of 46% (1974-1989) and 71% (1997-2002). The risk of event for >18 months of age versus <12 months decreased over time (hazard ratio; 4.61 (1974-1989) and 3.94 (1997-2002)). For children >12-18 months of age, 3-year EFS increased from 42% (1974-1989) to 77% (1997-2002). Outcome was worse if a) >18 months old (HR=4.47), b) bone marrow metastases (HR=4.00), and c) MYCN amplified-tumors (HR=3.97). For 1997-2002, the 3-year EFS for patients >18 months old with bone marrow involvement and MYCN amplification was 18%, but 89% for 0-12 months with neither bone marrow involvement nor MYCN amplification.
Conclusions: Although the adverse effect of increasing age on worsening outcome declined over 28 years, age remains a powerful indicator of unfavorable prognosis in children with NB. These results support the revised age cut-off of 18 months as a risk criterion in the INRG classification.

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Parallel session 9 – ALK OR65–OR71

OR65

Skewed distribution and oncogenic properties of *ALK* hotspot mutations in neuroblastoma

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Background: Recently, activating mutations were detected in the anaplastic lymphoma kinase (ALK) gene in familial and sporadic neuroblastomas (NB). We aimed to perform an in-depth analysis of the distribution of ALK mutations according to genomic and clinical parameters and establish an ALK expression signature as a first step towards understanding the nature of ALK signaling in NB.
Method/approach: Information on ALK mutation status, copy number and expression level was collected for 254 tumors and combined with data from 455 published cases. Oncogenicity of F1174L and R1275Q mutations was tested. An ALK signaling signature was obtained from expression data after pharmacological inhibition (NVP-TAE684) and shRNA knockdown of ALK mutant versus wild-type NB cells.
Results: ALK mutations were found in 6.9% of 709 investigated tumors, similarly distributed over favorable and unfavorable stages. Interestingly, one of the two hotspot mutations (F1174) occurred more frequently in MYCN amplified cases (P=0.001) and was associated with a particularly poor outcome, suggesting a cooperative effect between both aberrations. The skewed distribution and absence of the F1174 mutation in familial cases suggest possible differences in oncogenic signaling between F1174 and R1275 mutants. Further evidence for this hypothesis was provided by a Ba/F3 cell transformation assay where clearly stronger oncogenicity was noted for the F1174L than for the R1275Q mutant. Furthermore, an ALK gene signature was determined based on expression data from inhibition and knockdown experiments and ALK pathway activity scores were calculated in a set of 100 tumors following gene expression analysis. One ALK amplified case and an F1174 mutant exhibited a very high ALK activity score whereas three R1275 positive tumors showed no significant increase, further underscoring the difference in signaling properties between these two mutations.
Conclusion: The frequency of ALK mutations is similar in favorable and unfavorable stage tumors, but a skewed distribution was found for F1174 and R1275 hotspot mutations with evidence for higher oncogenicity for the F1174 mutation.

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OR66

High ALK receptor tyrosine kinase expression precedes ALK mutation as a determining factor of an unfavorable phenotype in primary neuroblastoma

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Background: Amplification and activating mutations of the anaplastic lymphoma kinase (ALK) have been postulated to contribute to neuroblastoma pathogenesis. This study aimed to determine the contribution of genomic ALK alterations and ALK expression levels to the clinical phenotype of neuroblastoma.
Method/approach: ALK genomic status and mRNA expression levels were determined by sequencing, quantitative PCR, and oligonucleotide-microarray analysis in 263 primary tumors from German neuroblastoma patients. Allele-specific ALK expression was determined by cloning and sequencing cDNA fragments. ALK protein expression was examined in western blots. The associations of genomic ALK alterations and ALK expression with survival were determined in log-rank tests and Cox regression models.
Results: Amplifications and non-synonymous mutations of ALK were detected in 2/263 and 21/263 neuroblastomas, respectively. Tumors with mutated ALK showed significantly elevated ALK mRNA and protein expression, and were associated with unfavorable patient outcome. Unexpectedly, the wild-type allele was preferentially expressed in most tumors with ALK mutations. In neuroblastomas without ALK mutations, ALK overexpression was strongly associated with prognostic markers of adverse outcome and with poor survival. Global gene expression patterns as well as clinical courses of patients with ALK mutations and patients with wild-type ALK showing comparably high ALK transcript levels were highly similar. In multivariate analysis, ALK expression but not mutation, was an independent factor of adverse outcome.
Conclusion: High ALK expression precedes ALK mutation as a determining factor of the clinical course in neuroblastoma, suggesting that elevated ALK expression in general should be considered as a specific target for novel therapeutic strategies in neuroblastoma.

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OR67

Risk stratification of neuroblastoma by genomic signature including *ALK* abnormality

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Background: We previously proposed the integration of genomic subgrouping system based on the array CGH analysis for the tumor risk classification of neuroblastoma (NB). There exist three genomic groups (GGs) of chromosomal aberrations in NB: silent (S), partial gains and/or losses (P) and whole gains and/or losses (W). Each GG was further segregated into subgroups with different clinical outcomes clearly defined by *MYCN* amplification (*MYCN*-amp), 1p loss, 11q loss and 17q gain. High-resolution array CGH also led us to identify a novel genomic alteration in NB, the activating mutation and amplification of *ALK* oncogene on 2p23. In this study, we conducted the clinical validation of our GG risk classification system together with the analysis of clinical impact of *ALK* genomic aberrations.
Method: Array CGH and mutation analysis of *ALK* gene of 343 sporadic NB samples in Japan (stage 1: 48; stage 2: 29; stage 4s: 27; stage 3: 60; stage 4: 179) were performed and the clinical impact of the genomic signatures was assessed.
Results: The results were fairly reproducible with our previous data, showing that GG-S or GG-W without *MYCN*-amp exhibited good prognosis (Ss and Ws, 8-year survival rates:>80%), while GG-P or GG-S accompanying *MYCN*-amp were very poor (Pa and Sa, 8-year survival rates:<33%). *ALK* gene mutations and amplification were found in 16 (4.7%) and 5 tumors (1.5%) in our dataset, respectively, which caused the patients' outcome worse (p=0.0061), especially those with *MYCN*-amp (*ALK*mut-, n=73 vs. *ALK*mut+, n=10, p=0.16). Notably, *ALK* abnormalities were frequently found in the certain GGs of the tumors, namely, GG-Pa with 1p loss, GG-Ps with 11q loss and GG-Ws with no partial 1p and 11q losses. Its nonrandom distribution will help find the *ALK* abnormality efficiently.
Conclusion: These results suggested that the combination of genomic signatures with *ALK* abnormalities can efficiently predict the patient prognosis without using conventional clinical markers, and will further improve the potential of the tumor risk classification system of NB. Prospective clinical studies of this classification are now underway.

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OR68

Analysis of human ALK neuroblastoma mutations in *Drosophila melanogaster*

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Mutations in the Anaplastic lymphoma kinase (ALK) receptor tyrosine kinase (RTK) have recently been described in neuroblastoma. Several studies have suggested that point mutations in particular residues in the kinase domain of the receptor result in constitutive, ligand independent, receptor activation and consequent pathological downstream signaling involved in neuroblastoma development. In order to understand the molecular cause of the disease we have generated transgenic *Drosophila melanogaster* expressing various mutant ALK present in neuroblastoma. Our results confirm that a number of the described point mutations in the kinase domain robustly activate ALK, as illustrated by the generation of a rough eye phenotype, suggestive of overproliferation, as a result of expression of these mutants in the fly eye. We are able to further conclude that this activation is ligand independent, since the wild type ALK RTK is unable to generate a rough eye phenotype, in contrast to mutant forms of ALK. Moreover, we are able to detect activation of downstream components of the ALK signaling pathway in tissues where the mutants are expressed. Our model system will enable further dissection of signaling transduction pathways affected in vivo by ALK gain of function mutations, with the aim of increasing our understanding of the molecular mechanisms behind neuroblastoma.

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OR69

Role of ALK and its ligands in neuroblastoma

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Background: Activating mutations in the tyrosine kinase domain of Anaplastic Lymphoma Kinase (ALK) receptor are found in the majority of familial neuroblastoma and in 10% of somatic cases. We have previously shown that ALK phosphorylation occurs in 50% of primary neuroblastoma samples. The ALK ligands, i.e. midkine (MDK) and pleiotrophin (PTN) were found to be expressed in 80% and 61% tumors respectively, suggesting that ALK activation could occur through mutation-independent mechanisms. The objective of the present study was to elucidate further the events leading to ALK activation.
Methods: In vitro, DNA transfection and siRNA were used to modulate expression of ALK and MDK in cell lines (IGR-NB8, IMR-32, LAN-1) expressing different levels of the receptor and its ligands. ALK pathway activation was evaluated by Western blot analysis and cell proliferation was measured by MTS assay. In vivo, MDK or PTN encoding DNAs were electrotransferred into mice skeletal muscle and tumor growth upon systemic delivery of either protein was evaluated on IGR-NB8 xenografts.
Results: In vitro, wild-type ALK over-expression in IGR-NB8 cell line expressing MDK led to phosphorylation of ALK and its downstream effector STAT3. Similarly, MDK knock-down reduced both ALK activation and expression, as well as STAT3 phosphorylation. Unexpectedly, MDK silencing dramatically decreased cell viability in three cell lines (from at least 50%). In vivo, electrotransfer of MDK and PTN led to opposite effects on both tumor take rate (87% in the group receiving MDK vs 80% in the group receiving PTN and 83% in controls) and on tumor growth (the delay to reach 1000 mm3 being 50 days in the group receiving MDK vs 63 days in the group receiving PTN($p<0.05$) and 54 days in controls). While MDK enhanced tumor take rate and tumor growth, PTN slowed-down both parameters.
Conclusion: Modulation of ALK ligands MDK and PTN has an effect on expression, activation and signaling of the ALK receptor. Moreover these cytokines regulate neuroblastoma cell proliferation in vitro and in vivo. MDK may therefore represent a new promising target for neuroblastoma treatment.

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OR70

Effects of selective ALK inhibitors to neuroblastoma

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Background: Neuroblastoma is one of the commonest solid cancers in childhood. In terms of the prognosis, it exhibits substantial heterogeneity; while some cases show spontaneous regression without any treatments, the prognosis of advanced neuroblastomas is still poor in spite of recent developments in treatments. We and others previously reported that anaplastic lymphoma kinase (ALK) is mutated or amplified in approximately 10% of advanced neuroblastoma cases. To evaluate a potential therapeutic role of ALK inhibition in advanced neuroblastoma, we examined the effect of different ALK inhibitors on a panel of neuroblastoma cell lines with or without ALK mutations.
Methods: In total, 30 neuroblastoma-derived cell lines were cultured at varying concentrations of ALK inhibitors, including TAE684, 2,4-PDD (pyrimidinediamine derivative) and PF02341066, and viable cells were counted by using the CellTiter-GloTM Luminescent Cell Viability Assay. NIH3T3 cells transduced with various ALK constructs were used as a control. The IC50 value of each ALK inhibitor against cell lines was calculated based on a nonlinear regression model (variable slope) using GrapfPad Prism 5 software. Effects of siRNA-mediated knock-down of mutated ALK on cell growth was also evaluated.
Results: All ALK inhibitors suppressed cell growth not only in ALK-mutated neuroblastoma cells but also in cells with wild-type ALK, which was accompanied by inhibition of downstream signaling molecules, including AKT, ERK, and STAT3. Cell growth of TGW carrying R1275Q mutation was not inhibited by these ALK inhibitors, which was similar to the IC50 for NIH3T3 cells with no ALK expression. siRNA-mediated knock-down of mutant $ALKF^{1174L}$ in SK-N-SH resulted in significant suppression of cell proliferation.
Conclusions Our findings suggested that ALK inhibition with low molecular weight compounds could have a therapeutic role to improve the outcome of advanced neuroblastoma.

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OR71

Therapeutic targeting of ALK on neuroblastoma cells by systemic delivery of GD_2-targeted liposomes entrapping siRNA

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Background and Aims: Anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase, which has recently been shown to contribute to oncogenesis in human neuroblastoma (NB) through mechanisms of gene mutation, amplification and/or deregulated expression. On the basis of functional studies demonstrating that ALK is clearly involved in NB cell growth/progression, we investigated on its potential as therapeutic target in NB.
Methods: Various NB cell lines stably transfected with an inducible lentiviral vector expressing short hairpin RNA (shRNA) against ALK were tested *in vitro* for proliferation and apoptosis by MTT and Annexin-V assays. The same cells were subcutaneously and orthotopically injected in nude mice to study tumor growth *in vivo*. A novel anti-GD_2-targeted nanodelivery system for siRNA was developed for *in vitro* and *in vivo* inhibition of NB growth and induction of apoptosis. *In vivo* effects on proliferation, apoptosis and angiogenesis were also analyzed by immunohistochemistry.
Results: ALK knock-down by lentiviral sh-RNA in human NB cells carrying wild-type or mutated gene sequence led to cell proliferation arrest and apoptosis *in vitro* and tumor growth inhibition *in vivo*. The targeted-liposomal formulation of ALK-directed siRNA showed increased siRNA stability and plasma concentration, and hence, improved binding, uptake, silencing and induction of apoptosis specifically in GD_2-expressing NB cells. In a subcutaneous mouse model of NB, intratumoral injection of the GD_2-targeted ALK-siRNA liposomes inhibited cell proliferation by inducing apoptosis and, concomitantly, decreasing blood vessel density. In pseudometastatic mouse models of NB, i.v. administration of the GD_2-targeted ALK-siRNA liposomes showed gene-specific antitumor activity with no side effects on repeated administrations.
Conclusion: ALK is important for NB cell growth/progression and angiogenesis, and is expected to impact on the design of innovative therapeutic strategies. ALK-selective siRNA entrapped into novel GD_2-targeted nanoparticles could be systemically delivered and used as a new modality for NB treatment.

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Parallel session 10 – Genomics, candidate loci OR72–OR80

OR72

A regulatory BCL2 promoter polymorphism (-938C>A) is associated with outcome in neuroblastoma

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Background: Expression of the BCL-2 anti-apoptotic protein correlates with adverse outcome of neuroblastoma and confers chemoresistance to neuroblastoma cells. BCL2, therefore, serves as a bona fide drug target in neuroblastoma. With the development of BCL-2 inhibitors, a new therapeutic option is now available for evaluation. We hypothesized that a regulatory BCL2 -938C>A promoter polymorphism, which significantly affects promoter activity and Bcl-2 expression in different malignancies, may influence survival of neuroblastoma patients.
Method/approach: Genotypes of the -938C>A BCL2 promotor SNP were determined in a cohort of 174 patients with neuroblastoma using PCR amplification and pyrosequencing (PSQ). Genotypes were correlated with relapse-free survival using Kaplan-Meier analysis.
Results: Kaplan-Meier analysis showed that the -938AA genotype was a favorable prognostic factor for relapse-free survival (p=0.04). Multivariate Cox regression analysis incorporating INSS stage and MYCN amplification revealed an increased risk of recurrent disease in patients with the CC genotype (hazard ratio of 2.5 [95% CI, 1.1 to 5.4; p = 0.021]) compared to patients with the AA genotype.
Conclusion: The BCL2 -938C>A polymorphism is a predictor of relapse-free survival in neuroblastoma patients. We are currently investigating its functional implication in neuroblastoma tumor biology. The BCL2 -938C>A promoter SNP has potential as a prognostic marker, and might be useful as a biomarker guiding optimal choice of patients who could benefit from treatment with BCL2-targeted drugs.

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OR73

Identification of critical domains that mediate the transcriptional and growth-inhibiting functions of the neuroblastoma tumor suppressor gene CASZ1

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Background: Chromosome 1p36 is frequently lost in the tumors of neuroblastoma (NB) patients with unfavorable prognoses and harbors the gene locus for the transcription factor CASZ1. Low CASZ1 expression is significantly associated with lower overall patient survival, and CASZ1 has been shown to suppress NB cell growth and to regulate key genes in neural development (e.g. TrkA, NGFR and TH). Despite prior studies implicating CASZ1 in neural development and NB growth suppression, the key domains that mediate its function, in any species, have never been elucidated. To address this question, we performed a detailed structure-function analysis of the most evolutionarily conserved CASZ1 isoform (CASZ1b).
Method/approach: Loss of function was determined by loss of the ability to induce endogenous TrkA, NGFR and TH mRNA transcription as well as by loss of the ability to activate a TH promoter-luciferase reporter construct.
Results: Each of the five zinc fingers was individually destroyed, and the loss of any one of ZF1-4 resulted in a 58-79% loss of function compared to WT CASZ1b ($p < 0.05$ for each sample). However, mutation of ZF5 or deletion of the C-terminal sequence AA728-1166 (a truncation of 38% of the protein) did not appreciably alter transcriptional function. A series of N-terminal truncations revealed a critical transactivation domain at AA31-185. Loss of this transactivation domain resulted in a complete loss of CASZ1b function ($p < 0.001$). There was no decrease in protein stability or nuclear localization. In order to assess whether loss of CASZ1b transcriptional activity affects suppression of NB cell growth, tetracycline-inducible WT or ZF4m CASZ1b expression vectors were stably cloned into SY5Y cells. There was an 81% suppression of growth in WT CASZ1b-expressing cells ($p < 0.001$), while two independent ZF4m-expressing clones failed to suppress NB cell growth.
Conclusion: Whether via 1pLOH, epigenetic silencing or genetic alterations, this study demonstrates the potential impact that loss of CASZ1 transcriptional activity has on the induction of genes important in NB cell differentiation and control of NB cell growth.

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OR74

KIF1Bβ tumor suppressor, identified from the homozygous deletion at chromosome 1p36.2, interacts with YME1L1 metalloprotease to induce apoptosis through mitochondrial morphogenesis and cytochrome c release

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Background: We have previously identified KIF1Bβ, a member of the kinesin superfamily genes, as a tumor suppressor from the homozygous deletion at chromosome 1p36.2 in NB1 neuroblastoma (NB) cell line (Oncogene, 2000; JBC, 2008). KIF1Bβ was also identified as a downstream target tumor suppressor of the dysregulated NGF signaling in familial pheochromocytoma (Genes Develop., 2008). Here we found that KIF1Bβ modulates mitochondrial morphogenesis and regulates cytochrome c release to induce apoptosis.
Methods: The yeast-two hybrid system was used to identify the interacting proteins. The mitochondrial morphology and functions were examined by immunofluoresence using MitoTracker Red® and anti-cytochrome c antibody. To quantify the rate of mitochondrial fusion we used the JC-1 probe.
Results: The yeast two-hybrid screening using the pro-apoptotic region of KIF1Bβ as a bait identified three interacting proteins, one of which was the mitochondrial YME1L1 metalloprotease. YME1L1 is known to regulate mitochondrial fusion by cleaving OPA1 which controls cytochrome c release from mitochondria during induction of apoptosis. Both NB1 cells homozygously deleted with KIF1Bβ as well as Hela cells with siRNA knockdown of KIF1Bβ showed increased mitochondrial fusion leading the cells to more survival. Those cells were resistant to doxorubicin and other pro-apoptotic stresses. On the other hand, overexpression of KIF1Bβ induced a split of mitochondrial morphology, enhanced cytochrome c release into the cytoplasm, and eventually promoted apoptosis. Indeed, YME1L1 physically interacted with OPA1. Interestingly, high expression of *YME1L1* was marginally associated with better survival of NB patients (n=101; p=0.07), while high expression of *OPA1*, which was significantly decreased in stage 4s tumors ($p<0.05$), was correlated with poor outcome (n=101; p=0.04).
Conclusion: KIF1Bβ is a downstream target of NGF signaling pathway which is attacked by downregulation of TrkA/p75 in NB as well as mutations of *NF1*, *RET*, *SDH* and *VHL* in familial pheochromocytoma. Our present data first unveiled the whole pathway how KIF1Bβ functions as a tumor suppressor in cancers like NB.

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OR75

Coordinate expression of Let-7 family members in neuroblastoma and their dysregulation by DNA copy number loss

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Background: MiRNAs are differentially expressed in subtypes of neuroblastoma (NB), and can be correlated with genomic imbalances and clinical outcome. Here, we analyze the expression patterns of let-7 family members (13 members located on 9 different chromosomes) in a large set of NB tumors in order to identify mechanisms of regulation and to assess whether any of these miRNAs might contribute to NB pathogenesis.
Method: A set of NB (n=145) was analyzed for expression of 430 miRNAs by stem-loop RT qPCR and by array CGH. Functional characterization of miRNAs was performed on NB cell lines.
Results: Lower expression of several let-7 family members (let-7e, 7i, 7g and 7f) correlated with poor EFS or OS in our set of tumors (P = 0.009 to 0.046). Although let-7a did not correlate with survival in the entire tumor set, stratified analysis of the MYCN amplifed (MNA) tumors indicated a correlation between lower expression and poor EFS ($p<0.05$). Ectopic over-expression of let-7a in MNA Kelly cells caused reduced cell proliferation, whereas antimiR knockdown resulted in enhanced cell proliferation. Integrated analysis of the miRNA expression profiling with array CGH data indicated that large-scale genomic deletions have contributed to the lowered expression of let-7 family members in unfavourable tumor subtypes. We also demonstrate that nearly the entire let-7 family show remarkable coordinated expression in our set of neuroblastoma in spite of mapping to 9 different chromosomes. The coordinated expression extends to other miRNAs which map within 10 kb of let-7 family members. The only outlier was let-7e, which showed a very different pattern of expression in the tumors.
Conclusions: Lower expression of let-7a was found to be correlated with genomic deletions and with poorer event free survival for patients with MNA tumors. Consistent with these observations, ectopic over-expression of let-7a led to decreased cell proliferation of Kelly cells. We conclude that let-7 family members are regulating, or being regulated, by a common molecular pathway given their highly significant pattern of coordinated expression across a sizeable tumor set.

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OR76

A genome-wide association study (GWAS) of neuroblastoma

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Background: The genetic etiology of familial neuroblastoma (NB) has recently come into focus, but the genetic and environmental factors that cause sporadic NB remain largely unknown.
Methods: We are comparing germline genome-wide single nucleotide polymorphism (SNP) genotypes from 5,000 NB patients to 10,000 controls in order to discover SNP and copy number variation (CNV) associations. Independent case:control series from the UK and Italy are used for replication efforts. Clinical correlative and mechanistic studies are performed in primary tumor tissues and cell line models.
Results: To date, we have genotyped over 3,500 NB cases and have reported two loci harboring common risk variants and one with a single CNV, each highly associated with NB (*FLJ22536* NEJM 2008, *BARD1* Nat Gen. 2009, *NBPF23* Nature 2009). We have discovered additional SNP associations (see Diskin, et al. ANR 2010 for additional CNV associations) including *LMO1* at 11p15, *DUSP12* at 1q23 and *DDX4* at 5q11. These associations are phenotype specific, with *DUSP12* and *DDX4* risk variants being enriched in low-risk disease cases, while the other SNP risk alleles were enriched in high-risk disease, suggesting that NB may represent distinct genetic diseases at the level of tumor initiation. We next demonstrated that *BARD1* and *LMO1* function as oncogenic drivers, and that targeted depletion of specific SNP-risk allele associated transcripts results in decreased cell proliferation, restoration of contact inhibition and induction of programmed cell death. The SNP risk alleles show additive effect on susceptibility, and ongoing interaction analyses have identified other genetic variants that act in an epistatic fashion.
Conclusions: The NB GWAS has identified multiple susceptibility variants, both common and rare. Planned resequencing of the regions in both germline and tumor tissues will identify the disease causal variations, and lay the groundwork for understanding the mechanism by which germline and somatic alterations at these loci impact tumor phenotype.

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Acquired segmental copy number changes in relapsed neuroblastoma

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Background: Stage 4 neuroblastoma is typified by initial response to chemotherapy and radiation therapy, followed by recurrence, a brief response to salvage therapy, and ultimately further disease progression and death. Whereas the underlying aberrations that drive neuroblastoma emergence are the subject of intense study, the genomic changes associated with chemoresistance and treatment failure are largely unexplored. Here, we probed the basis of therapy resistance by performing DNA copy number analysis in matched diagnostic and relapse neuroblastoma samples.
Method/approach: DNA was extracted from tumor sections comprised of >90% neuroblastoma cells. Samples included diagnostic adrenal masses from two patients diagnosed at >18 months of age, a lung mass detected 21 months after diagnosis in Patient 1, and a paraspinal mass detected 5 years after diagnosis in Patient 2. Diagnostic and relapse DNAs were competitively hybridized to an Agilent 1M CGH array and copy number changes determined.
Results: Direct comparison with the corresponding diagnostic tumors indicated that both relapse tumors harbored multiple segmental and focal copy number changes. Changes included aberrations that are prevalent at diagnosis (e.g. 17q gain), as well as partial losses of segments that are commonly gained in diagnostic samples (e.g. partial losses of 1q, 2p, and 17q). Also detected were additional gains and losses in the vicinity of an amplified *MYCN* indicative of remodeling of the original *MYCN* amplicon, rare segmental or whole chromosome copy number changes, and recurrent loss of a 3q segment in both of the relapse tumors. Focal gains or losses included genes that have been directly implicated in DNA repair, chemoresistance, and cancer progression.
Conclusion: Neuroblastomas acquire numerous segmental copy number changes as they evolve during chemotherapy and radiation treatment. Identification of genes within specific regions of copy number alterations may provide clues to the basis of acquired resistance to neuroblastoma therapy.

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OR78

Identification and characterization of somatic rearrangements in neuroblastoma cell lines using genome-wide massively parallel sequencing

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Background: The genetic alterations of neuroblastoma (NB) cell lines and tumors have been, up to now, characterized using conventional strategies including cytogenetic and molecular methods, providing a picture of genomic rearrangements at a quite low resolution. In order to genuinely characterize somatic rearrangements in NB samples, we used massively parallel sequencing.
Methods: For two NB cell lines, a mate-pair library was constructed and paired-end sequencing was performed using the Illumina Genome Analyzer II system. For one of the two cell lines, a normalized random primed cDNA library was prepared for subsequent sequencing by GS FLEX Titanium series chemistry.
Results: For both samples, almost 60 millions of pairs were obtained from the mate-paired librairies and aligned against the reference genome. There were 416,376 and 375,972 reads anomalously mapped by Bowtie in cell line A and cell line B, respectively, suggesting possible inter- or intra-chromosomal rearrangements. Various criteria were applied in order to identify aberrant links with the highest relevance and prioritize them for confirmatory screening. The majority of the unbalanced translocations previously detected by spectral karyotyping and/or array-CGH were detected amongst the inter-chromosomal rearrangements. Experimental validation by PCR allowed to characterize these structural variants to the base-pair level. For cell line A, analysis of a lymphoblastoid cell line derived from the same patient confirmed that the analyzed rearrangements were somatic. A high number of intra-chromosomal rearrangements was also identified in both cell lines. For RNA-Seq, ~ 500 000 reads of around 400 pb were obtained. These reads were mapped to the reference genome and analysis is ongoing to search for chimaeric transcripts and mutations.
Conclusion: Genome-wide massively parallel sequencing provides a more exhaustive and precise characterization of somatically acquired rearrangements in tumor cells as compared to conventional strategies. It represents a powerful approach to get insights into the mechanims that underlie NB oncogenesis.

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OR79

Genome/transcriptome analysis of metastatic neuroblastoma, reveals an increase of structural aberrations and deregulation of rho/ras and telomerase pathways associated with poor patients outcome

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Background: About 50% of patients with neuroblastoma (NB) show a metastatic disease at diagnosis and their clinical behavior ranges from spontaneous regression to fatal progression. The present work identifies genome/transcriptome signatures associated with disseminated NBs of patients at different age and outcome.
Method: Patients were selected according to age, stage and outcome. G1: stage 4S; G2: stage 4, ≤ 18 months (m), with survival > 3 years; G3: stage 4, ≥ 19 m, dead of disease. The genome of 132 NBs (G1: 49; G2: 36; G3: 47) was analyzed by aCGH. Gene Expression Profiles (GEP) of 142 NBs (G1: 60; G2: 30; G3: 52) were performed using a custom 11K oligonucleotide array.
Results: The aCGH showed different numerical (NA) and structural aberrations (SA) among the three groups. G1 NBs are characterized by a higher frequency of NA; G2 have NA and SA in 70% of cases and exclusive SA in only 5%; 17% of G3 have SA and 83% both NA and SA. Comparison among the groups showed that the average of NA occurrence significantly decreases from G1 to G2 to G3 (9.6 G1 > 7.2 G2 > 3.6 G3). In contrast, the SAs significantly increase in G3 (0.7 G1 < 3.7 G2 < 7.0 G3). We observed that some chromosomes have more than one SA in G3 NBs. The GEP showed a deregulation of Rho/Ras gene pathway. It has been also observed a progressive down regulation of development and adhesion genes and an increase of cell cycle genes from G1 → G2 → G3. Moreover, telemerase genes were significantly over-expressed in G3 with respect to remaining groups.
Conclusion: Present data show that frequency of SA significantly increases from stage 4S to 4 and are associated with a more aggressive phenotype. The deregulation of Rho/Ras pathway genes may explain the increase of tumor aggressiveness from G1 → G2 → G3. The increase of cell cycle and telomerase genes expression associated with G3 provides a possible explanation for the unlimited replicative potential of NB and may be responsible for the increase of SA. Finally, tumor progression in older patients in relation with SA accumulation and gene regulation is discussed.

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OR80

Irregular chromosome segregation by tripolar divisions; mechanisms for heterogeneity of karyotypes in neuroblastoma

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Neuroblastoma (NB) shows a distinct characteristic in ploidy and can be classified into diploid and triploid tumors, which are closely related with respect to prognosis. Diploid tumors are found in both infants and children; however, the prognosis is favorable in infants but not in children. MYCN amplification is a poor prognostic factor in diploid tumors and usually accompanied by 1p terminal deletion. Triploid tumors are found mainly in infants and show good prognosis despite 50 % increase of DNA contents. The range of chromosomes in triploid cells is defined as 58-80 chromosomes and karyotypes of triploid tumors vary in individuals. These triploid cells have gains or losses of certain whole chromosomes and do not show a complete set of 3 of each chromosome. A hypothesis describes that triploid cells are derived from tripolar divisions of tetraploid cells and this tripolar division produces two triploid and one diploid cells. In this study, we confirmed that tripolar divisions were observed in 3% of anaphases in a NB cell line and the chromosome complement in daughter cells was examined by fluorescence in situ hybridization (FISH) using centromeric probes. The number of FISH signals for chromosomes 1, 2 and X in the daughter cells was different depending on each chromosome, indicating that two identical triploid cells could not be formed through tripolar division. Chromosome complements based on these three chromosomes in daughter cells formed by a tripolar division varied between mitotic cells. Some of the daughter cells did not contain X chromosome, however no metaphases without X were found. Our results suggest that tripolar division is not a perfect cell division and might involve in chromosome losses or gains, and all the 3 daughter cells cannot always survive because of lack of certain chromosomes. Tripolar divisions change chromosome complements in triploid cells, and could be a cause of heterogeneity in NB.

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Parallel session 11 – Prognostic factors and markers OR81–OR89

OR81

Is subtotal resection sufficient for treatment of ganglioneuroma and localized ganglioneuroblastoma intermixed?

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Background: Ganglioneuroma (GN) and ganglioneuroblastoma intermixed (GNBI) form the mature end in the range of neuroblastic tumors (NT). We studied the clinical characteristics of GN/GNBI and the impact of resectability on long term outcome.
Methods: Clinical characteristics of patients with GN and GNBI were analyzed retrospectively and compared to patients with immature localized neuroblastoma (NB) and ganglioneuroblastoma nodular (GNBN) registered between 2000 and 2009.
Results: Of 874 consecutive registered patients with localized NT, 790 were reviewed centrally according to the International Neuroblastoma Pathology Classification. Of 790 tumors 159 (20%) were classified as GN (10% GN mature, 90% GN maturing) and 52 as GNBI (6.6%). Patients with GN/GNBI had more often stage 1 disease (69% vs. 37%, p<0.001) and presented less frequently an adrenal tumor (31% vs. 41%, p=0.02), positive mIBG-uptake (33% vs. 90%, p<0.001) and elevated urine catecholamine metabolites (HVA 38% vs. 63%, p<0.001, VMA 26% vs. 66%, p<0.001) than patients with more immature NT. The median age at diagnosis is increased with the grade of differentiation (NB/GNBN: 9; GNBI: 62; GN maturing: 70; GN mature: 120 months, p<0.001). Of 211 patients with GN/GNBI, the tumor was completely resected at diagnosis in 150 patients (71%; 116/159 GN, 34/52 GNBI) and in delayed surgery after 4 to 33 months in 11 patients (5 GN, 6 GNBI). Chemotherapy was given to 11 patients (3 GN, 8 GNBI). In 45 patients (34 GN, 11 GNBI) a residual tumor is currently under observation (Median observation time: 40 months). Only 2 patients (1 GN, 1 GNBI) showed local progression so far. Two patients died of treatment related complications (surgery: 1; chemotherapy: 1), but none of tumor progression.
Conclusions: GN and GNBI account for about one quarter of neuroblastic tumors and differ from more immature tumors in age at diagnosis, stage, localization, mIBG-uptake and catecholamine metabolism. Surgery alone seems to be sufficient for the treatment of GN and most patients with GNBI and does not need to be radical.

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OR82

Survival variability by race and ethnicity in neuroblastoma: A Children's Oncology Group (COG) Study

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Background: Survival probabilities according to race and ethnicity have been reported in a number of cancers, but little is known about outcome disparities in neuroblastoma.
Methods: We compared disease presentation and survival probabilities among white, black, Hispanic, Asian, and Native American children enrolled on the COG NBL biology protocol ANBL00B1. Disease outcome was measured as overall (OS) and event-free survival (EFS), and was adjusted for prognostic factors.
Results: 3,923 children with NBL consented and enrolled between 2001 and 2009 were included. Non-hispanic whites ("white") constituted 73% of the cohort; 11% were Hispanic, 11% non-hispanic black ("black"), 4% Asian, and 1% Native-American. When compared with whites, blacks were more likely to present >18 months of age (blacks: 62% vs. 48%, p<0.001), have stage 4 disease (blacks 53% vs 46%, p=0.003), unfavorable histology (blacks: 49% vs 40%, p<0.001), and high-risk disease (blacks: 56% vs. 43%, p<0.001). Native Americans also had a higher prevalence of high-risk disease than whites, but the incidence of high-risk disease in Asian and Hispanic children was similar to that of white children. Overall three-year EFS was: Hispanics, 74%; whites, 69%; blacks, 63%; Asians, 62%; and Native-Americans, 38%, (p<0.001). When compared with whites, EFS was worse for blacks (HR 1.28; p=0.01) and Native-Americans (HR 2.33; p=0.003), However, adjustment for risk group abrogated these differences. Examination of EFS by follow-up time (<2 years vs. 2+ years from diagnosis) revealed a higher prevalence of late-occurring events among blacks. This was confirmed in the multivariate analyses restricted to 2+ year survivors, where blacks had significantly worse EFS (HR=1.5, p=0.04) as compared with whites.
Discussion: Black and Native-American children have a higher prevalence of high-risk NBL and worse outcome than other ethnic groups. The propensity for delayed events in blacks suggests that this population may be more resistant to chemotherapy. Studies are being planned to delineate the role of genetic predisposition in the racial differences in prevalence of high risk NBL and survival.

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OR83

Stable incidence of neuroblastoma during 28 years in Sweden with significant sex differences and improved survival, in particular for children with high-risk disease with MYCN amplification

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Background: Over 28 years, 1982-2009, 384 children with neuroblastoma were included in the population based Swedish Childhood Cancer Registry.
Methods: All children were characterized according to age at diagnosis, sex, chromosomal aberrations with FISH/CGH, INRG stage, treatment and overall survival.
Results: 207 children had localized stage (L), 153 metastatic stage (M) and 21 infants metastatic special stage (MS) (three missing stage, 2 girls). Standardized annual incidence was 1/100000 children <15 years and did not change over time with boys:girls ratio 1.15:1. Boys had worse outcome than girls (61.3% vs. 70.2% 5y survival) due to more stage M (ratio 1.39:1, 1.5:1 >18 months). Sex ratio for stage L was 0.99:1 with boys significant younger (p<0.01). 17q-gain was more common in boys (p=0.04) but no sex difference for MYCN-amplification (MNA) or 11q-deletion. Age (>12 or >18 months), stage M, MNA, 1p- or 11q-deletion and 17q gain were all related to worse outcome. Children with 11q-deleted tumors were older (median 41 months; boys and girls equal) than those with MNA (median 22.5; boys older than girls, median 28 vs. 20). Five year survival for all children improved from 57.7% for those diagnosed 82-90 to 62.1% for 91-99 and 78.6% for 00-09 (p<0.001). The most improved prognosis was achieved in high-risk patients (stage M >18 months or MNA, n=148) from 11.1% (82-90, n=36) to 17.9% (91-99, n=56) and 61.6% (00-09, n= 56, p<0.001). Among 101 high-risk patients outcome improved for children with MNA (from 11.1% for 82-95 to 48.9% for 96-09) but not for those with 11q-deletion (37.5% for 82-95, and 42.9% for 96-09).
Conclusion: Population-based neuroblastoma incidence was stable over 28 years with significant sex differences with respect to stage, age and biology. Outcome improved over time related to risk-based therapy, in particular for children with high-risk disease where the majority now can be long-term survivors. However, children with MYCN amplification benefit most from current intensified multimodal treatment, whereas our results identify the need of reconsidered therapy for children with 11q-deleted neuroblastoma.

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OR84

Long term outcome and impact of biology within risk adapted treatment strategies: The Austrian neuroblastoma trial A-NB94

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Aim: Analysis of risk adapted treatment according to MYCN amplification (MNA), age, stage and response for children with localized or metastatic neuroblastoma investigated with pan- and/or multigenomic techniques.
Patients and Methods: Between July 1994 and January 2002, 164 patients (pts) were registered (87 males; median age 1.37yrs). Depending on stage, age (cut-off 1yr) and MYCN pts were stratified either to surgery only (stages1&2) or a total of 6 cycles of chemotherapy of adapted intensity (CV, CBDCA-Vp16,CDDP-Vp16,CAV,HD-CAV). All MNA pts >stage1 received local radiotherapy (age adopted 24-30Gy). Stage 4 pts received one course of high dose treatment (HDT) followed by autologous stem cell rescue (SCR) (MEL,CBDCA,VP16) if <1yr and MNA or if >1yr in case of complete skeletal response; if >1yr and skeletal residues prior HDT the triple HDT/SCR concept [TTP-CYC, TTP-CBDAC, MEL-VP16] was applied. The median observation time is 9 yrs (range, 8.35 to 10 yrs).
Results: The 5-yr overall survival (OS) of all pts is 85%±0.03. Outcome by age showed an OS of 94% for 63pts <1yr, 90% for 22pts between 1-1.5 years and 77% for 80pts >1.5 years. Pts with localized disease (LD) any age and any biology achieved OS rates of 98% for 63 pts stage1, 100% for 20 pts stage2, 100% for 26 pts stage3. Ten stage 4s pts had an OS of 90%. Stage 4 pts>1yr had an OS of 46% while pts <1yr had an OS of 75%. Thirty-two MNA pts had an OS of 63% as opposed to 132 non-MNA with an OS of 90% (p<0.001). Segmental Chromosome Aberrations (SCA) were present in 69pts without MNA in increasing frequencies according to age and stage: in localized pts up to 44%, but 93 % in stage 4 pts. In pts >1.5yrs 71% had SCA, but only less than 33% in pts<1.5yrs. SCA in 16 pts>1.5yrs resulted in an OS of 94% which was not significantly different to 36pts without SCA whilst SCA in pts >1.5yrs was highly correlated with stage 4.
Conclusion: Risk adopted treatment achieved excellent results in LD pts any age and acceptable outcome for high risk stage 4 disease. While known prognostic markers were confirmed, an age dependent frequency and prognostic role for SCA in non-MNA pts was newly identified.

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OR85

Exon-Level gene expression analyses of primary neuroblastoma improves risk prediction and identifies MYCN status as major determinant of alternative transcript use

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Background: Assignment of patients to adequate treatment regimens according to their individual risk is a major goal in cancer therapy. On the route to clinical application of array-based classifiers to predict the risk profile of a patient with neuroblastoma (NB), significant progress has been made over the past years.
Method/approach: We here present data on a cohort of 138 primary NB using a novel approach including information for all human coding exons described to date (Affymetrix ExonST Array). Prediction of outcome and identification of alternative transcript use was achieved using machine learning algorithms and conventional statistics, respectively. Validation of alternative transcript use was performed using PCR- and siRNA-based approaches.
Results: Using a classifier trained on 100 NB tumor samples and then used to predict the outcome of the remaining 38 NB patients, we were able to achieve prediction accuracies >80% in cross-validation using support vector machine (SVM) learning algorithms, which is superior to the current clinical risk stratification. Interestingly, exon-based prediction proved to be superior to gene-based prediction. Of all clinical and biolocial parameters analysed, the status of the MYCN oncogene was most tightly linked to the number of alternatively used transcripts. Classification of these transcripts revealed an association of MYCN amplification with up-regulation of genes involved in cell cycle control and down-regulation of genes involved in DNA repair. These findings could be verified in independent primary NB samples as well as in NB cell lines. Using siRNA-based approaches, transcript-specific knock-down could be verified for CCNB1/Cyclin B1, for which a longer and shorter isoform were identified in varying ratios in NB cell lines with single copy or amplified MYCN.
Conclusion: Exon-level analysis turned out to be a powerful tool for prediction of outcome in NB. This technique also facilitates the identification of alternative transcript use. The knowledge gained in NB should be readily transferable to other tumor entities and offers a deeper understanding of gene regulation in embryonal tumors.

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OR86

High expression of KIF1Bβ-interacting protein MAP1A and its family member MAP1B significantly correlates with favourable prognosis of neuroblastoma

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Background: Previously we identified *KIF1B*β as a haploinsufficient tumour suppressor gene at the 1p36.2 locus in neuroblastoma (NB). To better understand the regulation of KIF1Bβ at the molecular level we performed a yeast two-hybrid screening assay using its unique apoptosis inducing ROD-region as a bait and identified the light chain 2 (LC2) of MAP1A (Microtubule Associated Protein 1A) as a new binding partner. MAP1 family members consist of 3 proteins, MAP1A, MAP1B and MAP1S and they are almost exclusively expressed in neuronal cells. They stabilize the neuronal cytoskeleton and play an important role in neuronal development.
Method/approach: In this study we investigated the mRNA-expression levels of *MAP1A* and *MAP1B* in 25 NB cell lines and 106 primary NBs by RT-PCR and real-time PCR, respectively.
Results: Our RT-PCR data showed that both genes are highly expressed in all examined NB cell lines. Furthermore, our quantitative real-time PCR analysis has illustrated that high levels of *MAP1A* expression are significantly associated with the favourable prognostic indicators, such as low tumour stages (p<0,001), high *TrkA*-expression (p<0,0005), *MYCN* single copy number (p<0,0001), Shimada histology of favourable NBs (p<0,04) and the tumour origin (p<0,004). High *MAP1B* expression correlated with *MYCN* single copy number (p<0,029), ploidy (p<0,0013) and Shimada histology of favourable NBs (p<0,028). Furthermore, high expression of both genes markedly prolonged the overall survival of NB patients (*MAP1A* p<0,018; *MAP1B* p<0,031). Additionally, in the multivariate analysis, *MAP1B* expression was identified as an independent prognostic parameter (p<0,048) for NB.
Conclusion: Our results demonstrate that high expression of *MAP1A* and *MAP1B* are significantly correlated with a better overall survival of NB patients. Moreover, *MAP1B* was identified as an independent prognostic parameter for NB. The preferential expression of both genes may contribute to differentiation and/or enhanced regression of favourable NBs. Further experiments will be needed to elucidate their functional roles in NB development.

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OR87

Determination of 17q gain in neuroblastoma patients by analysis of circulating DNA

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Purpose: Retrospective studies have demonstrated the prognostic impact of genomic profiles in neuroblastoma. Segmental chromosome alterations have been found useful for identifying tumors with a high risk of relapse. As the gain of chromosome arm 17q is the most frequent chromosome alteration reported in neuroblastoma primary tumors, we attempted to evaluate the presence of this 17q gain in the peripheral blood of neuroblastoma patients.
Patients and Methods: Using duplex quantitative real-time PCR we quantified simultaneously on the one hand, MPO (17q.23.1) and a reference gene, p53, and on the other hand, Survivin (17q25) and p53. Then MPO and Survivin copy numbers were evaluated as MPO/p53 and Survivin /p53 ratios in 143 serum or plasma samples in which 17q status had been determined by array-based comparative genomic hybridization (aCGH) or Multiplex ligation-dependent probe amplification (MLPA).
Results: In patients less than 18 months of age, serum-based determination of 17q gain in DNA sequences had good specificity (93%) and 55% sensitivity (p<0.001). In contrast, for patients over 18 months of age the approach exhibited low specificity (69%) and 51 % sensitivity (p= not significant). Similar results were observed in neuroblastoma tumors without MYCN amplification.
Conclusion: Our results show that 17q gain determination in circulating DNA is possible and suggest that this non invasive test could be useful for infants and toddlers with neuroblastoma for whom the "wait and see" strategy is often recommended. This test is complementary to previously developed techniques for detecting circulating MYCN DNA sequences.

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OR88

Phox2B but not TH mRNA detected by QRT-PCR in peripheral blood stem cell harvest predicts time to relapse in randomised children with high risk neuroblastoma; a SIOPEN molecular monitoring group study

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Aim: The aim of this study was to determine whether the presence of neuroblastoma cells detected by quantitative reverse transcriptase polymerase chain reaction (QRT-PCR) for Phox2B or tyrosine hydroxylase (TH) mRNA in peripheral blood stem cell (PBSC) harvest from children with high risk neuroblastoma entered on the HR-NBL1/SIOPEN trial predicts outcome.

Methods: PBSC (0.5ml) were collected into PAXgene™ Blood RNA tubes (PreAnalytix) after induction therapy and processed according to standard operating procedures (Viprey et al, 2007, EJC, 43, 341-350; Viprey et al, 2008, J. Pathol, 216, 245-252). Blinded quality control was utilised to ensure the sensitivity, specificity and reliability of data across participating reference centres and countries. QRT-PCR data are reported on PBSC samples from 133 children for TH mRNA and from 119 children for Phox2B mRNA. RNA extraction and QRT-PCR results are recorded in the SIOPEN-R-NET database and reviewed by the central reference laboratory in Leeds.

Results: The frequency of Phox2B and TH mRNA detection by QRT-PCR in PBSC is 13% (16/119) and 49% (65/133) respectively. Of these children 74 were randomised to high dose therapy; TH was measured in all 74 and Phox2B in 65/74. Eighty-eight % (14/16) of PBSC samples positive for Phox2B were also positive for TH mRNA. The level of Phox2B and TH mRNA was low in all positive PBSC samples with Ct values of 38.9 (34.9-39.9) and 37.7 (31.0-39.8) respectively. In children randomised for high dose therapy Phox2B predicted time to relapse; HR 4.39 (95% CI 1.32-14.61; p =0.02). There was no significant correlation between TH mRNA in PBSC and overall survival or time to relapse.

Conclusions: Phox2B mRNA detected by QRT-PCR in the PBSC harvest (n=65) from children with high risk neuroblastoma randomised for high dose therapy predicts time to relapse. This requires further investigation in a larger patient group.

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OR89

Clinical utility of minimal residual disease marker panel during sequential phases of a multi-modality treatment of high-risk neuroblastoma

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Background: At MSKCC, induction for high-risk neuroblastoma (NB) patients (pts) included dose-intensive chemotherapy, surgery and local radiation. Consolidation consisted of either (1) anti-GD2 monoclonal antibody 3F8 followed by I-131-3F8 radioimmunotherapy (RIT) [NCT00002634] and autologous marrow/stem cell rescue, or (2) 3F8 plus granulocyte-macrophage colony-stimulating factor (GM-CSF)± 13-cis-retinoic acid (CRA) [NCT00002560], or (3) myeloablative chemotherapy plus autologous marrow/stem cell transplant (SCT)± 3F8/GMCSF/CRA. Since minimal residual disease (MRD) in the bone marrow (BM) portends poor outcome, we utilized a four-marker panel to measure MRD during different phases of this multi-modality treatment in 149 high risk pts to assess when the presence of MRD would correlate with clinical relapse.

Method/approach: BM samples from 4 groups of pts: (1) at diagnosis and after induction (43 pts), (2) before and following RIT (42 pts), (3) before and after SCT (57 pts), (4) before and after 2 cycles of 3F8/GMCSF (63 pts), were tested for MRD by qRT-PCR using a marker panel consisting of CCND1, GD2 synthase, PHOX2B, and TH. MRD analyses were based on a logistic regression model using marker panel positivity as a predictor of relapse. MRD response was scored by the marker panel.

Results: Except for the BM at diagnosis, all other BM tested in this study were histologically negative in 2/2 biopsies and 4/4 aspirates. Marker panel positivity correlated significantly with progression-free survival (PFS) in only 3 time points: after induction (p=0.03) in group 1; after 4 cycles of 3F8 (without GM-CSF) and right before RIT (p=0.002) in group 2; and after 2 cycles of 3F8/GMCSF, measured at a median time of 2.8 months from first day of 3F8 treatment (p=0.03) in group 4. MRD response scored by the marker panel following 2 cycles of 3F8/GMCSF was also strongly correlated with PFS (p=0.004).

Conclusion: MRD marker panel has clinical utility in the management of high risk NB pts. It can measure NB in histologically-negative BM, and depending on the time of BM sampling, the presence of MRD correlates with eventual clinical relapse.

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Selected posters
SEL1–SEL48

SEL1

Omics analysis and evolution for identification of candidate genes in progression of neuroblastoma

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Background: Neuroblastoma (NBL) is biologically heterogeneous and demonstrates both favorable and unfavorable outcomes. Genome-wide genetic aberrations and expression using microarray have already been reported. In this study, proteome and microRNA (miRNA) data were combined to evaluate the mechanism of tumor progression.
Methods: From 200 NBL samples analyzed by Affymetrix SNP and expression arrays, 10 NBL cell lines and 40 tumor samples were selected, including 20 favorable cases which regressed or matured spontaneously and 20 unfavorable cases who died of tumor progression. The miRNA expression levels were examined by Agliment microarray, using total RNA and LC-MS analysis using cell extracts . This was performed by mass spectrometers (QSTAR Elite or LTQ Orbitrap XL) with ESI module. MS/MS data of the specific peaks was matched with the data in the MassBank.
Results: About 2,000 peaks were extracted from the LC-MS data. The comparison between the gene expression and LC-MS data showed that *MYC*-induced and cholinergic pathways are activated in unfavorable tumors, while apoptosis pathways, including neuro-differentiation and glycolipid metabolites were activated in the favorable ones. The data of SNP array showed that the genes including *DDX1, NAG, NME1* located in the amplified and genetic gained loci activated this pathway in unfavorable tumors. The data of miRNA showed that the activated pathways in favorable tumors were mainly down-regulated by mi-RNA located in the genetic aberrated loci in unfavorable tumors. Gene dosage is correlated to located gene and miRNA expression levels, consequently regulating the products of these pathways. Cell lines showed the specific activation of miRNA and genes, which might correlate with *in vitro* proliferation.
Conclusions: Omics analysis from gene to protein revealed the activated pathway in unfavorable and favorable NBLs. The aberrations of gene dosage might regulate the expression of genes and miRNA and consequently regulate the malignant grade of NBLs. Further pathway analysis provided important candidates of indicators for risk assessment and of therapeutic targets for unfavorable NBLs.

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SEL2

Impaired activation of the tumor suppressor p14^{ARF} impedes its oncosuppressive impact in neuroblastoma

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Background: The p14^{ARF}-MDM2-p53 pathway is essential for the activation of cell cycle arrest and apoptosis upon oncogenic insults. The frequency of mutational or deletional *p53* inactivation is low in neuroblastoma (NB), whereas a functional loss of the p53 path during malignant NB progression is indicated. Low expression levels of the p53 stabilizer p14^{ARF} led us to analyze its role and regulation in NB.
Method/approach: Gene expression was measured via real-time quantitative RT-PCR. The genomic status of *CDKN2A (p16^{INK4a}/p14^{ARF})* was determined via array-based comparative genomic hybridisation (A-CGH). Methylation specific PCR served to assess *p14^{ARF}* promoter methylation. Chromatin immunoprecipitation on chip (ChIP chip) detected the activity of the *p14^{ARF}* promoter as indicated by activating/inactivating histone modifications. Functional characterizations were carried out by conditional overexpression in NB cell lines.
Results: Expression of *p14^{ARF}* mRNA was low but differential in NB cell lines and in a set of 81 NB tumors. One homozygous *CDKN2A* deletion was found in 194 primary NBs. Heterozygous losses either by whole (28 cases) or by partial chromosome loss (14 cases) occurred in 22% of 194 NBs. No evidence was found for *p14^{ARF}* promoter methylation in 60 NBs. ChIP chip analyses revealed weak *p14^{ARF}* promoter activities in NB cell lines. Promoter activity states differed among cell lines and were positively correlated with *p14^{ARF}* expression. Conditional p14^{ARF} overexpression in SH-EP and IMR5-75 cells significantly reduced cell viability. Expression and cell cycle analyses revealed an induction of the p53 machinery and a doubling of the apoptotic cell fraction after 48 h of p14^{ARF} overexpression.
Conclusion: Low expression levels indicate impaired activation of *p14^{ARF}* in NB. A-CGH data argue in favour of a reduced gene dosage due to genomic loss in a subset of NBs. ChIP chip analyses hint at epigenetic silencing of the gene in NB cells. Functional analyses revealed a high oncosuppressive potential of p14^{ARF} in NB cells. Currently the elucidation of *p14^{ARF}* regulation is refined to determine intervention points for targeted activation of the gene in NB.

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SEL3

The FRAGILOME Project to discover new biomarkers for neuroblastoma

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Background: Structural chromosome aberrations in cancer cells are frequently caused by DNA damage at particular regions of the genome, known as common fragile sites (cFS). cFS are chromosomal loci that are especially prone to forming site-specific gaps or breaks in response to DNA-replication stress. The human genome contains approximately 120 cFS regions that have been cytogenetically mapped to specific sites existing on basically all chromosomes. Instability at cFS believed to be a critical initiating event in the generation of genomic DNA damage during tumor development. We have initiated the project known by the acronym FRAGILOME, which aims at identification of the genetic information of the repertoire of cFS, definition of persisting cFS gene damage and determination in which way the damaged genetic information might contribute to tumorigenesis. As an essential part of this project, Neuro-FRAGILOME, focuses on the contribution of cFS genes to neuroblastoma development.
Method/approach: For detection of genetic aberrations leading DNA copy-number alterations, such as deletions, gains, and amplifications, we designed FRA-CGH chip targeting sequences of 21 cFS genes with resolution of ~90 bp. Structural chromosomal rearrangements within cFS genes resulting from incorrect joining of DNA segments, such as translocations, insertions or inversions, are determined by FISH.
Results: We identified new cFS genes, which exhibit genomic rearrangements in neuroblastoma cell lines and primary tumors. Five cFS genes, *NBAS (FRA2C), CENTL (FRA9G), LINGO2 (FRA9C), GPHN (FRA14B)* and *WWOX (FRA16D)* were involved in genomic rearrangements in different samples. Our data show that the chromosomal rearrangements that drive *MYCN* amplification occur non-randomly within the sequence of the *FRA2C* cFS.
Conclusion: Analysis of neuroblastoma tumor samples has pointed to a number of cFS genes that have undergone genomic damage. In perspective, it will be tested if such damaged genetic information could be used as a new biomarker for deciphering DNA rearrangements associated with the different neuroblastoma stages or even as a therapeutic target.

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SEL4

Expression profiling in neuroblastoma identifies a fourth subgroup with high expression of ERBB3

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There are currently three postulated genomic subtypes of the childhood tumour neuroblastoma (NB); Type 1, Type 2A, and Type 2B. The most aggressive forms of NB are characterized by amplification of the oncogene MYCN (MNA), low expression of the favourable marker NTRK1, and mutation or high expression of the familial predisposition gene Anaplastic Lymphoma Kinase (ALK). The present study aimed to explore subgroups defined by gene expression profiling of NB tumours from three published microarray studies (i.e. De Preter et al., 2006, McArdle et al., 2004, Wilzén et al., 2008). Principal Components Analysis (PCA) was performed on two parallel data sets of 17 and 30 NB tumours respectively. Four distinct clusters were identified by PCA in both data sets, and the expression signature of six NB associated genes, i.e. ALK, BIRC5, CCND1, MYCN, NTRK1, and PHOX2B, significantly distinguished these clusters ($p<0.05$, one-way ANOVA test). Three of the PCA subgroups were found to correspond well to the postulated subtypes Type 1, 2A, and 2B. Remarkably, a fourth novel group was detected in both independent data sets based on its unique expression profile. This group comprised mainly 11q deleted tumours with no MNA, and with low expression of MYCN and ALK, and was significantly associated with higher tumour stage, poor outcome and poor survival compared to the Type 1-corresponding favourable group in both data sets (INSS stage 4 and/or dead of disease, $p<0.05$, Fischer's exact test). By a set of 74 genes which were differentially expressed between groups, the existence of four subtypes could be verified by an unsupervised hierarchical clustering of an independent data set (101 NB samples, Wang et al., 2006). By Significance of Microarray (SAM), the cancer-associated tyrosine kinase gene ERBB3 was among the most highly expressed genes within the fourth subgroup. In conclusion, this meta-analysis study evidently shows that there are at least four molecular subgroups of neuroblastoma, which can be distinguished by gene expression profiling of only six genes. Efforts are currently made to further investigate the fourth novel subgroup's specific characteristics.

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SEL5

A SP1/MIZ1/MYCN ternary complex induces repression of TRKA and p75NTR neurotrophin receptors and affects neuroblastoma malignancy through inhibition of the cell apoptotic response to NGF

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Background: Good expression levels of the TRKA and p75NTR neurotrophin receptor genes are considered important markers of favorable prognosis in neuroblastoma. Notably, the expression of TRKA and p75NTR genes is often reduced or silenced in MYCN amplified tumors, suggesting a causal link between MYCN activity and repression of TRKA/p75NTR transcription. Here, we investigate the molecular mechanism by which MYCN induces repression of these two genes.
Method/approach: MYCN regulation of the TRKA and p75NTR gene promoters was studied through expression profiling and chromatin immunoprecipitation (ChIP) assays. Activity of MYCN on TRKA and p75NTR gene transcription was examined by transient trasfections of TRKA and p75NTR luciferase vectors. Interaction of MYCN with promoter-bound proteins was determined through protein co-immunoprecipitation, GST-pulldown assays and siRNA-mediated depletion of MYCN interactors. Apoptosis was assayed through Facs and biochemical analyses.
Results: Our results show that: 1) MYCN intracellular levels are critical for repression of TRKA and p75NTR; 2) MYCN binds the proximal/core promoter regions of the two genes by forming a ternary complex with SP1 and MIZ1 transcription factors; 3) This complex recruits HDAC1 to the two core promoters determining local histone modifications typical of repressed chromatin, which can be reversed with epigenetic drugs. 4) Forced re-expression of endogenous TRKA and p75NTR levels sensitizes neuroblastoma cells to NGF mediated apoptosis.
Conclusion: Our findings provide a mechanistic rationale to the inverse correlation often observed between MYCN expression and that of TRKA and p75NTR receptors and highlight the anti-oncogenic role of the NGF/TRKA/p75NTR pathway in neuroblastoma.

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SEL6

NCYM, a protein product of an antisense *MYCN* gene co-amplified with *MYCN*, targets MYCN for functional modulation and affects the prognosis of neuroblastoma

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Background: *NCYM* was first reported as a natural antisense transcript of *MYCN* (Mol. Cell Biol., 1990). However, the functional significance of *NCYM* has long been elusive. Recently, we have found that a TATA-less transcription factor TLP directly transactivates *TAp63*, a member of p53 family (J. Biol. Chem., 2009) and the presence of autoregulation of *MYCN* (Biochem. Biophys. Res. Commun., 2009). Here we show that TAp63 targets both *NCYM* and *MYCN* for transcriptional regulation and that the NCYM protein modulates MYCN to affect biology and clinical behavior of neuroblastoma (NB).
Methods: DNA and mRNA were obtained from 106 primary NBs. The mRNA expression was examined by a quantitative RT-PCR. Transcriptional activation was investigated by luciferase reporter assays and chromatin immunoprecipitation analysis.
Results: Based on the genomic database, *NCYM* could be translated to the protein only in human and chimpanzee but not in lower animals including mice. The expression of *NCYM* and *MYCN* mRNAs was highly significantly correlated ($p<0.00001$) and associated with poor outcomes ($p<0.001$) in 106 primary NBs. To confirm the endogenous expression of NCYM protein, we generated the specific antibody against NCYM and found the expression of 15 kDa NCYM protein in human NB cell lines, especially those with *MYCN* amplification. The retinoic acid treatment induced down-regulation of both *MYCN* and *NCYM* in CHP134 NB cells, suggesting that expression of both genes are closely regulated with shared upstream regulators. We then found that overexpression of TAp63 suppressed transcription of both *MYCN* and *NCYM*. Interestingly, among the p53 family genes, expression of only *TAp63* mRNA was negatively associated with that of *MYCN* in 106 NBs ($p=0.035$). Our further analyses revealed that TAp63 targets both *NCYM* and *MYCN* for transcriptional suppression, and that NCYM may directly transactivate *MYCN* and stabilize MYCN in cells. The generation of transgenic *NCYM* mice is on-going.
Conclusion: The TLP-TAp63-MYCN/NCYM pathway may regulate the genesis and stemness of human NB, that affects the clinical behavior.

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SEL7

Identification of a new fusion gene on 11q23 in neuroblastoma tumor samples

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Background: Chromosome 11q shows frequent LOH (Loss Of Hetrozygosity) and the SRO (Shortest Region of Overlap) has been located at the 11q23 region. Many tumor samples used for deletion mapping of this region showed small interstitial deletions. These deletions are thought to cause inactivation or haplo-insufficiency of tumor suppressor genes at this region. Alternatively these deletions could lead to gene fusion products that are tumor driving.
Methods and result: We used DNA copy number analysis techniques (CGH and SNP array) of a large series of neuroblastoma and integrated these data with Affymetrix mRNA expression data using the R2 bio-informatic tool. We identified 4 neuroblastoma tumor samples with small interstitial deletions of the same region at 11q23. In each case, the region upstream of the forkhead box transcription factor FOXR1 was deleted. FOXR1 was fused to genes on the opposite end of the deletion, resulting in fusion transcripts of MLL-FOXR1 and PAFHA1B2-FOXR1. Affymetrix microarray analysis showed that FOXR1 mRNA was highly expressed in these neuroblastoma tumors and sporadically in some other malignancies including an osteosarcoma cell line. To study the functional role of the FOXR1 fusion gene, we generated expression constructs of the MLL-FOXR1 fusion transcript and the wild type FOXR1 messenger. These sequences were transferred into non malignant mouse neuroblasts and were able to maintain proliferation of these cells, whereas control neuroblasts ceased proliferation. shRNA mediated silencing of the FOXR1 gene in the osteosarcoma cell line resulted in a strong growth inhibition and increase in apoptosis.
Conclusion: We conclude that FOXR1 is recurrently activated in neuroblastoma by intrachromosomal deletion/fusion events, resulting in over expression of fusion transcripts that increase the proliferative potential of the cells. This is the first report of intrachromosomal fusion genes in neuroblastoma and the first evidence of an oncogenic activation of a forkhead-box transcription factor in neuroblastoma.

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SEL8

Characterization of amplicon junction sequences in genomic regions surrounding the MYCN gene in neuroblastoma tumors; implications for clinical follow-up of high-risk patients

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Background: Amplification of the MYCN (2p24) gene region is a common feature among one group of unfavorable neuroblastoma tumors. This aberration has been known for more than 20 years, but the prognosis for these patients remains poor. The aims of this study were to characterize the amplified DNA segments (amplicons) in detail and to obtain tumor-specific PCR fragments to be used for minimal residual disease (MRD) detection in these patients.
Method/approach: High-density SNP arrays were used to map the endpoints of the MYCN amplicons in a subset of neuroblastoma tumors. As we sought to clone novel junctions between amplicons, outward-facing primers were designed, giving rise to a PCR product only in the case of a rearrangement. DNA sequencing then revealed information about the junction region and confirmed the validity of the primers. Successful primer combinations were also tested for MRD detection in a semi-quantitative PCR assay, comparing DNA from bone marrow or blood samples with a serial dilution of tumor/control DNA to estimate the amount of tumor DNA in the sample.
Results: A tumor-specific amplicon junction fragment was detected in each of the four cases hitherto analyzed, albeit different in each case. The junctions consisted of small microhomology regions of only a few bases and mapped to the reference genome as two separate hits on either side of MYCN, confirming a head-to-tail orientation of the amplicons. Our approach to MRD detection was also found to be sensitive enough to detect the junction fragment in a 1/10^6 dilution of Tumor/Control DNA. We were also able to estimate the MRD tumor-DNA content in the patient samples tested that were obtained at diagnosis and during follow-up.
Conclusion: We have shown that the MYCN amplicon junctions can be used as a tumor-specific target for PCR amplification, and that this PCR assay is sensitive enough to detect MRD in children with neuroblastoma. Thus, this method is suitable for patient-specific monitoring of treatment response and early detection of relapse.

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SEL9

Causal inference, a novel approach to disentangle the effects of off-protocol therapy from the primary effects of interest in COG protocol P9462: Topotecan vs. Topotecan+cyclophosphamide in relapsed neuroblastoma

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Background: Responders to Phase II therapy for relapsed disease often undergo subsequent off-protocol therapies. Novel statistical methods are needed to elucidate the effect of protocol therapy on long-term outcome. Using such methods, outcome for single agent topotecan (TOPO) and combination topotecan and cyclophosphamide (TOPO/CTX) was compared after completion of a Phase II randomized trial in relapsed/refractory neuroblastoma.
Patients and Methods: Children with refractory/recurrent neuroblastoma were randomized to daily 5-day TOPO (2 mg/m^2) or combination TOPO (0.75 mg/m^2) and CTX (250 mg/m^2). A randomized two-stage group sequential design enrolled 119 eligible patients. Patients could go on to other therapies, and some underwent ASCT. If ASCT results in increased survival, and if patients exposed to TOPO/CTX are more likely to undergo ASCT, then our ability to test for the hypothesized benefit of TOPO/CTX was confounded by ASCT. Long-term outcome of protocol therapy was assessed using novel methods -- causal inference -- which allowed adjustment for the confounding effect of off-study therapies.
Results: Seven more responses were observed for TOPO/CTX (CR+PR: 18/57 (32%)) than TOPO (CR+PR: 11/59 (19%)), (p=0.081); toxicity was similar. At 3-years, progression-free survival (PFS) and overall survival (OS) were 4%±2% and 15%±4%, respectively. PFS was significantly better for TOPO/CTX (p=0.029); there was no difference in OS. Adjusting for randomized-treatment-effect and subsequent autologous stem cell transplant, there was no difference between TOPO and TOPO/CTX in terms of the proportion alive at two years.
Conclusions: TOPO/CTX was superior to TOPO in terms of PFS, but there was no difference in OS. After adjustment for subsequent therapies, no difference was detected in the proportion alive at 2 years. Causal inference methods for assessing long-term outcomes of Phase II therapies after subsequent treatment can elucidate effects of initial therapies.

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SEL10

Persistence of disease in long-term survivors of high-risk neuroblastoma. Analysis of ENSG5 cooperative trial

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Background: There is no evidence available regarding the long-term outcome of children that do not achieve remission, especially for a small group of patients whose disease persists after several lines of treatment and have persistence of neuroblastoma during follow-up. We reviewed remission status and presence of active disease after End of Treatment (EOT) in long-term survivors of ENSG5 trial that treated high-risk neuroblastoma in Europe from 1990 to 1999.
Methods: Patients were randomised to receive the same induction drug doses but in one arm the dose intensity was 1.8 times greater (OPEC/OJEC vs. COJEC), surgical removal of primary tumour and high-dose melphalan with haemopoetic stem cell rescue. We identified children that were alive at five years from diagnosis who were not in remission after induction therapy and surgery. Patients were grouped in: 1) Persistent metastatic disease, 2) Persistent primary disease. Data were verified by sending questionnaires to registering centres (82.9% responses).
Results: Out of 262 children randomized in ENSG5, 62 were alive at five years and 19 of them showed persistence of disease after induction therapy and surgery. 177 children were not randomised but were treated according ENSG5, 36 were long-term survivors, seven with persistence of disease were included in the analysis. Seventeen children (median age 2.49 years) had persistent metastatic disease after induction chemotherapy. Two patients had persistent bone marrow disease (up to 9 years after diagnosis) and six had persistent MIBG skeletal positivity (up to 16 years after diagnosis). Seven children had persistent primary tumour during follow-up with residual masses (up to 16 years after diagnosis). There was no obvious difference between the characteristics of these groups and the whole ENSG5 cohort.
Discussion: Some patients can be long-term survivors despite of persistent disease after end of treatment (large primary tumours, bone or bone marrow metastases). Our findings show that a clinical course similar to infants with stage 4 disease is possible for this group of older patients. This may be explained in the future by more knowledge of tumour biology.

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SEL11

High dose MIBG and haploidentical stem cell transplantation with cell therapy in therapy resistant neuroblastoma

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Background: The prognosis for relapsing/refractory neuroblastoma (RRNB) remains dismal and no effective salvage treatment has been identified so far. We evaluated the feasibility and efficacy of using high-dose 131-I-MIBG (HDMIBG) followed by reduced-intensity conditioning (RIC) and transplantation of T cell-depleted haploidentical peripheral blood stem cells (haplo-SCT) to treat RRNB.
Methods: Ten children with RRNB were enrolled: 6 with relapse (4 after autologous SCT) and 4 with primary resistant disease. The preparative regimen included HD- MIBG on day –20, followed by fludarabine (Flu), thiotepa, and melphalan (Mel) from day –8 to –1. Granulocyte-colony stimulating factor (G-CSF)-mobilized, T cell-depleted haploidentical parental stem cells were infused on day 0 together with donor (n=7) or third party (n=3) mesenchymal stem cells. A single dose of rituximab was given on day +1. After cessation of short immunosuppression (MMF, OKT3), 7 children received donor lymphocyte infusion (DLI).
Results: Treatment was well tolerated. Two children developed primary acute graft-versus-host disease (aGVHD). Five children developed aGVHD after DLI and were successfully treated. Analysis of immunologic recovery showed fast reappearance of potentially immunocompetent natural killer (NK) and T cells, which might have acted as effector cells responsible for the graft-versus-tumor effect. Eight children are alive. Four patients are doing well with no evidence of disease 53, 52, 8 and 5 months haplo-SCT. Four other children are alive 52, 17, 5 and 4 months after haplo-SCT having stable/slowly progressive disease. There were no transplant related mortalities. Two children died because of progression 5 and 12 months after haplo-SCT.
Conclusion: HD MIBG followed by RIC and haplo-SCT for RRNB is feasible and promising. Eight of 10 children on that regimen are alive, four of them in complete remission. Large number of patients and a longer observation time are needed to evaluate the role of this approach including immune mediated graft versus neuroblastoma effect in the treatment of high-risk therapy resistant neuroblastoma.

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SEL12

Combined radioimmunotherapy and anti-angiogenic therapy for resistant neuroblastoma

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Background: Using preclinical mouse models, we demonstrated synergy between 131I-3F8-mediated radioimmunotherapy and bevacizumab (BV)-mediated anti-angiogenesis (ANR 2006 Abs 193). We translated our findings into a phase I study for patients with resistant NB.
Method/approach: Heavily pretreated (median of 4 prior therapeutic regimens; 10 with progressive disease [PD]) patients (pts) with recurrent or refractory stage 4 NB were treated on an IRB-approved study (NCT00450827) investigating the toxicity and effectiveness of the combination of 131I-3F8 and BV. Each cycle consisted of a single dose of 131I-3F8 on day 0 and a fixed dose of BV at 15mg/kg on days 1 and 15. 131I-3F8 was escalated at 4-8mCi/kg in cohorts of 6 pts. Patients could receive a maximum of 4 cycles in the absence of >grade 2 non-hematopoietic toxicity, human antimouse antibody response, severe myelosuppression and/or PD.
Results: 6 pts each received 4,5,6 and 8mCi/kg 131I-3F8. 39 cycles were administered. 2, 3, 3 and 16 pts completed 4, 3, 2 and 1 cycles respectively. All were evaluable for toxicity and response. 131I-3F8 targeting to NB was demonstrated in all pts by scintigraphy. Maximal tolerated dose (MTD) for 131I-3F8 was not reached. All pts developed grade 4 myelosuppression. 9 pts required autologous stem cell rescue (ASCR), with engraftment at 4-15 (median 11) days. 4 expected but treatable toxicities led to withdrawal from study: anaphylaxis (1) , grade 3 BV-related intestinal perforation (1) and sepsis requiring ASCR (2) . Responses by International NB Response Criteria were: 1 complete response, 1 mixed response, 16 no response and 6 PD. Objective responses were observed in 13/24 patients. 3-year event-free survival was 39±12%; median 7 months.
Conclusion: Multiple cycles of 131I-3F8 and BV resulted in no unexpected side-effects and showed anti-NB activity. MTD for 131I-3F8 was not reached. BV did not impair 131I-3F8 targeting to NB or bone marrow recovery after ASCR.

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SEL13

MYCN amplified neuroblastoma differ in clinical features at initial presentation

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Background: Molecular markers like MYCN amplification and chromosome 1p aberration are known to influence prognosis in neuroblastoma patients. We asked whether patients with these markers might differ regarding features at initial presentation.
Methods: We analysed features at initial presentation in all neuroblastoma patients registered between 2000 and 2009. Patients were grouped by molecular marker: group 1: neither MYCN amplification nor 1p aberration; group 2: MYCN not amplified, but 1p aberration: group 3: MYCN amplified and 1p aberration.
Results: Of 1095 patients with complete molecular data, the tumor showed neither amplification nor 1p aberration in 775 patients (71%), 1p aberration without MYCN amplification in 135 patients (12%), and MYCN amplification and 1p aberration in 185 patients (17%). Patients without aberrations were younger at diagnosis (median 11 months) and showed less frequently stage 4 (24%) than patients with 1p aberration (median: 33 months, stage 4: 61%) and patients with MYCN amplification (median: 24 months, p<0.001; stage 4: 71%, p<0.001). Patients with MYCN amplification presented more frequently with elevated LDH (98%) and elevated NSE (100%), while urinary vanylmandelic acid was elevated only in 45% (no aberration: 79%, 1p-aberration: 87%, p<0.001) and mIBG-uptake was seen only in 84% (no aberration: 91%, 1p-aberration: 97%, p<0.001). Undifferentiated histology according to INPC was found in 28% of patients with MYCN-amplification (no aberration: 4%, 1p-aberration: 7%, p<0.001). In stage 4 tumors, the presence of bone marrow or bone metastases at diagnosis did not differ between the groups. Liver metastases were found more frequently in patients with 1p aberrations and in patients with MYCN amplification (24% each, p=0.007). Patients with MYCN amplification presented more frequently distant lymph node metastases (32%; p=0.001), pleural or lung metastases (14%, p=0.001) and orbita infiltration (24%, p=0.03).
Conclusion: The different genotype of MYCN amplified neuroblastomas translates into a different phenotype at clinical presentation with respect to age, metastatic pattern, histology and metabolic features.

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SEL14

Diffusion-weighted whole-body imaging with background body suppression (DWIBS) in pediatric oncology patients - a feasibility assessment

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Background: For oncology patients repeated imaging scans are necessary for diagnosis, staging and treatment response monitoring. This may lead to considerable ionizing radiation doses to the patient, depending on the imaging method. Minimizing or avoiding ionizing radiation is especially important regarding pediatric patients. Diffusion-weighted whole-body imaging with background body suppression (DWIBS) is a free-breathing diffusion weighted (DWI) whole body sequence with multiple acquisitions, resulting in images resembling Positron Emission Tomography (PET) images. The potential value of this method in oncology imaging as a substitute for PET has been suggested in the literature. We propose that the method should be evaluated in pediatric oncology, to reduce the radiation dose caused by medical imaging.
Materials and Methods: To gain experience and to develop the method, we added a DWIBS sequence to the standard MRI examinations of pediatric oncology patients. The MRI scans were performed on a 1.5 T Philips Achieva MRI scanner, with diffusion encoding in three directions, and a b-factor of 800 s/mm2. The body coil was used, and for greater coverage the data was acquired in blocks, with the table position shifted, and later combined using MobiTrak (Philips, Best, NL). The average scan time for the DWIBS sequence was between 5 and 6 minutes per patient. Several of the patients also underwent a PET exam, with images acquired after an injection of 4 MBq/kg [18F]-2-fluoro-2-deoxy-D-glucose (FDG).
Results: DWIBS images of pediatric oncology patients with neuroblastoma as well as several other diagnoses were aquired. The findings on the DWIBS images corresponded well with the results of the conventional MR images, and with the PET exams. A prospective study to further evaluate the potential of DWIBS in pediatric oncology is planned.
Conclusion: DWIBS is a promising method with a potential to replace other imaging modalities in pediatric oncology imaging. Using DWIBS involves no ionizing radiation which makes it especially suitable for imaging young children with neuroblastoma.

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SEL15

Analysis of toxicity and efficacy of high dose chemotherapy with Busulfan and Melphalan followed by stem cell transplantation in high risk neuroblastoma patients: a retrospective study of a large cohort in a single institution

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Background: The impact of high dose chemotherapy (HDC) and hematopoietic stem cell transplantation (HSCT) in improving the survival of high risk neuroblastoma patients has been well established. We previously presented the positive impact of the Busulfan-Melphalan (BuMel) regimen in these patients. In this study, we analyze the toxicity and survival of a large cohort of patients treated with BuMel at the Pediatric Department, Institut Gustave Roussy.
Method/approach: We evaluated comprehensive data prospectively collected between 1980 and 2008 concerning all patients aged more than one year treated with HDC and SCT for high risk neuroblastoma. Patients enrolled on the HR-NBL1/ESIOP protocol were excluded.
Results: From October 1980 to December 2008, 209 patients aged more than one year were treated with BuMel and HSCT for high risk neuroblastoma. The median age at diagnosis was 40 months (range 12-218), the sex ratio was 1.4 and 88% of patients had an abdominal primary tumor. Bone marrow involvement was detected in 80% of cases and N-MYC amplification was present in 30% of tumors. HDC was followed by autologous HSCT. Grafts consisted of bone marrow, peripheral stem cells and both in 50%, 46% and 4% of the patients respectively. Mean duration of hospitalization and neutropenia was 48 and 18 days respectively (range 15-143 and 3-66 days). Grade 3/4 mucositis occurred in 75% of patients and veno-occlusive disease complicated 40% of grafts. Overall, treatment related toxicity significantly decreased with time. The 5-year EFS and OS post-diagnosis was 50 % and 44 % respectively, with a median follow-up of 41 months (range 5-231).
Conclusion: Analysis of this large series is encouraging with an improved EFS at 5 years than the cohort published by our team in 1999 (O. Hartmann *et al.*). BuMel is currently being compared to the CEM combination in the ongoing HR-NBL1 European protocol. We present complete toxicity analysis of Busulfan with particular attention to timing, schedule and administration route. The impact of supportive care and the benefit of retinoic acid maintenance therapy are also discussed.

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SEL16

Natural history of infantile neuroblastoma under "wait and see" observation — current status of patients after long term follow up for 5 - 15 years

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Background: A nationwide screening for 6 month-old infants was performed in Japan between 1985 and 2003. What we learned from experiences of mass screening (MS) for neuroblastoma was that infantile neuroblastomas were mostly favorable and sometimes regress spontaneously. Actually "wait and see" observation programs had been performed in several hospitals in Japan and spontaneous regression was often observed. Now those patients became 6 to 15 years old. The aim of this study was to evaluate the current status of those patients under long term observation, in order to know natural history of infantile neuroblastomas.
Method/approach: Of 88 infantile neuroblastoma patients diagnosed between 1991 and 2004, 29 were entered in the observation program. Of 29 patients, 11 patients underwent surgery because of tumor growth, elevation of tumor markers or upstage. Remaining 18 patients are still under observation. We retrospectively evaluated clinical feature at the disease onset, changes in tumor markers and size, and current status of their tumors.
Results: Of 18 patients, 17 were detected by MS and only one patient was diagnosed incidentally by chest X-ray. The mean age at diagnosis was 8.2 months (6.9 -10.8). Mean follow up time from the diagnosis to the latest hospital visit was 9.4 years (5.2 -14.9). Tumor markers had already been normalized in all patients. Mean periods required for normalization of all tumor markers was 3.8 months (0 -11.0). No tumor re-growth was observed after normalization of tumor markers. All patients currently have at least rudimentary tumors detected by ultrasound, chest X-ray, CT or MRI. The mean diameter of current tumors was 23mm (1 - 65). All patients are alive without any symptom.
Conclusion: At least 60% of the patients who underwent observation did not require any treatment and were safely observed for 5 to 15 years. Although there is possibly a minimum risk for tumor re-growth, rudimentary tumors were identified in all cases and those tumors are still a source of concern for the patients, parents and doctors.

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SEL17

Polyamine inhibition blocks initiation and progression of neuroblastoma

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Background: Polyamines (PAs) are upregulated in cancer, essential for cell survival, and controlled by Myc activity. We've shown MYCN co-ordinately enhances PA synthesis in neuroblastoma (NB), and inhibiting this pathway with DFMO delays NB onset in tumor-prone mice and synergises with chemotherapy to improve survival (Cancer Res., 2008). TH-MYCN NBs arising after DFMO had reduced putrescine but maintained spermidine/spermine suggesting compensatory Amd1 upregulation or PA import. We now show targeting multiple steps in PA homeostasis potentiates depletion and improves efficacy.
Methods: To evaluate PA homeostasis we studied radiolabeled-PA import, and rescue of PA pathway stress with supplemental PAs in vitro. To evaluate the TH-MYCN model we compared mRNA expression of PA regulatory enzymes via histologic audit and compared with human NBs. Finally, TH-MYCN NBs and xenografts in Balb/c nu/nu mice were treated variably with DFMO (Odc1 inhibitor), SAM486 (Amd1 inhibitor), celecoxib (augments PA export), and cyclophosphamide.
Results: NBs uptake PAs but do not augment transport during PA depletion. In vitro effects of combined PA depletion are on target as PA add-back rescues the phenotype. TH-MYCN NBs show alterations in PA regulators similar to human NBs, suggesting this is a conserved pathway. The TH-MYCN+/+ model is 100% penetrant for lethal NB. Pre-emptive SAM486 reduced NB penetrance ($p<0.001$). Remarkably, Dual DFMO-SAM486 pre-emptive therapy blocked NB initiation in >40% of +/+ mice ($p<0.001$), demonstrating a requirement for PAs for MYCN-mediated NB initiation. Next, TH-MYCN mice with palpable NBs were treated with DFMO, +/-celecoxib, +/-SAM486, +/-cyclophosphamide (CPM). The DFMO/celecoxib/CPM arm had superior TFS compared with any agent alone ($p<0.0001$). Effects were not unique to the murine model as BE2C NB xenografts treated with this triple drug combination had better survival than any other combination ($p<0.01$).
Conclusion: Our data support PA depletion as a valuable therapeutic approach for NB, as MYC deregulation provides a unique vulnerability. A Phase 1/2 trial is planned to assess the safety and efficacy of DFMO combined with celecoxib and CPM.

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SEL18

Blocking Galectin-1 function reduces growth of aggressive neuroblastoma cells *in vitro* and *in vivo*

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Background: Galectin-1 (Gal-1) has emerged as an interesting cancer target, as it is involved in modulating cell proliferation, death and migration, all of which are linked to cancer initiation and progression. We identified up-regulation of Gal-1 mRNA in patients with aggressive, relapsing neuroblastoma (NB). Gal-1 protein was up-regulated in human SY5Y NB cells upon activation of ectopically expressed TrkB neurotrophin receptor (SY5Y-TrkB), a known marker of aggressive biology and poor prognosis in NB. In contrast, NB cells expressing the biologically favorable TrkA receptor did not express Gal-1.
Method/approach: Gal-1 mRNA and protein expression was analyzed in primary NB. Antibody- or siRNA-based approaches were used to assess the role of impaired Gal-1 function in vitro. Effects of exogenous Gal-1 were studied using recombinant Gal-1. In vivo, Gal-1 function was studied in an NB mouse model using a small molecule inhibitor of the Gal-1/H-Ras interaction, farnesyl thiosalicylate, or siRNA directed against Gal-1.
Results: In a cohort of 102 primary NB, Gal-1 mRNA levels correlated positively with TrkB and negatively with TrkA expression. Immunohistochemistry of 92 primary NB revealed high Gal-1 protein expression in stromal septae as well as in neuroblasts. Activation of TrkB by its ligand, BDNF, enhanced invasiveness and migration of NB cells in vitro, both of which were impaired in response to Gal-1 siRNA or a Gal-1 neutralizing antibody. Subsequent addition of recombinant Gal-1 partially restored migration and invasive capacity of SY5Y-TrkB cells. Inhibition with the Trk-inhibitor, K252a, revealed a dependence of Gal-1 up-regulation on activation of the TrkB receptor in SY5Y-TrkB cells. Treatment of established tumors in an NB mouse model with a Gal-1 blocking siRNA resulted in reduced tumor size. Efficacy was comparable to treatment with temozolomide as a single agent, while no additive effect was seen using a combination of both therapies.
Conclusion: Gal-1 is highly expressed in NB and correlates with TrkB in vitro and in vivo. Our data suggest that targeting Gal-1 might be a promising strategy for the treatment of aggressive NB.

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SEL19

Bortezomib delays neuroblastoma tumor growth while impairing bone growth, testis development and fertility in a male xenograft mouse model

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Background: The proteasome inhibitor, bortezomib (PS-341/Velcade™), is approved for treatment of adult multiple myeloma, and in current pediatric phase-II clinical trials. The experience of bortezomib effects on neuroblastoma tumor growth is limited and secondary long term effects on normal tissues in young, growing individuals are so far unknown.
Aim: To study if bortezomib treatment can abrogate tumor growth in a mouse model of human neuroblastoma, investigate the underlying mechanisms, and possible secondary side effects on normal tissues.
Methods: In vitro: MTT screening of bortezomib effects at various concentrations in six human neuroblastoma cell lines. In vivo: Five weeks old, male NMRI nu/nu mice were used for establishment of neuroblastoma (SK-N-BE2) xenografts. When tumors reached 0.1 mL, mice were randomised to treatment with one cycle of a clinically relevant dose of bortezomib (1 mg/kg; 2 injections/wk; 2-wks treatment) or vehicle. Tumor volumes were followed longitudinally by daily calliper measurements. Bone lengths were measured by X-ray, at start of treatment and at time of autopsy, and fertility was evaluated as quantitated fecundity by counting the number of littermates after mating.
Results: Bortezomib in vitro caused a significant time- and dose-dependent inhibition of cell viability. Bortezomib impaired the ubiquitin/proteasome system and importantly, delayed tumor size doubling time to 22.3 days (interquartile range 11.5-38.6) vs 5.8 days in vehicle-treated animals, (interquartile range 2.5-9.4). Bortezomib-treated tumors showed a trend towards increased apoptosis, decreased proliferation and angiogenesis compared to vehicle. Consequently, bortezomib affected growth plate cartilage leading to significantly impaired bone growth, testis weight, and the number of littermates was reduced.
Conclusions: Bortezomib delayed tumor growth of human neuroblastoma in vitro and in vivo. However, bortezomib also causes permanent long term side effects on linear bone growth, testis development and fecundity. Our findings have important implications for the use of proteasome inhibitors in the treatment of neuroblastoma and other childhood cancers.

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SEL20

Evaluation of the effect of acetyl l-carnitine on experimental cisplatin ototoxicity and neurotoxicity

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Background: Cisplatin is effective and widely used chemotherapeutic agent for the treatment of pediatric solid tumors including neuroblastoma. Ototoxicity is one of the serious dose-limiting side effect of cisplatin.
Aims: To investigate the protective role of acetyl L-carnitine (ALCAR) on cisplatin-induced ototoxicity in rats by audiologic tests, and ultrastructural examination using electron microscopy (TEM), and also to investigate the mechanisms of ototoxicity including apoptotic pathways.
Methods: Adult Wistar albino rats (n:28) were studied. At the begining of the study audiologic assessments including transient oto-acoustic emissions, auditory brainstem response testing were done. There were 4 groups: G1; Saline supplemented control rats, G2; ALCAR administered rats, G3; Cisplatin administered rats; G4; Cisplatin administered rats following ALCAR pretreatment. Rats were sacrificed after control audiological assessments were done at the 3rd day of the study. Brain and inner ear specimens including hairy cells and spiral ganglia were examined by TEM, and caspase 3, 8, 9 activities were studied by immunohistochemical methods.
Results: Hearing tresholds by 6 and 8 kHz tone burst stimulus were found significantly low in G4 when compared to G3. The number of TUNEL positive cells, and caspase 3, 8, 9 immunostaining cells were significantly increased in G3 when compared to G1, G2, G4. There was no significant difference between G1, G2, G4. Ultrastructural findings of hairy cells, spiral ganglia and brain were normal in G1, G2, G4. In hairy cells of G3; mitochondrial effacement, mitochondrial crystalysis, intracellular degenerative areas, and damage of intercellular junctions were observed. In spiral ganglia of G3; changes in cells shapes, cell membrane irregularity, secession from satellite cells and increased in cytoplasmic degenerative areas were seen. The ultrastructural damage of brain, spiral ganglia and the organ of Corti was prominent in G3 when compared to G1, G2 and G4.
Conclusions: ALCAR improves cisplatin-induced audiologic impairment and also antioxidative, antiapoptotic properties of ALCAR on cisplatin ototoxicity were supported by the findings.

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SEL21

BTK expression is critical in neuroblastoma tumor initiating cells

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Background: Patient-derived tumor initiating cells (TIC's) have been characterized by the expression of CD24 and CD34 and we have confirmed that these cells also express CD20 and signal through the BTK pathway. Inhibition of the BTK pathway has been shown to be important for survival of B-cell malignancies and may be a potential target for inhibiting proliferation of neuroblastoma (NB) TIC's. Initial testing of the BTK inhibitor PCI-32765 shows cytotoxicity in NB TIC's and holds potential to be a promising new therapy.
Methods: : mRNA expression profiles of TIC's (NB12 and B011SC) and neuroblastoma cell lines (SMSKCNR, Be2C, SY5Y) were analyzed with U133 2+ arrays. Immunofluorescence of TIC's were characterized using CD20, CD24, CD34, nestin, p75, NB84, TrkB, synaptophysin, GD2. BTK phosphorylation was measured by Western blot. In 96-well plates, TIC's and NB cell lines were treated with BTK inhibitor PCI-32765 at concentrations ranging from 0-20uM, for 24-48 hours. Cell viability was assessed by calcein AM assays. Western blot evaluated caspase-3 and PARP cleavage. Nude mice were injected with 107 TIC's (B011SC or NB12 cells) subcutaneously and treated with daily doses of 3 and 30 mg/kg PCI-32765. Tumors were measured weekly.
Results: Immunofluorescence of TIC's as compared to NB cell lines showed: the presence of CD20, CD 24, CD34; comparable levels of nestin, p75, synaptophysin, NB83; and a decrease in TrkB and GD2. TIC lines showed increased mRNA expression of CD20 and BTK as compared to NB cell lines. Western blot analysis confirmed the presence of phosphorylated BTK in these cells which is ablated with the addition of PCI-32765. These cells showed decreased cell viability after treatment with PCI-32765 with IC50's < 15uM. The TIC xenograft mouse models showed a decreased tumor volume in mice treated with PCI-32765 as compared to control.
Conclusion: TIC's in neuroblastoma may play an important role in preventing treatment relapse in NB. Thus, inhibition of the CD20/Syk/BTK pathway warrants further study and may illuminate a potential therapeutic target for neuroblastoma.

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SEL22

Inflammatory prostaglandin E_2 induces neuroblastoma cell proliferation and survival in an autocrine and/or paracrine manner

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Background: Cyclooxygenases (COX) catalyze the conversion of arachidonic acid to prostaglandins. There are two COX isoforms, COX-1 that is ubiquitously expressed in most tissues and COX-2 which is induced by inflammatory stimuli. We have previously shown that COX-2 is highly expressed in neuroblastoma, and treatment of established neuroblastoma xenografts with COX-2 inhibitors reduces tumor growth in vivo. To further elucidate the importance of COX-2 in neuroblastoma we have investigated the role of prostaglandin E_2 (PGE_2) signaling. PGE_2 is an inflammatory lipid mediator that mediates its effect through four G-protein coupled receptors EP1- EP4 that activates distinct intracellular signaling pathways.
Method/approach: PGE_2 receptors EP1-4 were analyzed in clinical primary neuroblastoma tumors, using immunohistochemistry, and in cell lines, using RT-PCR, western blot, and immunofluorescence. Production of PGE_2 was measured using LC-MS/MS and the effect of PGE_2 on cell viability was assessed with MTT-assay. To investigate intracellular signaling pathways that are activated upon PGE_2 stimulation western blot were performed.
Results: All four PGE_2 receptor subtypes were detected in human neuroblastoma primary tumors, as well as in cell lines. In addition, neuroblastoma cells were shown to produce PGE_2 and exogenously added PGE_2 induced phosphorylation of both ERK1/2 and Akt. Importantly, PGE_2 promoted neuroblastoma cell survival and induced proliferation in a dose- time- and cell-line-dependent manner. Moreover, PGE_2 rescues neuroblastoma cells from NSAID-induced apoptosis, and inhibition of PGE_2 signaling by an mPGES-1 inhibitor or receptor antagonists against EP1, EP3 and EP4 reduced cell viability.
Conclusion: These results demonstrate the importance of prostaglandin E_2 for neuroblastoma cell proliferation and survival. They indicate a potential therapeutic significance of a more specific inhibition of prostaglandin E_2 signaling in neuroblastoma treatment. Complementary studies are in progress, to further elucidate the significance of prostaglandin E_2 and its receptors in neuroblastoma.

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SEL23

Inhibition of Fatty Acid Synthase (FASN) as a potential therapy in neuroblastomas with MYCN amplification

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Neuroblastoma is a common and lethal pediatric cancer. More than half of patients are at high-risk for relapse and die despite intensive therapy; thus presenting an urgent need for new, rationally designed drugs.
To address the need for new therapeutic targets we sought to identify metabolic genes that are critical to neuroblastoma growth and survival. We restricted our analysis to a set of 12 metabolic genes that can be targeted therapeutically and had previously been implicated in other cancers. Analysis of 188 primary human neuroblastomas genotyped on the Illumina HumanHap SNP Array identified high-level, unbalanced gain at the FASN locus (17q25) in 23% of tumors. Analysis of FASN mRNA expression in 99 of these primary neuroblastomas using the IlluminaHT Expression Array identified significant increases in FASN expression restricted to high-risk neuroblastomas with amplification of the MYCN oncogene (P=0.0001). Within the subset of high-risk tumors with MYCN amplification, those that harbored FASN DNA copy number gains showed greater FASN mRNA expression than those with two copy FASN DNA. siRNA inhibition of fatty acid synthase (FASN) in a panel of 12 neuroblastoma cell lines resulted in significant growth inhibition and apoptosis in 9/12 (75%) of lines, including 8/8 (100%) of cell lines with MYCN amplification, suggesting a synthetic lethal effect of FASN inhibition in neuroblastomas with MYCN amplification. Ongoing analyses of pharmacologic inhibitors of FASN in neuroblastoma cell line and animal models including the impact on global lipidomic profiles will be reported. Taken together, an integrated genomic and functional analytic approach has identified fatty acid synthase, a key enzymatic mediator of de novo fatty acid metabolism, as a candidate oncogene and therapeutic target in neuroblastomas harboring MYCN amplification.

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SEL24

Neuroblastoma-mononuclear phagocyte interactions promoting tumor growth are suppressed by lenalidomide

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Background: We observed that the most aggressive high-risk neuroblastomas have high expression of genes of mononuclear phagocytes and of inflammatory cytokines, suggesting their possible role in promoting tumor growth. Experimentally, co-injection of monocytes with neuroblastoma cells into NOD/SCID mice enhanced the growth of neuroblastoma cells, and an anti-IL-6 antibody suppressed this effect. The current research characterized the cytokine milieu created by neuroblastoma cells and mononuclear phagocytes and determined the effect of lenalidomide on cytokine secretion and tumor cell growth in vitro and in vivo.
Methods: 21 cytokines secreted by 8-15 neuroblastoma cell lines, by monocytes from normal adults, and by monocytes cultured with condition medium from cell lines were quantified with the BD?Cytometric Bead Array assay, and TGF? was measured by ELISA. Tumor cell growth was evaluated in vitro with digital imaging microscopy and BrdU incorporation and in vivo with bioluminescence imaging of luciferase labeled tumor cells in NOD/SCID mice.
Results: Neuroblastoma cell lines secreted TGF? (93%), VEGF (87.5%), and MCP-1 (62.5%) but not other cytokines including IL-6. Monocytes secreted IL-8 and low levels of MCP-1 and MIP1? Monocytes were stimulated to secrete cytokines by conditioned medium (CM) of neuroblastoma cell lines: 1) TGF? by 73%; 2) IL-6, MCP-1, IL-8 by 62.5%; and 3) MIP-1-?by 37.5%. These CM induced phosphorylation of STAT3 and SMAD2/3 in monocytes and tumor cells and BrdU incorporation in tumor cells. Lenalidomide suppressed secretion of TGF?, IL-6, MCP-1, IL-8, and MCP-1-?by monocytes cultured with neuroblastoma CM and activation of STAT3 and SMAD2/3 in tumor cells by IL-6 and TGF?. Monocytes stimulated neuroblastoma cell line growth in vitro and in vivo in NOD/SCID mice, and this was prevented by lenalidomide.
Conclusion: TGF?, IL-6, and mononuclear phagocytes are potential therapeutic targets in high-risk neuroblastomas. Lenalidomide inhibits monocyte stimulation of tumor cell growth, possibly by suppressing monocyte secretion of growth stimulating cytokines and activation of STAT3 and SMAD2/3 signaling.

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SEL25

Identification and molecular characterization of human neuroblastoma tumor-initiating cells

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Background: Neuroblastoma (NB) displays a cellular heterogeneity within the tumor. There is increasing evidence that at the top of this observed tumor cell hierarchy, there is a sub-population of tumor-initiating cells (TICs), responsible for initiation and maintenance of the tumor. Candidates TICs have been isolated in a variety of adult solid tumors, representing a powerful potential therapeutic target. However this population has not yet been identified nor characterized in NB. NB is the most common extracranial childhood solid tumour originates from neural crest-derived malignant sympathoadrenal cells. We have identified cells within primary NB tissues and cell lines that express markers of neural crest stem cells and their derivatives, leading us to postulate the existence of TICs in NB tumour that recapitulate the properties of sympathetic precursor cells.
Method/approach: We proposed a novel approach to identify and characterize NB TICs by prospectively identifying their self-renewal properties. From a very aggressive stage 4 NB sample, we selected self-renewing putative TICs by their sphere-forming capacity and analyzed their gene expression profiles by time-course micro-array analysis.
Results: Supervised and unsupervised analyses provided a list of sphere markers genes involved in embryogenesis and nervous system development (*CD133, EDNRA/B, NOTCH1/3, GPR177*…), and drug resistance (*MDR1, ABCA1*). To determine whether the sup-populations selected in spheres correspond to TICs, their tumorigenic potential was assayed by in vivo tumor growth analyses using subcutaneous and orthotopic (adrenal glands) implantations of tumor cells into nude mice. Tumors derived from the sphere cells were significantly more frequent and were detected earlier compared to whole tumor cells. In addition, a more detailed study of the potential NB-TICs revealed a phenotypic heterogeneity in the sphere sub-populations based on the exclusive expression of *CD133* and *MDR1*.
Conclusion: We identified new NB-TICS specific markers and we characterized heterogeneous sphere sub-populations that will be individually analyzed by functional assays.

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SEL26

Synergy of targeted GMCSF and IL2 to tumor microenvironments is mediated by an adaptive anti-neuroblastoma immune response

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Immunotherapies based on monoclonal antibodies targeting neuroblastoma emerge as important treatment options. Neuroblastoma microenvironments are characterized by the overexpression of ganglioside GD2 and transferrin receptor. We investigated strategies based on targeted cytokines capable of eradication of disseminated metastases in a preclinical model.
Two types of genetically engineered antibody cytokine fusion proteins, called immunocytokines (ICs), were engineered and used, i.e. transferrin receptor specific ch17217-GMCSF and ch17217-IL2 and ganglioside GD2 specific ch14.18-GMCSF and ch14.18-IL2. All ICs were characterized by the determination of binding to the target antigen and cytokine activity. Efficacy and mechanism of mono- and combined therapies with GMCSF- and IL2-ICs was determined in the NXS2 mouse model.
We demonstrated that monotherapies with both types of IL2-ICs specifically suppress disseminated neuroblastoma mediated by an NK-cell dependent immune response. A weak, but specific innate response was also observed with both types GMCSF-ICs mediated by granulocytes and NO radicals produced by macrophages in vivo.
Interestingly, a synergistic effect with simultaneous combinations of GMCSF-ICs and IL2-ICs was observed. This combination therapy can completely eradicate established experimental and spontaneous hepatic metastases in contrast to ICs used as monotherapy. It is important to note that this effect was specific and not achievable with mixtures of antibody and cytokine in equivalent doses. Anti-tumor effects of GMCSF-ICs and IL2-ICs combinations were abrogated in T-cell deficient SCID mice and mice depleted of CD8+T-cells in contrast to controls. Furthermore, splenocytes isolated from mice receiving the combined GMCSF-IC and IL2-IC therapy revealed MHC class I restricted target cell killing in contrast to monotherapy controls and adoptive transfer of these T-cells protected naïve mice from neuroblastoma.
These findings indicate a synergy effect combining GMCSF- and IL2 ICs in neuroblastoma explained by a switch from an innate anti-neuroblastoma immune response when used as monotherapy to an adaptive when used in combination.

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SEL27

Opposite roles of distinct caspase-10 isoforms in death receptor apoptotic pathway

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Background: Resistance to death receptor-induced apoptosis is common in aggressive NB. Both caspases-8 and -10 are often co-silenced in childhood tumour. In contrast to caspase-8, the physiological functions of caspase-10 remain poorly understood, and caspase-10 ability to substitute for caspase-8 in apoptosis initiation is still controversial. Here we analysed the particular contribution of caspase-10 in death receptor-mediated apoptosis by manipulating the relative expression level of caspase-8 and four caspase-10 isoforms.
Method/approach: Stable expression of caspase-8 and caspase-10 isoforms was performed by retroviral infections and caspase-8 silencing by stable shRNA lentiviral infections. mRNA quantification was carried out by real time PCR. Apoptosis was measured by the Propidium Iodide method.
Results: Overexpression of caspase-10-A or caspase-10-D isoforms strongly increased TRAIL and FAS-L sensitivity of caspase-8 expressing NB and colon carcinoma cells, whereas overexpression of caspase-10-B or -G has no effect or was weakly anti-apoptotic. Surprisingly, a complete opposite effect of caspase-10 isoforms was observed in Jurkat cells (acute T cell leukemia), where caspase-10-A and -D displayed an anti-apoptotic role in death receptor-induced apoptosis, caspase-10-B was weakly pro-apoptotic, and caspase-10-G had no effect. The ability of endogenous caspase-10 isoforms to substitute for caspase-8 was analysed by caspase-8 silencing in TRAIL sensitive NB cells (SH-EP-shC8, SK-N-AS-shC8), which resulted in their complete resistance to TRAIL-induced apoptosis. Interestingly, overexpression of caspase-10-A or -D in SH-EP-shC8 cells or in caspase-8 and-10-negative IGR-N91 cells partially restored their sensitivity to TRAIL-mediated apoptosis.
Conclusion: These data highlight a differential cell type-related pro- or anti-apoptotic role for the distinct caspase-10 isoforms in the death receptor apoptotic pathway. Moreover they suggest that, at endogenous expression level, caspase-10 may modulate the extent of the apoptotic response, while over-expression of caspase-10-A or -D isoforms can substitute for caspase-8 in downstream activation of apoptosis in NB cells.

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SEL28

The tumor suppressor candidate gene *APITD1/CENP-S* on chromosome 1p36 is involved in chromosome segregation and DNA damage repair

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Background: Increased chromosomal instability manifested as aneuploidy is a major driving force for tumor development and progression. Proper chromosome segregation in eukaryotes requires that a multiprotein structure termed the kinetochore assembles on the centromeres and conveys binding of spindle microtubules to chromosomes and subsequent chromosome movement. The *APITD1* (a.k.a. *CENP-S*) gene, in the neuroblastoma tumor suppressor candidate region on chromosome 1p36.2, was recently shown to be part of the CENP-A centromere associated complex that is essential for chromosome segregation during mitosis.
Method/approach: We used tandem affinity purification of GFP-tagged Apitd1 and mass spectrometry to identify new Apitd1 binding proteins. To study the involvement of Apitd1 in DNA damage repair, we used a multiphoton laser to induce DNA damage to a small local volume (~1 μ^3 or less) in living human cells. Apitd1 protein expression was also analyzed in different cell lines and in primary neuroblastoma tumors by western blot analysis.
Results: We confirm previous findings that Apitd1 binds to the centromere components CENP-U, CENP-T, and CENP-X and we show additional interactions with CENP-P and CENP-Q, further strengthening the finding that Apitd1 is part of the CENP-A centromere associated complex. We also show that Apitd1 interacts with the Fanconi Anemia associated protein M (FANCM). Since FANCM plays a key role in the Fanconi Anemia DNA damage-response pathway we wanted to test whether Apitd1 was also associated with DNA damage. By multiphoton laser induction and live cell imaging microscopy we could determine that Apitd1-GFP rapidly accumulates at sites of DNA damage. Furthermore, the level of Apitd1 protein is low or absent in primary neuroblastoma tumors with a high frequency of aneuploidy.
Conclusion: We have confirmed Apitd1 as a centromeric component and shown that protein levels are low in primary neuroblastoma tumors, possibly contributing to the high prevalence of chromosomal instability in these tumors. Furthermore, Apitd1 has a more direct role in sensing DNA damage which may be unrelated to its function as a centromere component.

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SEL29

Conditional MYCN knockdown using shRNAs encoded by lentivirus vectors

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MYCN amplified (MNA) neuroblastomas are thought to have developed an addiction to the oncogene MYCN, meaning that its proliferation and possibly long term survival is dependent on its high expression. Since the development of RNAi as a tool for target-specific knockdown of gene expression, several studies have shown reduced proliferation and increased differentiation when MYCN expression is reduced in MNA neuroblastoma cell lines. These studies have been performed using transient delivery of synthetic siRNAs or plasmid expressing shRNAs targeting MYCN mRNA.
We have previously developed an inducible H1 RNA polymerase III promoter for efficient conditional shRNA expression. In a transient transfection system using luciferase as the reporter target, this promoter showed no apparent transcriptional leakiness in the OFF-state (without doxycyclin-dox). Addition of dox to the media (ON-state) resulted in expression of shRNAs at levels similar to the wild-type promoter. We have now used this promoter in combination with shRNAs targeting MYCN, and stably introduced the construct to SK-N-BE (2) and Kelly MNA neuroblastoma cells using retroviruses as carriers.
Induced anti-MYCN shRNA expression in stably transduced MNA neuroblastoma cell lines, resulted in steady down-regulation of MYCN over a period of two days, before stabilizing at appr. 70-80 % MYCN protein knockdown as long as dox was present in the media. These cells showed excessive neuronal-like differentiation and reduced proliferation 3-4 days after induction. Removal of MYCN also abolished the cells ability to form CFU in clonogenic assays. Removal of dox from the media resulted in recovery of MYCN protein expression. Uninduced cells did show slightly reduced proliferation and morphological changes (SK-N-BE (2)) compared to scrambled shRNA control, indicating some transcriptional leakiness in the system. This did however not significantly reduce CFU in clonogenic assays. We have developed SK-N-BE (2) and Kelly MNA neuroblastoma cell lines with inducible expression of shRNAs targeting MYCN. These cell lines make it possible to study the long-term effect of removing MYCN from MNA neuroblastoma.

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SEL30

Integration of genome-wide ChIP-data of MYCN/MYC and histone marks with gene expression

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Background: The proto-oncogenes MYCN and MYC are implicated in both normal development and malignant transformation. These proteins appear to integrate environmental signals in order to modulate diverse, and sometimes contradicting cellular processes. Although many studies are available, a comprehensive view on MYCN/MYC function in cancer remains elusive. This is caused by an apparent contradiction between their broad effects on multiple cellular functions and their molecular delineation as a relatively weak transcriptional activator with a poorly characterised set of target genes. To address this issue we performed genome-wide expression profiling and ChIP-chip or ChIP-seq analysis of both transcription factors and three histone marks in neuroblastoma cell lines with high MYCN or MYC gene expression.
Method/approach: ChIP is performed on eight different NB cell lines. ChIP-chip and gene expression hybridizations were performed on their respective Agilent microarray platforms. ChIP-sequencing data was produced on the Illumina Genome Analyzer. After initial ChIP-seq peak finding with the MACS tool we applied a novel feature extraction algorithm, developed to produce continuous DNA-regulator scores. Input samples were included in all ChIP analyses to generate statistically significant binding peaks. All data analyses including the integration of the ChIP and expression data were performed in R.
Results: MYCN/MYC DNA binding is co-occurring with transcriptionally active (H3K4me3) or elongating (H3K36me3) as with inactive (H3K27me3) histone marks. The regulation appears fine-tuned by the binding affinity of MYCN/MYC and the interaction with other DNA-binding proteins. The detailed landscape of the DNA-interactome in combination with gene expression results shows that MYCN and MYC have different modes of action in NB.
Conclusion: This unique genome-wide dataset captures the complex system of gene expression regulated by the low and high affinity DNA-interactions governed by MYCN/MYC in a NB background.

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SEL31

RUNX3, mapped to chromosome 1p36, is a tumor suppressor functionally regulating p53 and MYCN in neuroblastoma

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Background: *RUNX3*, a runt-related transcription factor, is a tumor suppressor gene mapped to chromosome 1p36 and is epigenetically silenced in several human cancers. However, the role of RUNX3 in neuroblastoma (NB) has remained elusive. Thus, we examined whether RUNX3 is involved in the progression of NB and unveiled its novel molecular function.
Method/approach: *RUNX3* expression levels were determined utilizing quantitative real time PCR in 110 primary NBs. We also investigated biochemical properties of RUNX3 protein in the context of DNA damage response pathway.
Results: The *RUNX3* region of chromosome 1p36 was deleted in 12 out of 59 NBs examined by an array-based analysis. This hemizygous deletion was closely associated with decreased levels of *RUNX3* mRNA expression (p=0.02). Low *RUNX3* expression was strongly associated with poor outcome of NBs (p=0.016) and was significantly correlated with INSS stage (p<0.001) and *MYCN* copy number (p=0.047), but not with other prognostic factors such as age, tumor origin, Shimada pathology, *TrkA* expression, and DNA index. On the other hand, there was no significant correlation between the stages and the promoter methylation of *RUNX3* gene, suggesting that other epigenetic modulation in addition to gene dosage may participate in its transcriptional regulation. Interestingly, among the patients with NBs in which *MYCN* is highly expressed, only those with high levels of *RUNX3* expression could survive (15 out of 31, p=0.003). In support with this finding, our *in vitro* data indicated that RUNX3 and MYCN physically interact and mutually affect their expressions. Furthermore, overexpression of RUNX3 induced neurites outgrowth in SH-SY5Y NB cells. We have also found that RUNX3 translocates from the cytoplasm into the nucleus, physically interacts with p53, and acts as a transcriptional co-activator of p53 by enhancing phosphorylation at Ser15 to induce apoptosis after DNA damage.
Conclusion: Our results suggest that *RUNX3* functions as a tumor suppressor in NB and affects the prognosis. Genetic and molecular interactions among RUNX3, p53, and MYCN proteins could play an important role in the development and maintenance of NB.

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SEL32

The p53 target Wig-1 is a novel regulator of N-Myc at the mRNA level

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The p53 tumor suppressor triggers cell cycle arrest, apoptosis, or a variety of other responses after exposure to cellular stress. p53 exerts its biological function at least in part through transcriptional transactivation of target genes. One such gene is wig-1 (for wild-type p53 induced gene 1). Our biochemical studies have revealed that the Wig-1 protein binds double stranded (ds) RNA with high affinity in vitro and in living cells. We have also shown that Wig-1 binds to a U-rich region in the 3'UTR of the p53 mRNA and stabilizes it by preventing deadenylation, the first and rate limiting step of mRNA degradation. U-rich regions are a subgroup of the AU-rich elements (AREs) known for regulating mRNA stability and translation. There are a number of proteins known to bind and regulate mRNAs containing AREs, and these proteins generally target multiple ARE-containing mRNAs. The mRNA of the oncogene N-Myc contains several AREs, and we show here that Wig-1 binds to and regulates the N-Myc mRNA through its 3'UTR. Wig-1 knockdown using small interfering RNA (siRNA) decreases N-Myc protein and mRNA levels and induces morphological differentiation in SK-N-BE(2) neuroblastoma cells carrying N-Myc amplification, phenocopying the effect of knocking down N-Myc itself. In conclusion, Wig-1 is a novel regulator of N-Myc mRNA and appears critical for maintaining high N-Myc levels in neuroblastoma cells.

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SEL33

Neurocristopathy-associated Phox2b mutations cause Sox10 dysregulation and affects self-renewal, proliferation and differentiation of autonomic neural progenitors

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Background: Phox2b is a paired homeodomain transcription factor essential for the development of visceral neurons. Heterozygous mutations of the PHOX2B gene have been identified in a neurocristopathy syndrome associated with Congenital Central Hypoventilation Syndrome (CCHS), Hirschscprung's disease (HSCR, intestinal aganglionosis) and neuroblastoma.
Method/approach: To understand how these PHOX2B mutations affect development of the enteric and sympathetic nervous system, we introduced two of these mutations into the mouse Phox2b locus by gene targeting and examined the resulting phenotype in these mouse mutants.
Results: Mice heterozygous for these PHOX2B mutations developed to term but died soon after birth due to the lack of spontaneous breathing. These mice also displayed hypoplasia or absence of the enteric ganglia, and size reduction and ectopic formation of the sympathetic ganglia. No obvious neuroblastoma formation was observed in the mutant mice before birth. Developmental analyses revealed that Sox10 expression was abnormally high and persistent in the enteric and sympathetic neural progenitors of Phox2b mutant mice, which led to biased differentiation of those progenitors toward the glial lineage. Moreover, the numbers and size of neurospheres generated from the enteric and sympathetic ganglia of Phox2b mutant embryos were significantly reduced as compared to those of control embryos.
Conclusion: Phox2b mutant knockin mice displayed phenotypes reminiscent of the syndromic neurocristopathy in human, demonstrating that the pathogenetic effects of mutant Phox2b are conserved between human and mouse. Mutant Phox2b affects the maintenance and differentiation autonomic progenitors possibly through dysregulation of Sox10.

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SEL34

***In vivo* analysis of human neuroblastoma cell lines in a human embryonic stem cell derived microenvironment - Impact of cues from the microenvironment**

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We describe the use of a pre-clinical model for studies on Neuroblastoma (NB), based on a species-specific embryonic microenvironment, derived from human Embryonic Stem cell (hESC) Teratomas (the hEST-model), contributing an NB growth support. The lines IMR32, Kelly and SK-N-BE(2) were injected into mature benign hESC-derived teratoma tissues and tumour growth compared to xenografts from the same lines. A clear tropism was detected in the hEST-model with an exclusive integration into mesenchymal stroma. An impact from the micro-environment was detected on all three cell lines with individual variations in histology, morphology and marker profiles. In general, despite cell-line dependent variations, a trend towards a higher inter-tumour heterogeneity was found in the hEST-model. This notion was also further supported from IHC with focal variations of markers of aggressiveness and differentiation. Furthermore, expression of Cox-2, an enzyme correlating with proliferation and apoptosis of NB, differed between the models. While the hEST-model showed similar findings to the primary NB tumour mass, ie homogenous staining of 100% Cox-2+ cells, xenografts showed a focal expression. Variation in frequencies of vimentin expressing cells was a further difference between the microenvironments, with a tendency for a biased support towards a vimentin high (mesenchymal) phenotype in xenografts. Similar to previous reports a vascularisation of hESC origin was induced by injections of NB tumour cells. A small minority of cells in the In SK-N-BE(2) xenografts expressed human CD31, indicative of an endothelial differentiation from the NB cells them selves. In summary, this species-specific embryonic in vivo model offers a higher maintenance of heterogeneity of the NB tumour growth, in this respect more resembling clinical neuroblastomas. Altogether, we propose that the model offers potentially more relevant preclinical studies on biological features of NB, as well as therapy response, enabling higher efficacy of clinical translation. Considering the high frequency of therapy resistance, and high number of deaths in this malignancy, this is of outmost importance.

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SEL35

Tenascin-C$^+$/Oct-4$^+$ perivascular neuroblastoma cells serve as progenitors of tumor-derived endothelial cells

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Background/Aims: Primary neuroblastoma (NB)-associated endothelial micro-vessels (EM) may be lined by tumor-derived endothelial cells (TEC), that are genetically unstable and chemoresistant. Here we have addressed the identification of TEC progenitors in NB focusing on the Oct-4 and Tenascin-C (TNC) markers.
Methods: Twenty-three primary NB tissue samples, 10 metastatic bone marrow (BM) aspirates, and five human NB cell lines were tested for the presence of TNC$^+$/Oct-4$^+$ cells by immunofluorescence combined with *MYCN* specific fluorescence in situ hybridization (FISH). Two *MYCN* amplified NB cell lines (HTLA-230 and GI-LI-N) were injected orthotopically in the adrenal gland of immunodeficient mice. TEC were detected by human CD31 immunofluorescence staining and *MYCN* FISH. NB-bearing mice were treated with anti-human CD31 or anti- human and mouse prostate specific membrane antigen (PSMA) monoclonal antibodies (mAbs), that target exclusively endothelial cells . Tube formation was investigated using a Matrigel 3D System (Trevigen) containing VEGF.
Results: HTLA-230, ACN, LAN5, SHSY-5Y, but not GI-LI-N, cell lines co-expressed TNC and Oct-4 (range 2-30%). All NB tumors and BM aspirates contained TNC$^+$/Oct-4$^+$ cells (ranges 2-30% and 0.2-1.5%, respectively). *MYCN* amplified TEC lined a half of EM from tumors formed by HTLA-230 cells. No TEC were detected in tumors formed by GI-LI-N cells. *MYCN* amplified TNC$^+$/Oct-4$^+$ NB cells were detected in perivascular niches and expressed cancer stem cell-related (SOX-2, CD133, CD24, Nestin, III tubulin, p75, hTERT and HIF-2) and NB-related (GD2, NB84, CD56) markers. Number and proliferating fraction of TNC$^+$/Oct-4$^+$ NB cells increased significantly (P=0.0012 and <0.0001, respectively) in tumors from mice treated with mAbs to CD31 or prostate specific membrane antigen (PSMA) suggesting involvement of these cells in TEC differentiation. Indeed, TNC$^+$/Oct-4$^+$ cells isolated from human HTLA-230 cells, i) formed tubes in a Matrigel assay and ii) differentiated in vitro into VE-cadherin$^+$, PSMA$^+$, CD31$^{+/-}$ EC-like cells.
Conclusion: TNC$^+$/Oct-4$^+$ NB cells represent a source of TEC and a novel potential therapeutic target.

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SEL36

NVP-BEZ235 a dual PI3K/mTOR inhibitor destabilises Mycn *in vitro* and is growth inhibitory in the TH-*MYCN* murine neuroblastoma model

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Background: MYCN oncogene amplification in Neuroblastoma (NB) is associated with advanced disease stage and poor prognosis. Mycn is a critical regulator of neuroblast fate and survival, and is specifically expressed in NB tumour tissue, making it an ideal candidate for targeted therapy, we therefore examined methods that destabilize Mycn through inhibition of phosphorylation and proteasomal degradation. In previous work we showed that broad-spectrum phosphatidylinositol-3-kinases (PI3K) inhibitors destabilise Mycn and inhibit growth of native murine tumours (Chesler and Weiss, 2006). Refined compounds such as NVP-BEZ235 (Novartis Inc.) selectively inhibit class I PI3K and mammalian target of rapamycin (mTOR), with IC50 values in the nanomolar (nM) range and have entered adult phase I clinical trials.
Methods/Results: Using a panel of N-myc wild-type (wt) and N-myc phosphomutant expressing cell lines, we show that NVP-BEZ235 inhibits PI3K and/or mTOR-dependent phosphorylation of key PI3K pathway components. Treatment with NVP-BEZ235 destabilises wt N-myc and cell proliferation and cycling is effectively inhibited as shown by both GI50 (MTS and SRB) and flow cytometry assays with significant apoptosis detected by caspase cleavage assay. N-myc mutants treated with BEZ235 exhibit no decrease in the steady state levels of the N-myc protein and show cell cycle and proliferation inhibition at higher GI50. To test the in vivo effectiveness of BEZ235 we used the TH-MYCN model in which MYCN overexpression induces spontaneous NB (Weiss, 1997). Mice were treated with 45mg/kg of NVP-BEZ235 PO, daily and tumour progression was monitored using MRI. BEZ-235 was nontoxic and induced good partial responses against a majority of large tumours and a statistically valid regression of tumour size. Intratumoral PI3K pathway expression was inhibited concomitant with ablation of Mycn protein.
Conclusion: Inhibition of PI3K/mTOR signalling by BEZ235 is highly effective against Mycn protein in both *in vitro* and *in vivo* models of *MYCN* driven NB. It could therefore provide a promising therapeutic strategy for Mycn-targeted treatment of NB.

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SEL37

Development of novel therapeutic strategy for neuroblastoma: Reactivation of the p53 tumor suppressor function by small molecules

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Background: Upon development of metastasis and therapy resistance neuroblastoma (NB) becomes the most deadly tumor of childhood. The tumor suppression function of p53 is crucial for the prevention of tumor development as well as for the response to anticancer therapy. Mutations of p53 gene occur in around 50% of human tumors. In tumors that retain wild type p53 its tumor suppressor function is inhibited via overexpression of p53 destructor HDM2 which is a crucial regulator of p53: it binds and targets it for the ubiquitin-dependent degradation. Restoration of the p53 function in tumors should trigger massive apoptosis in presensitized tumor cells. In NB, p53 is inactivated mainly via deregulated expression of its inhibitors MDM2 and MDMX. N-myc, the oncogene which overexpression is a driving force of a large fraction of NB to inactivate p53 by inducing the expression of p53's own destructor MDM2.
Methods: We have previously identified a small molecule RITA that disrupts p53's interaction with several inhibitory proteins, including MDM2 and MDMX (1,2,3). Here we used cell proliferation and apoptosis assays to investigate reactivation of p53 in NB tumor cell lines by RITA. We also assessed p53-target gene activation using qPCR and western blot.
Results: We found that 1μM RITA efficiently kills NB cells, as assessed in nine NB lines with or without MYCN amplification. We studied the expression of p53 and its target genes in NB cell lines and found that RITA induced p53 accumulation and expression of p53 target genes, suggesting that p53 is reactivated by RITA in NB lines. After 48 hours of RITA treatment several p53 target genes were induced, including pro-apoptotic BAX and PUMA as well as cell-cycle arrest p21 gene. Our studies using SHEP cells in which the expression of N-myc is regulated by doxycycline showed that cells overexpressing N-Myc oncogene are efficiently killed by RITA. We are planning to address the effect of RITA on the growth of NB xenografts in mice.
Conclusions: Taken together, our results suggest that RITA or its analogs might serve as a good strategy to target therapy-resistant NB.

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SEL38

Modeling neuroblastomagenesis from neural crest stem cells *in vitro* and *in vivo*

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Background: Accumulating evidence suggests that neuroblastoma (NB) originates from neural crest stem cells (NCSC). MYCN has been shown to promote cell cycle progression of NB cells in vitro, and transgenic overexpression of MYCN is sufficient to induce NB-like tumors in mice. However, the origin of NB has still not yet been clearly assigned to a defined cell population.
Method/approach: To further address this question, we established an immortalized multipotent NCSC line, JoMa1, isolated from a transgenic mouse expressing a conditional 4-OHT (Tamoxifen) inducible allele of c-Myc (c-MycERT). JoMa1 cells express p75 and Sox10 stem cell markers, proliferate robustly depending on 4-OHT induced c-MycERT activity, and can be differentiated into all derivates of the neural crest. JoMa1 cells were stably transfected with a MYCN cDNA, and their tumorigenicity was analyzed in nude mice. Tumors were histopathologically evaluated, MYCN expression analyzed by RT-qPCR and genomic aberrations assessed using aCGH. Cell lines established from tumors were serially propagated in mice, and the effect of the Myc inhibitor, NBT-272, on NB cell growth was analyzed.
Results: In contrast to parental JoMa1 cells, JoMa1-MYCN cells were able to grow in cell culture independent of c-MycERT activity, and their s.c. injection into nude mice caused formation of NB-like tumors, as deduced from histology. Tumorigenicity of the cells was enhanced upon serial transplantation. Tumors originating from JoMa1-MYCN cells expressed variable levels of MYCN, which were inversely correlated with time to tumor formation. A focal amplification on chromosome 8 was identified by aCGH analysis in tumors with high MYCN expression. Interestingly, cell lines derived from xenografted JoMa1-MYCN tumors were highly susceptible to the Myc-inhibitor, NBT-272, indicating that cell growth depended on functional MYCN.
Conclusion: We here show for the first time that transformed NCSC cells can give rise to neuroblastic tumors, supporting that NCSC are the precursor cells of NB. We are currently analyzing the transforming capacity of other potential NB oncogenes in the JoMa1 model system.

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SEL39

FOXO3/FKHRL1 is activated in high-risk neuroblastoma and contributes to chemotherapy-resistance and angiogenesis

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Background: FOXO transcription factors control programmed cell death, stress resistance and longevity in normal and malignant cells. We investigated the expression, subcellular localization and phosphorylation of FOXO3/FKHRL1 in tumor sections of post chemotherapy neuroblastoma (NB) patients and analyzed its effect in cultured neuroblastoma cells.
Methods: Paraffin-embedded sections from 27 NB patients were analyzed for FOXO3 expression, localization and phosphorylation. Effects of chemotherapeutic agents on FOXO3 subcellular shuttling were assessed by live cell fluorescence imaging in ECFP-FOXO3 transgenic NB cells. To study how FOXO3 modulates survival of NB cells we generated cell lines expressing a conditional PKB-independent FOXO3 allele (FOXO3(A3)ERtm) that can be activated by 4OH-tamoxifen and studied the effects of FOXO3 activation in vitro and in vivo by xenograft transplantation into nude mice.
Results: We found that FOXO3 was localized in the nucleus and phosphorylated at the protein kinase B (PKB) phosphorylation site T-32 in tumor sections from high-risk NB patients. FOXO3 nuclear localization and phosphorylation significantly correlated with reduced patient survival. The chemotherapeutics etoposide and doxorubicin led to rapid nuclear accumulation and increased phosphorylation of FOXO3 at the PKB sites T-32 and S-253 in NB cell lines as measured by live cell fluorescence imaging and immunoblot, respectively. NB1/FOXO cells expressing the conditional FOXO3(A3)ERtm allele became resistant to chemotherapy-induced cell death whereas NB15/FOXO cells underwent spontaneous apoptosis upon FOXO3 activation. However, when transplanting NB15/FOXO cells into nude mice, low-level activation of FOXO3 strongly induced angiogenesis of NB tumors in vivo.
Conclusion: The combined data suggest that FOXO3 is activated in high risk NB tumors and contributes to chemotherapy resistance and tumor angiogenesis.

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SEL40

Segmental chromosome aberrations and ploidy in localized neuroblastomas without MYCN amplification – Report from the SIOP Europe Neuroblastoma (SIOPEN) Group on the LNESG I Trial

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Background: The prognostic impact of segmental chromosome aberrations (SCA) and DNA content was assessed for 123 patients with stage 2A and 2B neuroblastoma without MYCN amplification. The patients were included in a multinational protocol (the localized Neuroblastoma European Study Group trial 94.01, LNESG I, 1995-99) and were treated by surgery only irrespective of a tumour residual.
Methods: The neuroblastomas were analyzed by pan-genomic (array comparative genomic hybridization, aCGH) and/or multigenomic techniques (multiplex ligation-dependent probe amplification, MLPA), fluorescence in situ hybridization (FISH) and flow cytometry (FCM). All genetic data were quality controlled by the SIOPEN Biology Group.
Results: Pan-/multigenomic data were available for 67 tumours and FISH for an additional 39 tumours. SCA were more frequently associated with near-di-/tetraploidy than with aneuploidy ($p<0.00075$, Fisher's exact test, 65 patients). The most frequently affected loci were: 17q (21.7%), 1p/1q (12.3%) followed by 11q (8.1%). Relapse-free survival (RFS) was significantly related to 1p loss ($p<0.02$, Logrank test, 106 patients). OS and RFS were significantly lower if patients had diploid neuroblastomas ($p<0.02$ and $p<0.03$, respectively). Moreover, while OS and RFS were significantly associated with SCA in patients over 18 months of age at diagnosis ($p<0.0015$ and $p<0.00035$, respectively, 106 patients, FISH negative results included), this relationship was not observed for patients below 18 months ($p>0.28$ and $p>0.59$, respectively).
Conclusion: Based on the analysis of this patient cohort, it is suspected that in localised resectable neuroblastomas with normal MYCN status, the clinical impact of SCA depends on the age of the patient at diagnosis.

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SEL41

Segmental chromosome abnormalities and age over 36 months at diagnosis are associated with increased risk of relapse in localised unresectable neuroblastoma without MYCN amplification - A preliminary report from the SIOP Europe Neuroblastoma (SIOPEN) Biology Group

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Background/Aims: Recent reports suggest that segmental chromosome abnormalities (SCA) in neuroblastoma (NB) without MYCN gene amplification correlate with worse outcome. So far, no treatment modifications have been made for children with stage 2 and 3 disease over 1 year of age at diagnosis based on this information. The current study presents validated multigenomic data concerning the presence and the prognostic impact of SCA on a cohort of patients over one year with Localized Unresectable Neuroblastoma without MYCN amplification.
Methods: Between January 2001 and October 2006, a total of 161 newly diagnosed children were enrolled in the SIOPEN Unresectable Neuroblastoma study (EUNB). Out of 161 tumors, 157 were analyzed by Interphase FISH (I-FISH) for MYCN amplification and 1p deletion, while Multiplex Ligation-dependent Probe Amplification (MLPA) and array-Comparative Genomic Hybridization (array-CGH) were performed on 56 and 38 tumors respectively. Genetic data were reviewed by members of the SIOPEN Biology Group.
Results: One or more segmental chromosome abnormalities were detected in 49 (52%) tumors. Of the 7 recurrent chromosome aberrations (gain of 1q, 2p, 17q; loss of 1p, 3p, 4p, 11q), described in NB the most frequently observed were: 17q gain (33%), 11q loss (23%), 1p loss (22%) and 2p gain (12%). Compared to children at the age range 12-18 and 19-36 months, those>36 months showed the highest frequency of the number of SCA ($p=0.027$, Kruskal-Wallis test). In these patients the presence of at least one of these aberrations was associated with poor overall and progression free survival ($p=0.06$ and $p=0.043$, respectively). This effect was not evident in younger children.
Conclusion of the study: In this cohort of patients with not MYCN amplified tumors, the presence of at least one SCA increased with age at diagnosis and was associated to poor overall and progression free survival only in children >36 months. Our data suggest that other chromosome abnormalities than MYCN could play an important role in tumor development and progression.

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SEL42

Drug-induced senescence in *MYCN*-amplified neuroblastoma - gene expression profiling and functional consequences

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Background: Cellular senescence, a permanent state of proliferative arrest, provides a barrier to tumorigenesis *in vivo*. Activation as well as inactivation of oncogenes can cause senescence and are associated with inhibition of initial stages of tumor development or tumor "regression" at later stages. In *MYCN*-amplified (MNA) neuroblastoma (NB), we previously identified hydroxyurea (HU) as a substance that leads to the elimination of episomal MNA and senescence induction upon low dose long-term treatment *in vitro*. The aim of this study was to analyze senescence induction, characterize senescent NB cells and describe their functional role.
Methods: Two NB cell lines with MNA (STA-NB-9 and -10) were genetically characterized before and after senescence induction by MLPA and arrayCGH. In order to identify the sequential pathways involved in senescence induction, changes in the gene expression profile were analyzed and validated by qRT-PCR. Functional features of senescent NB-cells were assessed by co-cultivation with non-senescent NB-cells or PBMCs for their immune-stimulatory capacity.
Results: We found that MNA is the only chromosomal aberration that changes between non-senescent and senescent NB-cell lines, while all other aberrations remain unchanged. Senescent NB cells down-regulate unfavorable (survivin, NME1, BIRC1, cyclinE1) and up-regulate favorable tumor markers (NTRK1, ephrin receptor, CD44). Furthermore, the cell cycle-inhibitors p21, p15 and p16 and the tumor growth- and angiogenesis inhibiting factors DKK1 and 3, TIMP1 and 2, SERPINE1, IGFBPs, SPARC and INHBA were increased. Functional analyses revealed that senescent tumor cells may reduce cell growth of non-senescent tumor cells *in vitro* and - in line with increased expression of MHCI and other immune-response-related molecules - senescent NB cells allow T-cell and NKT-cell activation.
Conclusion: We hypothesize that HU induces a senescent, non-malignant, immunogenic state in neuroblasts. These data provide the basis for future studies using HU as senescence inducer in neuroblastoma patients to prevent tumor relapse.

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SEL43

Parvovirus H1 induces oncolytic effects on human neuroblastoma cells *in vitro* and in neuroblastoma xenograft-bearing nude rats

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Background: H1-PV is an oncolytic parvovirus of rodents that is non-pathogenic in humans. In vitro and in vivo, H-1PV has been shown to have relevant oncolytic effects on a broad variety of adult cancers. Here, we performed a pre-clinical evaluation of the therapeutic efficiency of H-1PV for the treatment of neuroblastoma in vitro and in vivo.
Methods: Neuroblastoma cell lines with different MYCN status as well as non-malignant primary cells of different origin were infected with H1-PV. We determined infection efficacy, viral replication, and lytic activity and cell viability in vitro. To determine the therapeutic efficiency of intra-tumoral H-1PV infection in vivo, human MYCN amplified or over expressing neuroblastoma cells were implanted subcutaneously in immunodeficient rats. Tumor volume was monitored by sonographical 3D-reconstruction, and body weight, clinical signs and survival were monitored.
Results: Non-neoplastic infant cells were unaffected by H1-PV. All neuroblastoma cell lines analyzed were infectable with H1-PV and competent for virus replication. In infected neuroblastoma cell cultures viral genomes copy numbers increased up to 10.000-fold within 48 to 96 hours. Parvovirus H1 induced lytic infection in all 11 neuroblastoma cell lines tested after application at MOIs between 0.001 and 10 pfu /cell. The cytotoxic effect of H-1PV on neuroblastoma cells could be shown to be mediated by G2-arrest and subsequent apoptosis induction and was independent of MYCN oncogene amplification status. In vivo no toxic side effects were observed in infant nude rats after injection of H-1PV. In human neuroblastoma xenotransplant bearing animals reduction of tumor growth and prolonged survival could be achieved by a single intra-tumoral H-1PV-infection. In some treated animals, we observed complete neuroblastoma regression and relapse-free survival of more than 6 months.
Conclusion: The efficiency of H-1PV infection, virus replication and relevant lytic effects on neuroblastoma cells in vitro and in vivo, together with the low toxicity of H-1PV for non-malignant infant cells, make this parvovirus a promising option for oncolytic virotherapy in neuroblastoma patients.

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SEL44

Targeting MYCN in neuroblastoma with small molecules *in vitro* and *in vivo*

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Background: *MYCN*-amplification is strongly related to poor clinical outcome in neuroblastoma patients, with low survival rates despite novel advances in treatment strategies. An alternative treatment option for children with *MYCN*-amplified neuroblastoma is therefore urgently needed. Our aim is to identify and characterize compounds that selectively induce cell death in neuroblastoma cells with high MYCN expression. Here we will present data on one such compound.
Methods: *MYCN*-amplified and non-amplified neuroblastoma cell lines were treated *in vitro* to test the effect of our candidate compound on cell death induction and growth arrest. Immunoblotting was used to monitor effect on MYCN protein levels in treated neuroblastoma cells. The effect on MYCN:Max interaction and MYCN:Max DNA binding was studied by co-immunoprecipitation and EMSA. To further evaluate the potential of our compound in targeting *MYCN*-amplified neuroblastoma, its *in vivo* efficacy was tested in a *MYCN*-amplified neuroblastoma xenograft model as well as in an MYCN-driven transgenic mouse model of neuroblastoma.
Results: Treatment of *MYCN*-amplified cell lines with our candidate compound resulted in decreased MYCN protein levels and induction of apoptosis in a concentration-dependent manner in all *MYCN*-amplified but not in the majority of non-amplified cell lines tested. *In vivo* treatment of established tumors delayed tumor growth of either xenografted *MYCN*-amplified human neuroblastoma cells or tumors arising in the *MYCN*-transgenic mice. In addition, in the *MYCN*-transgenic model, survival after initiation of treatment was significantly enhanced in treated as compared to control animals.
Conclusion: Here, we provide data showing that small molecules specifically targeting the cells over expressing MYCN would be beneficial for the treatment of neuroblastoma. Further analysis of the mechanisms of action and validation of structural analogs of our candidate compound will hence be important for the implication of using small molecules in the management of MYCN deregulated tumors.

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SEL45

Protein interactions of the *PHOX2B* variants identified in patients with neuroblastoma

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Background: Mutations in the *PHOX2B* transcription factor have been associated with neural crest disorders, namely, congenital central hypoventilation syndrome (CCHS), Hirschprung's disease, and neuroblastoma. While missense and frameshift mutations are mainly seen in neuroblastoma patients, in-frame expansion of the second polyalanine repeat in the *PHOX2B* gene is the most common aberration in CCHS. It is unclear whether these variants lead to a loss or gain of function. The goal of this study was to investigate the role of *PHOX2B* in development and disease by identification of interacting protein partners which could then be linked to known cellular pathways.
Method: We used a human ORFeome-based high-throughput yeast two-hybrid screening system containing 12,000 ORF (open-reading frame) clones. Wild-type (WT) and disease-related mutant constructs, R141G, R100L, K155stop, 676delG (neuroblastoma) and a +7-alanine (27 alanine) repeat mutation (CCHS) were transformed into yeast cells and mated against cells containing each of the ORF clones in the library. Potential interactors from the primary screen were verified by two rounds of pair-wise mating tests.
Results: We identified a number of protein-protein interactions for WT PHOX2B; which were also shared by the PHOX2B polyalanine expansion variant. However, these interactions were lost in all the neuroblastoma-associated variants. Moreover, the 676delG variant gained novel protein interactors that were not seen in any of the other variants. The interactions derived from the screen have been verified in vivo in mammalian cells. Moreover, proteins that were found to interact with WT PHOX2B enhanced the transactivation potential of PHOX2B in luciferase assays.
Conclusion: Our results show that the neuroblastoma associated PHOX2B variant, 676delG, not only abolishes normal PHOX2B binding but also acquires novel protein interactions, suggesting a dominant negative disease-causing role. These interactors serve as candidate genes whose role in tumorigenesis has yet to be elucidated.

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SEL46

Targeted therapeutics in chemotherapy-refractory neuroblastoma

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Amplification of the MYCN oncogene in neuroblastoma is one of the clearest indicators of poor prognosis and heralds a highly chemotherapy resistant and malignant form of the disease. Clinically, nearly all neuroblastomas respond to initial high-dose chemotherapy but a subset of high risk tumors relapse with very poor prognosis. Mycn protein expression is known to be tightly controlled at the post-translational level downstream of the PI3-kinase/AKT/mTOR pathway. Since MYCN is known to play such a central role in a subset of high-risk neuroblastoma, we have screened a panel of inhibitors to PI3K and other pathway members using neuroblastoma cell lines in search of potential candidate drugs which effect apoptosis and Mycn expression. Neuroblastoma tumors almost universally have wild-type p53 at diagnosis, but at relapse mutations in p53 and p53 pathway members are common indicating a potential role for p53 in the chemotherapy resistant phenotype of relapsed disease. In a now well established model of neuroblastoma developed in our lab, transgenic mice expressing human Mycn driven by the rat tyrosine hydroxylase promoter develop neuroblastoma tumors which are genetically and phenotypically similar to human disease. Expression of Mycn is also well known to drive apoptosis and therefore, in order to develop neuroblastoma driven by Mycn, tumor cells must bypass this Mycn apoptotic signal perhaps through p53. We have crossed THMYCN neuroblastoma with p53ER-TAM knock-in mice, in which p53 can be rapidly and reversibly restored. These THMYCN/p53ER-TAM mice have an increased penetrance of the neuroblastoma phenotype at a younger age. Preliminary results indicate that restoration of p53 function by tamoxifen injection causes the induction of apoptosis in neuroblastoma tumors. This THMYCN/p53ER-TAM may represent an improved model for therapy-resistant, relapsed neuroblastoma and is an ideal platform for testing potential future therapeutic modalities.

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SEL47

Validation of Survivin as a therapeutic target in neuroblastoma

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The inhibitor of apoptosis protein BIRC5 (Survivin) is a gene located on the SRO of chromosome 17q. Using Affymetrix mRNA expression data, we show that BIRC5 expression is strongly up-regulated in neuroblastoma compared to normal tissue, other adult malignancies and compared to non malignant fetal adrenal neuroblasts. The high BIRC5 expression is correlated to 17q gain indicating that the genetic aberration on 17q is contributing to the overexpression. Finally, the overexpression of BIRC5 strongly correlates to a bad prognosis, independent of 17q gain. To further validate BIRC5 as a potential drug target we used both LNA and lentiviral shRNA to inhibit BIRC5 in neuroblastoma cell lines. Both BIRC5 antisense techniques caused a specific knock down on mRNA and protein level. This resulted in massive apoptosis as indicated by PARP cleavage and an increase of the sub-G1 fraction on FACS analysis.
BIRC5 has two functions. One is as an inhibitor of the intrinsic apoptotic pathway; the other is stabilization of the microtubules in the chromosomal passenger complex. We investigated the mechanism by which apoptosis is caused after BIRC5 knockdown. We could not detect interaction of BIRC5 with DIABLO or XIAP, two proteins in the intrinsic apoptosis pathway. However, we could show an interaction between BIRC5 and Aurora Kinase B, a protein in the chromosomal passenger complex.
In addition we observed P53 activation after BIRC5 silencing, and we rescued apoptosis after BIRC5 silencing by CASP2 (caspase-2) inhibition. P53 and CASP2 are known to be activated as a result of chromosomal instability. Immunofluorescence showed multinucleated cells and aberrantly shaped nuclei after BIRC5 knockdown. This indicates that the cells could not finish the cell cycle properly.
We conclude that both BIRC5 LNA and shRNA cause a specific inhibition of BIRC5 which results in a pro-apoptotic effect on neuroblastoma cells via mitotic catastrophe.
We are currently determining the IC50 of YM-155, a small molecule BIRC5 inhibitor, in a panel of 25 neuroblastoma cell lines. The aim is to find YM-155 sensitivity markers and to find synergy with other inhibitors based on the results described above.

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SEL48

Rituximab is a novel neuroblastoma therapy with efficacy against neuroblastoma tumor initiating cells *in vitro* and *in vivo*

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Background: Tumour initiating cells (TIC) have been isolated from several solid tumours, and novel therapeutic approaches are under development based on the assumption that these cells are chemoresistant and may be responsible for tumour relapse. Neuroblastoma (NB) TICs isolated from bone marrow metastases express markers consistent with NB and a subset of surface markers characteristic of B-lymphocytes, including CD20 (L. Hansford, ANR 2010). We investigated the sensitivity of NB TICs to Rituximab, a monoclonal chimeric anti-CD20 antibody currently used for the treatment of CD20+ lymphoma.
Method/approach: Immunophenotype of NB TIC (NB12, NB88R, NB122R2) and NB adherent cell lines (SK-N-SH, SK-N-AS, SH-SY5Y) was performed by flow cytometry (FACS). In vitro Rituximab activity was tested by complement-mediated cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) assays. The effect of Rituximab +/- goat anti-human antibody (to induce hyper-crosslinking) and +/- chemotherapy was tested by Alamar Blue and FACS (apoptosis and cell cycle analyses). For in vivo studies, tumours established by injection of TICs intra dermally in BALB/c nu/nu mice were treated with Rituximab or control human IgG1 20 mg/kg intra-peritoneal days 1 and 4.
Results: NB TIC (but not adherent NB lines, even when grown in TIC conditions) express the B-cell surface molecules CD20, IgM, CD74, CD19, CD22, MHC class II and CD32. Rituximab induced CDC and ADCC in vitro for all tested NB TICs. Treatment with Rituximab alone or hyper-crosslinked reduced TIC viability in vitro, and pre-treatment with Rituximab significantly potentiated TIC chemotherapeutic agent (cisplatin, doxorubicin, irinotecan, rapamycine) sensitivity. Preliminary studies also demonstrate an in vivo response to Rituximab monotherapy.
Conclusion: NB TICs display a unique immunophenotype including B-cell surface markers and Rituximab has in vitro and in vivo efficacy against NB TICs. Since Rituximab is already in phase III paediatric lymphoma clinical trials in combination with chemotherapy, it represents an innovative immunotherapy approach with potential fast translation into clinical practice for NB patients

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POB1

Identification *in vitro* and *in vivo* of tumoral glial precursor cells in neuroblastoma

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Background: Neuroblastic tumors (NBT) are derived from multipotential neural crest stem cells, and composed by a neuroblastic component and Schwannian-like (glial) stroma. A correlation has been established between the degree of differentiation of the neuroblastic subtype, the amount of glia and patient outcome. However, the physiological relationship between Schwannian-like stromal cells and neuroblastic cells has not been clarified. We reported lineage specificity of membrane GD2 for neuroblastic component and the nuclear membrane S100A6 for glial cells (Acosta S, 2009). The aim of this work was to explore the existence of bipotential tumoral precursor cells in neuroblastoma.
Methods: Double immunofluorescence (IF) for GD2 and S100A6 was performed in 14 primary NBT and 8 metastasic bone marrow specimens. FACS analysis was performed in 7 enzymatically disaggregated tumor samples. The I-type cell line SK-N-Be2C differentiated with 1μM ATRA was used to model GD2+/S100A6+ cells in vitro.
Results: In NB specimens, GD2 staining was detected in all neuroblastic cells, while S100A6 was seen in the stromal-glial bundles and endothelial cells but also in dispersed neuroblasts. 12 (85%) of 14 diagnostic samples showed GD2+/S100A6+ neuroblasts, being more abundant (15%) in low-risk than (1%) high-risk tumors. GD2+/S100A6+ cells were also identified in some stromal bundles and blood vessels. All bone marrow specimens analyzed showed GD2+/S100A6+ representing less than 10% of the total. By FACS analysis, double stained cells represented 11-53% of total cells correlating with IF results. For MYCN amplified tumors, all FACS-sorted GD2/S100A6 differentially stained subpopulations showed MYCN amplification. Interestingly, during in vitro neuronal induced differentiation of I-type NB cells, rare GD2+ neuroblatic cells with concomitant S100A6+ staining appeared. Subsequently, these GD2+/S100A6+ cells changed morphology, displaying flat and enlarged cytoplasms, distinctive features of the S-like type cell phenotype.
Conclusions: GD2+/S100A6+ neuroblasts may represent a tumoral glial precursor subpopulation in NBT.

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POB2

Integrated analysis of DNA methylation, copy number and mRNA expression identifies novel candidate tumor suppressor genes in neuroblastoma

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Background: Bi-allelic inactivation of tumor suppressor genes has been previously reported in cancer through the concurrent loss of one gene copy through deletion and hyper-methylation of the remaining allele. The purpose of this study was to identify such concurrent events through the integrated analysis of DNA copy number, methylation and mRNA data sets for neuroblastoma (NB) tumors and cell lines.
Methods: A set of primary NB was analyzed by high resolution aCGH (n=160), and a subset (n=18) of these tumors was further investigated using methylated DNA immunoprecipitation applied to microarrays to assess genome-wide promoter hypermethylation status. NB cell lines treated with 5-Aza-2'-deoxycytidine (DAC) were profiled using mRNA expression microarrays (NimbleGen).
Results: Potential bi-allelic gene inactivation events were identified through an integrated analysis of MYCN amplified (MNA) and 11q- tumor sub-groups profiled for DNA methylation and DNA copy number. In total, 29 genes were both hypermethylated and deleted in a minimum of three MNA tumor subtypes while 51 genes were similarly identified in a minimum of three tumors from the 11q- subtype. The majority of genes that were both hemizygoulsy deleted and hypermethylated from the MNA subgroup mapped to either chromosome 1p or 19p, while the majority of such "two hit" genes in the 11q- subtype mapped to either chromosome 11q or 3p. In order to determine if the hypermethylation affecting the hemizygous allele might be functionally significant, we carried out mRNA microarray expression profiling on three different NB cell lines treated with DAC. In total, 37 genes out of the 80 genes (46%) that were concomitantly hypermethylated and deleted in the primary tumors were re-expressed by greater than 1.5 fold in at least one cell line.
Conclusion: Integrated analyses of high resolution DNA copy number, methylation and mRNA expression data is a powerful approach for candidate gene selection in neuroblastoma. Potential targets identified through this strategy will form the basis of a functional studies.

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POB3

Ganglioneuroblastoma, nodular subtype and MYCN amplification: the hospital for sick children experience

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Background: Nodular ganglioneuroblastoma (nGNB) represents ~10% of neuroblastic tumours. Despite its poor prognosis, only 2% of cases carry MYCN amplification. However, the presence of both stroma-poor and stroma-rich components makes biological studies prone to sampling error. We investigated MYCN copy number in stroma-rich and stroma-poor areas, to clarify MYCN amplification role and potential heterogeneity in nGNB.
Method/approach: Patients diagnosed with nGNB between 1987-2008 were identified through Health records. MYCN copy number was assessed by chromogenic in situ hybridization (CISH).
Results: 10 patients were identified and represent this study population. The M/F ratio was 2.3. Although 6/10 were older than 18m at diagnosis, only 2 had metastatic disease. VMA was elevated in 7/9 available cases. The 2 cases with stage 4 disease received chemotherapy and all others were treated with surgery only. 3 patients relapsed (1 with stage 4 disease, 1 with stage 1 multifocal, 1 with stage 2B) at a median time 12.5 months (1.4-31). 2 patients are lost to follow-up, 8 are alive, with (N=2 cases) or without (N=6) disease. Tumour tissue adequate to perform CISH was available for 8 cases. At the single neuroblast level, MYCN copy number was heterogeneous within any given sample and among different patients' samples. Similar results were obtained in ganglion cells (GC). Schwann cells had normal MYCN copy number. In all but one case the increase in MYCN copy number was related to aneuploidy, as determined by FISH using centromere probes for chromosome 1, 2 and 16. One case was defined as MYCN amplification, since the FISH identified a normal diploid number, while MYCN copy number ranged from normal to 5 in neuroblasts, and from normal to 15 in GC. We observed a complete concordance between neuroblasts from nodular areas and GC from stroma-rich areas.
Conclusion: Extensive investigation of MYCN copy number confirms that MYCN amplification is present only in a minority of cases of nGNB and does not account for the poor prognosis observed in nGNB. A genome wide approach by SNP-array is underway to identify genetic changes associated with this subgroup of neuroblastoma.<

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POB4

Interleukin-6-mediated activation of the signal transduction and activator of transcription (stat)3 contributes to chemoresistance and tumor progression in neuroblastoma

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Background: There is recent evidence that STAT3 activation plays a central pro-tumorigenic role in cancer progression as it is constitutively activated in a number of diverse tumors. Here we tested the hypothesis that STAT3 also contributes to neuroblastoma progression.
Method/approach: We analyzed a panel of 14 neuroblastoma cell lines including drug resistant and drug sensitive cell lines for STAT3 activation (phosphorylation) and its effect on drug resistance. STAT3 expression was also examined in metastatic bone marrow samples.
Results: We observed that in most neuroblastoma cell lines STAT3 was not constitutively activated. However we documented that it is rapidly activated (15 minutes) when neuroblastoma cells are treated with IL-6 and its soluble agonistic receptor sIL-6R. Twelve of the neuroblastoma cell lines examined expressed the 2 subunits of IL-6 receptor (IL-6R/gp80 and gp130) but do not make IL-6 and sIL-6R. Activation of STAT3 by IL-6 and IL-6 plus sIL-6R in drug sensitive CHLA255 and SK-N-SH neuroblastoma cells made them resistant to etoposide and melphalan. We then documented that treatment of neuroblastoma cells with IL-6 upregulates the expression of several anti-apoptotic proteins, in particular survivin, Bcl-$_{XL}$, and Bcl-2, and inhibits etoposide-induced cytochrome C released from the mitochondria and activation of the intrinsic apoptotic pathway. The protective effect of IL-6 on drug-induced apoptosis was abolished when the cells were pretreated with stattic (2.5 μM), a small STAT3 inhibitor, or upon STAT3 downregulation by siRNA. We also observed that sIL-6R, which enhances IL-6-mediated STAT3 activation and drug resistance, is produced by monocytes in the presence of neuroblastoma cells. Finally, we found elevated expression of phosphoSTAT3 and survivin in bone marrow specimens of neuroblastoma patients with bone marrow metastasis.
Conclusion: The data indicate that in neuroblastoma STAT3 activation is primarily mediated by the tumor microenvironment through the production of IL-6 and sIL-6R by stromal cells, and point to a critical role for the IL-6/IL-6R/STAT3 pathway in chemoresistance.

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POB5

Differential gene and pathway expression and alternate splicing in high-risk MYCN amplified and non-amplified neuroblastomas. A report from the Neuroblastoma TARGET (Therapeutically Applicable Research to Generate Effective Treatments) Initiative

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Introduction: Genomic amplification of MYCN in neuroblastoma (NB) is associated with an aggressive clinical phenotype. We used transcriptome profiling to identify differential gene and pathway expression and alternate splicing in MYCN amplified (MYCN-A) and MYCN non-amplified (MYCN-NA) high-risk metastatic tumors compared to MYCN-NA low-risk tumors.
Methods: 190 primary NBs from Children's Oncology Group patients were profiled using Affymetrix Human Exon arrays. The high-risk cohort included 52 MYCN-A (H-NBA) and 114 MYCN-NA (H-NBN) tumors, and the low-risk cohort included 24 MYCN-NA tumors (L-NB). Robust multichip analysis (RMA) was used for summarization of probe selection (PSR) and transcript regions. After unsupervised pre-filtering based on coefficient of variation, analyses of variance were conducted to identify differential transcript and isoform expression. Gene set enrichment analyses (GSEA) were conducted using unfiltered transcript data and the Molecular Signature Database's canonical gene sets to identify differentially expressed pathways.
Results: 1,733 of 22,011 core transcripts passed prefiltering criteria, and 1,672 had multiple PSRs (>1 exon). There was a larger number of genes with differential expression between H-NBA vs L-NB than H-NBN vs. L-NB (23% vs 4.5%, P<0.001). There also was a larger number of genes with alternative splicing between H-NBA vs L-NB compared to H-NBN vs L-NB (15.7% vs 1.7% of PSRs; P<0.001). NTRK1, which is known to be alternatively spliced in neuroblastoma, had lower expression in both H-NBA and H-NBN vs L-NB tumors but showed alternative splicing in only H-NBA tumors. GSEA identified more differentially expressed pathways in H-NBA vs L-NB than in H-NBN vs L-NB tumors (70 vs 27; P<0.001), with those related to cell cycle and axon guidance being among the top in the H-NBA vs L-NB tumors.
Conclusion: Differences in gene and pathway expression are identified among H-NBA vs H-NBN tumors, and frequent alternative splicing is shown in H-NBA tumors. These findings enhance understanding of gene expression networks in the two subgroups of high-risk neuroblastomas and reveal possible therapeutic target.

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POB6

Immunogenicity of neuroblastoma - insights from experimental models

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Background: The development of immunotherapeutic approaches to neuroblastoma (NB) has been generally hindered by the assumption of the non-immunogenic nature of this tumor, absence of specific antigens except for GD2 and modest success of therapies aiming to augment immune responsiveness. This particularly poses questions on the conceptual and pragmatic considerations of allogeneic bone marrow transplants (versus autologous), attempting to induce graft versus tumor reactivity (GVT).
Methods and results: As a prevalent model of murine MB, we showed that Neuro-2a cells express MHC antigens at lower, but considerable frequencies as compared to lymphocytes. Accordingly, Neuro-2a (H2Ka) elicit immune responses as effective as A20 murine lymphoblastoma cells (H2Kd) in immunized third party recipients (H2Kb), as demonstrated by direct cytolysis in vitro. This is the likely reason for suppression of tumor growth after allogeneic and haploidentical bone marrow transplants (BMT) as compared to syngeneic BMT. Although MHC antigens are involved in sensitization against NB, the immunogenic targets consist primarily of minor antigens: immunized F1 mice (H2Kb/a) develop cytolytic reactivity against NB as efficient as allogeneic cells (H2Kb). Furthermore, dendritic cells (DC) from congenic (H2Ka), allogeneic (H2Kb) and haploidentical (H2Kab) donors consistently promote anti-NB cytolytic responses in vitro, and reduce tumor growth in vivo.
Conclusions: It is therefore dual involvement of MHC and minor antigens in immune reactivity against NB, which might be used to attain effective GVT in allogeneic transplants. The low immunogenic nature of this tumor originates from multiple mechanisms of evasion from immune surveillance, which are currently under investigation.

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POB7

PHOX2B-mediated regulation of ALK expression in neuroblastoma pathogenesis

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Background and Aims: The heterogeneous and complex genetic etiology of neurobastoma (NB) has been confirmed with the identification of mutations in two genes, encoding for the receptor tyrosine kinase Anaplastic Lymphoma Kinase (ALK) and the transcription factor Paired-like Homeobox 2B (PHOX2B), in a limited proportion of NB patients. These two genes have also been found over-expressed in the majority of primary NB samples and cell lines. These observations led us to test the hypothesis of a regulatory or functional relationship between ALK and PHOX2B lying behind NB pathogenesis.
Methods: The effect of gene expression modulation was studied in vitro in NB cell lines by either siRNA-mediated knock-down or forced-over-expression of each target gene and transcription levels were evaluated by realtime RT-PCR and Western blot. PHOX2B binding to ALK promoter was detected by EMSA and is being confirmed by ChIP.
Results: Following the hypothesis of a cross-talk between PHOX2B and ALK, we confirmed a striking correlation between the transcription levels of ALK, PHOX2B and its direct target PHOX2A in a panel of NB cell lines. We observed that the PHOX2B- and PHOX2A-siRNAs efficiently down-regulated each other as well as the ALK gene while ALK down-regulation did not affect PHOX2 transcripts in NB cells. We also demonstrated that PHOX2B drives ALK gene transcription by directly binding its promoter, thus establishing ALK as a novel PHOX2B target gene.
Conclusion: The new finding of a concurrent involvement of ALK and PHOX2B genes in NB pathogenesis provides new insights into molecular biology of NB and opens new perspectives to design novel combined therapeutic approaches.

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POB8

Natural histone deacetylase inhibitor, sulforaphane, inhibits growth and survival of human neuroblastoma

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Sulforaphane(SFN), an isothiocyanate, found in cruciferous vegetables inhibits HDAC activity in colon and prostate cancer cell lines leading to growth retardaion and apoptosis. We determined the effect of SFN on short and long term growth and survival of human neuroblastoma (NB) and asked whether it involved HDAC inhibition. NB cell line(NUB-7), fetal lung fibroblasts (FLF; negative control) and breast cancer line MCF-7(positive control), were treated with 0.6-40uM SFN up to 7 days. Growth and cytotoxicity were determined by AlamarBlue and trypan blue exclusion assays. Proliferation,cell cycle, differentiation and induction of apoptosis were assessed using Ki67, phosphoH3,NF(150) staining, and PI/FACs respectively. Methylcellulose clonogenic assay and xenograft studies were used to determine the effect of SFN on NUB-7 tumorigenic potential. After 48-72h in 10u MSFN, NUB-7 showed a significant cytotoxic effect and decrease in growth. Longer 7d treatment with SFN(2.5uM,5uM, and 10uM) reduced survival by 87%,44%, and 11% ($p<0.05$) respectively, compared to MCF-7 inhibition of 82%-27%($p<0.01$) after 48h in 2.5-10uM SFN. Conversely, 2.5-10uM SFN had no significant effect on FLF cell proliferation. Reported 24h treatments of NUB-7(2.5uM,5uM, and 10uM SFN) over 7d showed G1,G2 and S phase cell cycle arrests that were concentration and time dependent. Increases in histone acetylation using anti-Histone H3/H4 were confirmed by immunofluorescence labeling and correlated with decrease in Ki67 staining. Clonogenic assay revealed a marked decrease in colony formation. SFN pretreated showed a significant decrease (87% at 10 uM;$p<0.01$) in NUB-7 tumorigenicity in nude mice. SFN administered i.p. inhibited significantly growth of established NUB-7 xenografts. Thus SFN at low doses reduced proliferation, survival and tumorigenicity of NB NUB-7 cells. The effects correlated with SFN inducible HDAC inhibitory activity in vitro and in vivo and suggest that such a mechanism might contribute to the cancer chemopreventive and therapeutic of SFN. As a dietary factor of very low intrinsic toxicity SFN alone or as an adjunct to other treatment should be considered for clinical evaluation.

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POB9

Promising effects of the PI3K/mTOR inhibitor PI-103 with currently applied chemotherapeutic drugs on neuroblastoma cell lines

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Background: Neuroblastoma is a childhood tumor with a poor prognosis and therefore, new therapeutical options are needed. The PI3K/AKT/mTOR pathway is a potent survival-signaling pathway that is aberrantly activated in a variety of human cancers. We investigated the efficacy of the PI3K/mTOR inhibitor PI-103 and determined if inhibition of the PI3K/AKT/mTOR pathway sensitized neuroblastoma cells towards currently applied chemotherapeutic drugs.
Method/approach: Six human neuroblastoma cell lines, three MYCN amplified and three MYCN single copy, were treated with topotecan, gemcitabine, cisplatin, etoposide or doxorubicine alone or combined with 4hr preincubation of PI-103 (80 or 400 nM). Viability in monolayers was measured using the MTS assay and dose-effect curves were generated to perform multiple drug-effect analysis. A combination index (CI) < 1.1 indicates a synergistic/additive effect of the combination. The effect of PI-103 was also studied in cells growing in spheroids, a three dimensional tumor model and as such a model for micrometastases. Spheroids were treated for 14 days with PI-103 (0 - 600 nM).
Results: In all six neuroblastoma cell lines growing in monolayers a dose- and time-dependent decrease in viability after treatment with PI-103 or currently applied chemotherapeutics was observed. The IC50 values for PI-103 ranged from 130 - 960 nM for the different cell lines. PI-103 showed an inhibiting effect on tumor-spheroids growth (IC50 = 400 nM). A synergistic effect in monolayers was observed for most combinations, with the most pronounced effect for the combination topotecan - PI-103 (CI< 0.65).
Conclusion: Our results showed that the combination of PI-103 with five currently applied chemotherapeutic drugs has a mainly synergistic effect with respect to viability in most neuroblastoma cell lines growing in monolayers, in particular for PI-103 combined with topotecan. Profound growth inhibition of spheroids incubated with PI-103 was observed. Therefore, combinations of PI-103 with currently applied chemotherapeutic drugs might be a promising new strategy in treatment of neuroblastoma.

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POB10

Expression of the B-cell-activating factor BAFF and its receptors in opsoclonus-myoclonus associated neuroblastoma

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Background/aims: Opsoclonus-myoclonus syndrome (OMS) is a rare paraneoplastic disorder, often associated in childhood with neuroblastoma (NB). In these patients, NB presents as localized tumor with favourable prognosis. The pathogenesis of OMS is still unknown but the detection of autoantibodies and lymphocytic infiltration in NB samples is suggestive of an ongoing autoimmune response. BCMA (B-cell maturation antigen), and BAFF-R (B-cell activating factor-Receptor) are members of the Tumor Necrosis Factor Receptor Superfamily (TNFRSF) that promote B-cell growth and survival upon interaction with BAFF ligand. Aim of this study was to investigate the role of BAFF, BCMA and BAFF-R in ectopic lymphoid neogenesis of OMS-associated NB.
Methods: Expression of BAFF, BAFF-R and BCMA was evaluated by immunohistochemistry in paraffin embedded specimens of NB patients associated or not with OMS by using Envision System Horse Radish Peroxidase (HRP) (Dako). In vitro expression of BAFF, BAFF-R and BCMA was analyzed by flow cytometry in different NB cell lines before and after treatment with Retinoic Acid or Interferon-γ (IFN-γ). Secretion of BAFF by NB cells was investigated by ELISA kit. In vitro effects of recombinant (r)BAFF were tested by counting the cells with Trypan Blue dye.
Results: BAFF and BCMA were detected both in tumor cells (neuroblasts and ganglion-like cells) and infiltrating B lymphocytes from NB patients with or without OMS. In contrast, BAFF-R was expressed only by lymphoid cells. Similarly, BAFF and BCMA, but not BAFF- R, were expressed in a large panel of NB cell lines. BAFF, BCMA and BAFF R expression was up regulated by differentiating agents such as RA and IFN-γ. Finally, BAFF was never secreted in NB cells supernatants. Preliminary experiments suggested that rBAFF did not support proliferation or survival of NB cells.
Conclusion: This study demonstrates that BAFF and their receptors are expressed in tumor tissues from NB patients with or without OMS, and are likely involved in attraction and survival of tumor-infiltrating B cells.

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POB11

Role of ATP and myeloid-derived suppressor cells in neuroblastoma microenvironment

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Background/Aim: The biochemical composition of tumor microenvironment is crucial for the modulation of cancer cell growth as well as for the functions of immune cells. Recent findings have shown that solid tumors have an increased concentration of adenosine and extracellular ATP that may exert a pivotal role in the regulation and homeostasis of immunosuppressive cell populations. Aim of this study was to investigate whether:
i) extracellular ATP is a component of neuroblastoma (NB) microenvironment,
ii) myeloid-derived suppressor cells (MDSC) are involved in NB cell growth
Methods: The NXS2 murine NB cell line was stably transfected with plasma membrane luciferase (pmeLUC) probe and inoculated in the tail vein or orthotopically in the adrenal gland of A/J mice. Bioluminescence imaging (BLI) was used to detect extracellular ATP in living animals. MDSC were phenotypically characterized in the blood, bone marrow (BM), spleen and tumor from healthy and NB-bearing mice by cytometric analysis of CD11b, Gr-1, F4/80, IL-4 receptor (r) and CD62L expression. Proliferation of unstimulated and CD3-stimulated splenocytes from healthy and NB-bearing mice was measured by 3H-thymidine incorporation.
Results: Extracellular ATP was specifically detected in the tumor microenvironment of NB bearing mice compared to healthy tissues. The percentage of CD11b+, Gr-1intermediate/low + cells was found to be higher in the spleen, BM, and blood from NB bearing mice compared to healthy animals. In addition, higher proportions of CD11b+, Gr-1+, IL-4r+ were found in the blood and BM from NB bearing mice vs healthy animals. In contrast, lower percentages of CD11b+, Gr-1+, F4/80+ and CD11b+, Gr-1+, CD62L+ were detected in the blood and BM from NB bearing than control mice. Finally, splenocytes from NB bearing mice proliferated less than those from healthy animals. Preliminary experiments indicate that two MDSC cell lines express the functional purinergic receptor P2X7, through which ATP may be released.
Conclusion: Extracellular ATP and functional MDSC were found to be a relevant component of NB microenvironment and may modulate NB cell growth.

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POB12

Non-transcriptional role of MYC and genomic rearrangements in neuroblastoma

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Background: The primary focus of cancer related MYCN and c-MYC research (hereafter called MYC) has been the identification of MYC transcriptional target genes. MYC also controls DNA replication origin activity through interaction with the pre-replicative complex. Still, non-transcriptional function of MYC in tumorigenesis is poorly understood. Aim of the study is to understand the role of MYC at replication origins in the context of fragile sites and genomic instability.
Method/approach: To localize coincidences of MYC binding to DNA, replication origins and double-strand breaks chromatin immunoprecipitation (ChIP) was performed in c-MYC (SH-SY5Y, SJ-NB12) and MYCN (Kelly) expressing cell lines. Antibodies against MCM3 and ORC2 (replication origin markers); MYCN and c-MYC as well as pH2AX (double-strand break marker) were used. ChIP enriched DNA fragments were hybridized to a fine tiling array, representing chromosome 2p. The correlation between MYC triggered replication origins and genomic rearrangements on 2p was determined.
Results: For MYCN (Kelly) number of binding peaks on 2p were 2908 and for c-MYC 1799 (SH-SY5Y) and 2557 (SJ-NB12). MYCN binding mapped to replication origins at 16 regions (Kelly), c-MYC binding mapped to replication origins at 64 regions (SH-SY5Y) and 256 regions (SJ-NB12). Phosphorylated H2AX coincided with replication origins that were bound by MYC at 13 regions in Kelly (MYCN), at 29 regions in SH-SY5Y and at 123 regions in SJ-NB12 (c-MYC). The genomic overlap of marker binding for replication origin formation and double strand breaks as well as MYC binding mapped to characteristic genomic rearrangements in NB cell lines, in Kelly ($p=0.006$), SH-SY5Y ($p<0.001$) and SJ-NB12 ($p<0.001$). These genomic rearrangements coincide with fragile sites on 2p.
Conclusion: Collectively, number of MYC binding correlated with the genetic background of the cell lines. Correlation analyses suggest that MYC triggered origin formation leads to genomic rearrangements. In consequence, deregulated MYC may contribute to tumorigenesis in a non-transcriptional manner by causing genomic instability in cancer cells.

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POB13

Two common fragile sites *FRA2Ctel* and *FRA2Ccen* map to the borders of *MYCN* amplicons in neuroblastoma

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Background: Common fragile sites (cFS) represent chromosomal regions that are prone to breakage after partial inhibition of DNA synthesis. They are associated with various forms of DNA instability in cancer cells, and cFS activation is thought to be an initiating event in the generation of DNA damage in early stage tumorigenesis. One well-documented genomic alteration departing from cFS is DNA amplification in cancer cells, but its molecular basis remains poorly understood.
Method/approach: The main focus of this study is to map the cFS *FRA2C* distal of chromosome 2 using six-color FISH and the analysis of its correlation to *MYCN* amplicon boundaries in neuroblastoma (NB) cell lines and primary tumors by array CGH.
Results: *FRA2C* consists of two cFS spanning 747 kb (*FRA2Ctel*) and 746 kb (*FRA2Ccen*) at 2p24.3 and 2p24.2, respectively. Both cFS flank a 2.8 Mb non-fragile region containing *MYCN*. *FRA2Ctel* and *FRA2Ccen* are highly enriched in flexibility peak domains compared to non-fragile regions. A perfect 23-23.5 bp (AT/TA) stretch spans the maximum flexibility peak domain in *FRA2Ccen*. *MYCN* amplicon borders and gains clustered in *FRA2Ctel* or *FRA2Ccen* in approximately 70% of NB cell lines and primary tumors.
Conclusion: These observations raise the possibility that initiation of *MYCN* amplification is related to the activation of *FRA2Ctel* and/ or *FRA2Ccen*.

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POB14

Expression and clinicla relevance of melanoma-associated antigens in neuroblastoma

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The high molecular weight melanoma-associated antigen (HMW-MAA) and the cytoplasmic melanoma-associated antigen (cyt-MAA) are expressed by melanoma cells; their serum levels are increased in melanoma patients, and correlate with clinical outcome.
The expression of these molecules was evaluated by flow cytometry in Neuroblastoma (NB) cell lines and in short-term cultures of metastatic neuroblasts (mNB) from NB patients' bone marrow. cyt-MAA was strongly expressed in 3 out of 5 NB cell lines and in 3 out of 5 mNB. Its expression was weaker in 2 NB cell lines and undetectable in 2 mNB. HMW-MAA was strongly expressed only in 1 out of 5 NB cell lines and in 2 out of 5 mNB. Its expression was weaker in 2 out of 5 NB cell lines and in 3 out of 5 mNB, and undetectable in 2 NB cell lines. Release of CYT-MAA was evaluated by ELISA. CYT-MAA was released in vitro by 5 NB cell lines and by 3 mNB.
Serum levels of cyt-MAA were significantly higher in NB patients (median 23,86 ± 2,59 U/ml) than in healthy donors (median 5,73± 3,12 U/ml, $p=0.0005$). The incidence of relapse was significantly higher in patients with serum levels of cyt-MAA >25 U/ml ($p=0.0041$).
Serum levels of HMW-MAA were higher in NB patients (median 2.65 ± 1.2 U/ml) than in healthy controls (median 0.86 ± 0.15 U/ml, $p=0.035$), without correlations with clinical outcome.
CYT-MAA expression was next evaluated by RT-PCR analysis on i) freshly isolated GD2+ cells from 5 NB patients, ii) mNB from 5 NB patients; iii) 5 stroma poor NB samples and iv) 5 stroma rich ganglioneuroblastomas. CYT-MAA was expressed in stroma-rich ganglioneuroblastoma but not in stroma poor samples, suggesting that CYT-MAA is released predominantly by stromal cells. Moreover, metastatic GD2+ cells freshly isolated by NB patient's bone marrow expressed low levels of CYT-MAA. The expression is significantly up-regulated after few passages of colture in vitro.
We conclude that HMW-MAA and cyt-MAA are expressed by NB cell lines and metastatic NB cells, and serum levels of these molecules are increased in NB patients. High serum levels of cyt-MAA correlate with a poorer clinical outcome of NB patients.

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POB15

Heterogeneous *MYCN* amplification - amplicon, genomic background and genome instability

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Background: Among 110 *MYCN* amplified (MNA) neuroblastomas (NB) analyzed at the CCRI in the last decade, 26 tumors contained a varying number of tumor cells/areas without MNA, defined as heterogeneous *MYCN* amplification (hetMNA) according to INRG biology guidelines. Since the genomic background of hetMNA tumors has not yet been described, we looked for common segmental and/or numerical chromosome aberrations (SCA and/or NCA) and genetic instability as initiators for amplicon formation.
Methods: Up to 4 tumor pieces of the same hetMNA tumors from 5 patients were cryosectioned. In total, 13 pieces were analyzed by interphase FISH (I-FISH) and their DNA was analyzed by MLPA and 250k SNP arrays to investigate the genomic background of hetMNA tumors. *MYCN* I-FISH was performed to detect *MYCN* heterogeneity and combined with *DDX1* probes to assess amplicon composition. γH2AX staining and subsequent *MYCN* FISH was performed to correlate DNA double-strand breaks (DSB) occurrence with MNA. Quantification of signals on sections was done with an automatic device.
Results: All analyzed hetMNA tumors showed NCAs, 2 samples from 2 patients each showed only NCAs, 6 pieces from 4 tumors from 2 patients displayed a variety of inconsistent, additional SCAs, and 3 of 4 pieces from a patient had only NCAs. Numerous chromosomes were unitarily found overrepresented and chromosome 19 was the only disomic one. MNA was confirmed in 4 tumor pieces of 4 patients by MLPA/SNP arrays, the fifth showed MNA only in its *MYCN* FISH results. One tumor revealed different amplicon compositions in the same piece and another showed two 1p-breakpoints in different pieces. γH2AX staining pattern only partially correlated with MNA cells.
Conclusion: Our data indicates the existence of multiple tumor cell clones and different amplicons in a single hetMNA NB. At the applied resolution, no common SCA is found to be underlying MNA (subtle genomic changes and mutations not tested for). A conclusion on genetic instability as a cause for MNA is premature. Our data strongly supports a detailed tumor work up and the use of both I-FISH and DNA-based techniques as recommended by the INRG.

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POB16

Immunocytochemical study of bone marrow in neuroblastoma patients - Polish experience

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The presence of disseminated neuroblastoma cells in bone marrow is important for clinical staging and risk assessment at diagnosis and for therapy monitoring. Reliable detection of tumor cells in bone marrow may be factor of great prognostic significance.
In Department of Pediatric Oncology/Hematology in Krakow, from 1 October 2006 to 31 January 2010, frequency of GD2-antigen occurrence were analyzed in 138 bone marrow samples from 41 NBL patients. Among all 41 patients evaluated at diagnosis, 23 (56%) had GD-2 positive bone marrow immunocytology. In 17 patients, NBL cells were found in classic cytological evaluation. In all cases immucytology confirmed bone marrow infiltration. In all patients without evidence of NBL cells in immunochemistry, there were no blasts found on smears. However, in 6 patients with bone marrow involvement found in immunocytochemistry, classical cytology did not reveal any pathological cells. The number of positive cells in immunocytochemistry ranged between 1 and 180 out of total 1x106 total cell analyzed. The concordance of evidence of bone marrow metastases in classical cytology with bone marrow immunocytology was 84% (44% - negative in both methods, 40% - positive and 16% - discordant between methods), with higher sensitivity of immunocytochemistry.
Despite the fact that cytomorphological screening of bone marrow smears is still the only accepted method for the detection of disseminating neuroblastoma cells, there is a need for checking prognostic value of immunocytchemical assay.

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POB17

***ChIPaway*: A tool for visualization and analysis of high-throughput microarray based immunoprecipitation data**

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Background: The development of high-throughput microarray based techniques have allowed the routine generation of vast amounts of biological data. The current software pipeline for analysing data generated from high-throughput array based immunoprecipitation experiments such as chromatin immunoprecipitation (ChIP) and methylated DNA immunoprecipitation (MeDIP) is generally limited to peak calling and provides limited support for genome-wide visualization and for further downstream analysis such as experimental cross analysis, inter-class comparisons and marker selection. In the course of our research into the involvement of the DNA binding protein MYCN and the impact of DNA methylation in neuroblastoma we have developed a software application called ChIPaway which aims to fulfil the above needs.
Methods: ChIPaway is a user friendly, cross platform java based, point and click application that allows the user to visualize, interact and compare multiple experimental samples both from the perspective of raw enrichment values and post processed peak data.
Result: As well as basic functionality such as export and import of various formats; genomic visualization and annotation; and co-ordinate and gene name based search methods, ChIPaway also enables rapid assessment of replicates and comparison of peaks generated across alternative sample conditions. Furthermore, ChIPaway also allows comparison between sample classes using machine learning techniques such as cluster analysis and feature selection methods to aid in the discovery of disease markers.
Conclusion: ChIPaway has been developed to support several studies into DNA binding and methylation in neuroblastoma. Ultimately, ChIPaway allows researchers to assess many genome scale immunoprecipitation experiments simultaneously and rapidly distil these data into usable information to support understanding of disease mechanism and marker selection.

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POB18

Genome-wide DNA methylation profiling reveals extensive and complex epigenetic alterations in neuroblastic tumors

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Background: Although a number of studies have reported aberrant methylation and inactivation of selected genes in neuroblastoma (NB), the extent of genome-wide promoter hypermethylation is poorly understood. We have applied methylated DNA immunoprecipitation (MeDIP) to genomic microarrays representing all known promoter and/or CpG islands in the human genome to more fully characterize the epigenome of neuroblastic tumors.
Methods: MeDIP analysis was applied to NB primary tumors (n=18), cell lines (n=7), ganglioneuroblastoma (GNB) (n=4) and ganglioneuroma (GN) (n=6).
Results: The total number of hypermethylated sites per sample ranged from 1,462-5,197). Consistent differences in DNA methylation patterns were identified between cell lines and tumor subtypes, indicating that epigenetic changes play a significant role in adapting cells to in vitro proliferation. Unsupervised hierarchical clustering of methylation data revealed a distinct split between the GN/GNB and NB groups. mRNA microarray expression analyses of cell lines following treatment with 5'-aza-2-deoxycytidine allowed us to explore the functional significance of the hypermethylation. The number of genes which were consistently hypermethylated in the GN/GNB group relative to NB was far greater (199 genes) than the opposite comparison (2 genes). Gene ontology analysis carried out on genes hypermethylated in >90% of GN/GNB displayed a statistically significant enrichment for protein kinases, growth factors and mitosis. In total, 70 recurrent large-scale blocks of contiguously hypermethylated promoters/CpG islands were identified, consistent with other studies of breast and colon cancer. The size of these regions ranged from 12.5 kb to 590.5 kb, with a mean length of 96.4 kb, with nearly one-third of the blocks clustering within telomeric regions.
Conclusion: Our results indicate that genome-wide hypermethylation in neuroblastic tumors is highly complex and plays important roles in many cellular processes, including in vitro cell growth and differentiation. We also identify many candidate genes which are potentially silenced through methylation and which will form the basis of functional studies.

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POB19

Identification of epigenetically regulated genes that predict patient outcome in neuroblastoma

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Background: Epigenetic mechanisms such as DNA methylation and histone deacetylation are important regulators of gene expression and are frequently involved in silencing tumour suppressor genes. The aim of this study is to detect genes abnormally methylated in neuroblastoma, using genome-wide approaches.
Method/approach: In order to identify genes that are epigenetically regulated in neuroblastoma tumors we treated four neuroblastoma cell lines with the demethylating agent 5-Aza-2'-deoxycytidine (5-Aza-dC) separately and in conjunction with the histone deacetylase inhibitor trichostatin A (TSA). Expression was analyzed using whole genome expression arrays (Illumina Human-6 v2 Expression BeadChip) to identify genes that were activated by treatment. These data were then combined with data from whole-genome DNA methylation arrays (Illumina Human Methylation27 DNA analysis BeadChip) to identify candidate genes silenced in neuroblastoma due to DNA methylation.
Results: We present eight genes (*KRT19*, *PRKCDBP*, *SCNN1A*, *POU2F2*, *TGFBI*, *COL1A2*, *DHRS3* and *DUSP23*) that are methylated in neuroblastoma, most of them not previously reported as such, some of which also distinguish between biological subsets of neuroblastoma tumours. A high methylation frequency of *SCNN1A*, *PRKCDBP* and *KRT19* is significantly associated with adverse outcome in neuroblastoma.
Conclusion: Identification of biomarkers will be important in risk stratification of patients with neuroblastoma in the future and for this, methylation status of genes would be a suitable marker. It is essential to make the best prognosis possible to ensure that patients receive the best available treatment, as well as to avoid unnecessary treatment, which can lead to severe side effects in this group of young patients.

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POB20

Factors affecting the outcome of the p53 mediated DNA damage response in neuroblastoma

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Background: *MYCN* oncogene amplification occurs in 25-30% of neuroblastoma and is associated with poor prognosis. We previously reported that *MYCN* amplified (MNA) p53 wild-type neuroblastoma cell lines failed to G_1 arrest in response to irradiation, but this could not be attributed to MYCN alone. We hypothesised that a) Genes co-amplified with MYCN and/or b) the predominant cell type (N or S) determines the downstream response to DNA damage in neuroblastoma cell lines.
Method/approach: The *MYCN* amplicons of five MNA and two non-MNA cell line were mapped using 50K SNP arrays. One MNA (NBL-W) and one non-MNA neuroblastoma cell line (SKNSH) were sub-cloned into N-type and S-type and characterised for expression of BCL-2 and Vimentin, markers for N and S type cells respectively. The response to 4Gy irradiation induced DNA damage was assessed using western blotting for expression of p53 regulated genes, FACS analysis for cell cycle arrest and a sub-G_1 peak for apoptosis.To determine the role of p53 in this process p53 was knocked down using siRNA.
Results: No genes with a potential role in cell cycle regulation were consistently co-amplified in the MNA cell lines excluding a role for co-amplified genes. Mixed N and S type non-MNA SKNSH cells underwent a G_1 arrest following irradiation and mixed N and S type MNA and high MYCN expressing NBL-W cells failed to G_1 arrest following irradiation. N-type SHSY5Y and NLBLW-N cells failed to G_1 arrest in response to DNA damage, and showed impaired induction of p21 and MDM2, whereas S-type SHEP and NBLW-S cells did undergo a G_1 arrest with induction of p21 and MDM2. MYCN was downregulated in NBLW-S cells suggesting that the effect of MYCN cannot be excluded. Conversely N type cell lines underwent higher levels of apoptosis than S type cell lines. Following p53 knockdown in SHSY5Y N-type cells there was a decrease in apoptosis confirming the partial dependence of this effect on p53.
Conclusion: The downstream response to DNA damage in p53 wild-type neuroblastoma cell lines is p53 dependent, and determined predominantly by the morphological sub-type.

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POB21

Characterization of *ALK* rearrangements in neuroblastoma

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Background: Neuroblastoma (NB) is an embryonal cancer of the peripheral autonomous nervous system. Recently, activating mutations of the *ALK* gene, highly expressed in NB, have been identified in sporadic and familial NB cases. The *ALK* gene encodes a tyrosine kinase receptor that is preferentially expressed in the central and peripheral nervous system during embryonic development. Translocations, leading to fusion proteins containing the tyrosine kinase domain of ALK and involving various partners, have already been shown to be oncogenic in several human cancers, including anaplastic lymphomas, myofibroblastic tumors and a subset of lung carcinomas.
Method/approach: Chromosomal rearrangements were searched by Comparative Genomic Hybridization experiments using an oligonucleotide array covering the entire *ALK* gene. Inverse PCR experiments were then performed to identify the sequences involved at the breakpoint junctions. In one cell line a NGS (Next Generation Sequencing) strategy was performed to characterize chromosomal rearrangements.
Results: Rearrangements within the *ALK* gene have been identified in several cell lines and tumors. These rearrangements consist in partial gains or amplifications of *ALK*, with breakpoints occurring in introns at various positions. Different types of rearrangements have now been characterized, including coamplifications and inverted duplication structures. In one cell line, exhibiting multiple breakpoints within the *ALK* gene, NGS allowed to pinpoint several of these breakpoints. Further Northern blot experiments will allow to determine whether the rearranged alleles are expressed. Moreover, a protein variant of ALK, identified in a NB cell line, is currently being analyzed by mass spectrometry in order to fully characterize its structure.
Conclusion: The complete structural and functional characterization of these rearrangements will allow to determine whether they lead to an oncogenic property of *ALK* in NB. Such abnormalities of the *ALK* gene may constitute an alternative mechanism to ALK activation, therefore implicating the ALK receptor in a higher

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POB22

Clusterin interacts with HSP60: implications in neuroblastoma development

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Background: Nuclear factor kappa B (NF-κB) is a transcription factor with an established role in the cellular inflammatory immune response and is constitutively activated in different types of human cancers. Previously, a highly conserved heterodimeric sulfated glycoprotein known as Clusterin (CLU) was shown to be a negative regulator of NF-κB activity in neuroblastoma. Suppression of CLU could elicit NF-κB activation and increase markers for the epithelial-to-mesenchymal transition (EMT) process in a MYCN transgenic neuroblastoma model.
In our laboratory, we have shown that CLU expression is negatively regulated by the proto-oncogene MYCN, which is widely associated with aggressive types of neuroblastoma tumours. Therefore, we hypothesized that CLU is a tumour and neuroblastoma suppressor protein and aim to investigate the molecular mechanisms by which CLU regulates NF-κB activity in neuroblastoma.
Methods: Luciferase assay was used to investigate which CLU subunits control NF-κB activity. CLU-interacting proteins were identified and confirmed using GST-pull down assay, Mass-Spectrometry and Co-immunoprecipitation.
Results: Only the intracellular form of CLU (cytoplasmic CLU) showed to be a negative regulator of NF-κB activity with the most effective suppression of NF-κB activity within the alpha chain region near the N-terminal. Extracellular CLU (secreted CLU), on the other hand, is not involved in the regulation of NF-κB activity but appeared to be a specific key regulator of AKT activation in the Phosphoinositide-3 Kinase (PI3K) survival pathway.
Data from GST-pull down assay, mass-spectrometry analysis and co-immunoprecipitation demonstrated that a specific chaperone protein named Heat Shock Protein 60 (HSP60) directly interacts with the N-terminal region of intracellular CLU. HSP60 levels correlate with poor neuroblastoma patients' survival.
Conclusions: This report is the first to describe a novel and direct interaction between intracellular CLU and HSP60, which may play an important role in the regulation of NF-κB activity during neuroblastoma development.

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POB23

Human mesenchymal stromal cells (hMSCs) enhanced migration and invasion of neuroblastoma cells via SDF-1/CXCR4 and SDF-1/CXCR7 axes

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Background: Neuroblastoma is one of the cancers that commonly invade the bone and bone marrow. The SDF-1/CXCR4 axis has been proposed as an important pathway involved in bone marrow metastasis for various cancers including neuroblastoma. But the role of hMSCs & CXCR7, the other known receptor for SDF-1, in metastatic neuroblastoma is yet to be explored.
Materials and Methods: In this study, we first used a chemokines miniarray to screen for the chemokines secreted by the hMSCs and confirmed that SDF-1 was one of the secreted factors. W then collected the serum poor conditioned media from hMSC cultures derived from 3 different healthy donors (stored hMSCs previously with IRB approval and written informed consent) and evaluated their SDF-1 levels respectively by ELISA. The expression of CXCR4 and CXCR7 on 5 known neuroblastoma cell lines (BE2C, BE2M17, IMR32, SK-N-LP & SY5Y) with metastatic potential was then determined by flow cytometer. We then performed migration and invasion assay with hMSCs and neuroblastoma co-culture under a transwell system.
Results: SDF-1 can be found in all hMSCs serum poor culture media. Flow cytometry analysis revealed that all of the 5 neuroblastoma cell lines expressed CXCR7, and 4/5 expressed CXCR4. We then found that the migration and invasion of neuroblastoma cells was enhanced by MSCs co-culture or SDF-1 under the transwell migration & invasion assay. The migration efficiency of neuroblastoma in response to hMSCs conditioned media and SDF-1 treatments was considerably higher than that of the control medium (n=3, $p<0.01$), and such effect could be significantly but not totally blocked by AMD3100, an inhibitor of CXCR4.
Conclusion: Our preliminary data suggested that hMSCs played a significant role in the metastatic potential of neuroblastoma possibly through the release of SDF-1. SDF-1 might act on both CXCR4 and CXCR7 and the blocker of CXCR4 could significantly but not totally block the migration and invasion of neuroblastoma cells.

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POB24

Alterations of NDP kinase A/ NM23-H1 deregulate c-myc transcription

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Altered expression and mutations of NDP kinase A (NDPK-A), encoded by nm23-H1, have been detected in patients with metastatic tumors. One of new functions of NDPK-A is to participate in gene regulation. However, its role in regulating c-myc transcription remains unclear. Here we demonstrate for the first time that NDPK-A specifically bound nuclease hypersensitive element (NHE) III_1 of the c-myc promoter in vitro and in vivo. Neuroblastoma metastasis-associated S120G mutation of NDPK-A (NDPK-A^{S120G}), but not S120G and N82S double mutations (NDPK-$A^{S120G/N82S}$), retained the DNA-binding activity. In human NB69 neuroblastoma and HeLa cervical cancer cells, a high level of ectopic NDPK-A or NDPK-A^{S120G} suppressed c-myc transcription based on the CAT reporter and RT-PCR. In contrast, shRNA-mediated knockdown of NDPK-A enhanced c-myc transcription. Without the DNA-binding activity, however, NDPK-$A^{S120G/N82S}$ did not display an effect on c-myc transcription. NDPK-A appears to exert transcriptional suppression via not only NHE III_1 but also upstream cis-element(s) of c-myc promoter. The role of NDPK-A in regulating c-myc transcription was supported by nuclear localization of NDPK-A in cells and by negative correlation of NDPK-A and c-myc mRNA levels in several human cancer subtypes. Our findings provide a link between NDPK-A alterations and c-myc transcriptional deregulation in cancer.

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POB25

Overexpression of β1,4-N-acetylgalactosaminyltransferase III suppresses the malignant phenotype of neuroblastoma cells via β1-integrin signaling

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β1,4-N-acetylgalactosaminyltransferase III (B4GALNT3) promotes formation of GalNAcβ1,4GlcNAc (LacdiNAc or LDN). Drosophila β1,4-N-acetylgalactos- aminyltransferase A (B4GALNTA) contributes to synthesis of LDN that helps regulate neuronal development. In this study, we investigated the expression and role of B4GALNT3 in human neuroblastoma (NB). We examined 87 NB tumors by mmunohistochemistry to determine correlations between B4GALNT3 expression and clinicopathologic factors including patient survival. Effects of recombinant B4GALNT3 on cell behavior and signaling were studied in SK-N-SH NB cells. We found that increased expression of B4GALNT3 in NB tumors correlated with differentiated histologies ($P < 0.001$) and early clinical stage ($P = 0.041$). B4GALNT3 expression was an independent favorable prognostic factor for survival by both univariate and multivariate analyses. Reexpression of B4GALNT3 in SK-N-SH cells suppressed cell proliferation, colony formation, migration, and invasion. Moreover, B4GALNT3 increased LacdiNAc inβ1 integrin leading to decreased phosphorylation of focal adhesion kinase (FAK), Src, paxillin, Akt and ERK1/2. Effects of B4GALNT3 to suppress cell migration and invasion were substantially reversed by concomitant expression of constitutively active Akt or MEK. We conclude that B4GALNT3 is a useful prognostic factor for NB and that B4GALNT3 suppresses the malignant phenotype via decreasing β1 integrin signaling.

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POB26

The role of protein tyrosine-phosphatases in neuroblastoma

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Neuroblastoma (NBL) is the most common and deadly extracranial solid tumour of childhood, accounting for approximately 7-10% of malignancies in patients under the age of 15, and 10-15% of paediatric oncology deaths. Although the causes of NBL are unclear, its formation and progression are likely governed by a number of signaling pathways. One of the key events in cell-cell signalling is reversible phosphorylation of tyrosine-residues on proteins, controlled jointly by protein tyrosine kinases (PTKs) – which phosphorylate proteins, and protein tyrosine phosphatases (PTPs) – which dephosphorylate proteins. There is past evidence that PTPs may have diverse influences over NBL cells. For instance, the inhibition of PTPs has been shown to induce either cell cycle arrest or neuritogenesis in NB41 or SH-SY5Y NBL cell lines, respectively. In contrast, evidence suggests that the PTPRD gene that encodes PTP∂ is frequently deleted in NBL, suggesting that some PTPs may potentially harbour tumour-suppressive functions.
We tested the hypothesis that PTP inhibition with sodium orthovanadate (VA) would induce differentiation and suppress the growth of a range of NBL cell lines, while combination treatment with retinoic acid (RA) would increase these effects. We show that treatment with VA decreased proliferation rates, and caused subtle cell-line specific changes to the cell cycle distribution of 4 NBL cell lines. Treatment with RA, but not VA caused an increase in the percentage of cells in G0, as shown by a reduction in the expression of the proliferative marker Ki67. Furthermore we showed that treatment with VA induced significant increases in neuritogenesis, a cell-structural parameter of differentiation, and that combination treatment with VA and RA greatly enhanced neuritogenesis beyond the effect of either chemical alone. A general inhibitor of PTPs therefore can induce a more differentiated phenotype in a range of NBL cell lines and can enhance the effects of RA.

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POB27

Identification of hypoxia signatures in neuroblastoma cell lines by l1-l2 regularization and data filtering

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Background: Gene expression signatures are clusters of genes discriminating different statuses of the cells and their definition is critical to understand the molecular bases of diseases. The identification of a gene signature is complicated by the high dimensionality of the data and by the heterogeneity of cells response. The l1-l2 regularization is an embedded feature selection technique that fulfills all the desirable properties of a variable selection algorithm with the potential to generate a gene signature even in biologically complex settings. We applied this algorithm to detect the signature characterizing the transcriptional response of neuroblastoma tumor cell lines to hypoxia, a condition of low oxygen tension that occurs in the tumor microenvironment.
Method/approach: We determined the gene expression profile of 9 neuroblastoma cell lines cultured under normoxic and hypoxic conditions. We studied a heterogeneous set of neuroblastoma cell lines to mimic the in vivo situation and to test the validity of the l1-l2 regularization. Hierarchical and k-means clustering and supervised approach have been applied to divide the cell lines on the bases of genetic differences. However, the disturbance of this strong transcriptional response masked the detection of the more subtle response to hypoxia. In order to address this issue, data filtering techniques based on prior knowledge have been applied.
Results: The algorithm distinguished the normoxic and hypoxic statuses defining signatures comprising 3 to 38 probesets, with a leave-one-out error of 17%. This signature is composed by 11 probesets representing 8 genes known to be modulated by hypoxia. We demonstrate that l1-l2 regularization outperforms more conventional approaches allowing the identification of a gene expression signature under complex experimental conditions.
Conclusion: The l1-l2 regularization, coupled with data filtering, generates an unbiased output with a low classification error. We feel that the application of this algorithm to tumor biology will be instrumental to analyze gene expression signatures hidden in the transcriptome that, like hypoxia, may be major determinant of the disease.

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POB28

Autophagy and its regulation in neuroblastoma

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Background/aims: Neuroblastoma (NB) is a frequent paediatric tumour. After combined treatments of chemotherapy, bone marrow transplantation, surgery or radiotherapy, the metastatic tumours still have a low success rate. Finding new therapeutic strategies to increase the survival rate of patients with NB is therefore essential. Autophagy is a self-degradative process that insures a constant protein and organelle turnover. By suppressing altered organelles, autophagy maintains cell survival by adapting to stress, but in some cases can induce cell death. The aim of this study was to determine if autophagy is present and could be regulated in NB.
Methods: Five Tissue Micro Array containing 184 NB diagnosed from 1977 to 2008 were studied. An immunohistochemistry method was used to identify the expression of LC3, a cytosolic protein required for autophagic vacuole (autophagosome) formation, and beclin 1, a positive regulator of autophagy. In addition, two NB cell lines (SK-N-SH and SK-N-DZ) were treated in vitro with six drugs (temozolomide, LY294002, rapamycin, vincristin, doxorubicin and cisplatin). Cell survival was measured by MTT cell proliferation assay. The autophagic vacuoles were labelled with monodansylcadaverine and the result was measured by fluorescence. Autophagosome formation was monitored by immunodetection of LC3 cleavage and beclin 1.
Results: Our study demonstrates that low levels of autophagy are present in a majority of NB. Also, LC3 expression is not correlated with clinical pathological features. By contrast, higher levels of Beclin 1 are expressed in NB of children over one year of age (with poor prognostic). Beclin 1 has a higher expression level in primary tumours than in metastases. Our in vitro study demonstrates that autophagy could be modulated by chemotherapy that inhibits cell survival. This inhibition was correlated with increase of the autophagic process as shown by the cleavage of LC3, the augmentation of autophagosomes and the expression of beclin 1.
Conclusion: Autophagy is present and could be modulated by chemotherapy in NB. Regulation of autophagy represents a very attractive target to develop new therapeutic strategies for NB.

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POB29

All-trans retinoic acid induced differentiation of SK-N-BE cells results in extensive DNA methylation alterations of gene promoter regions

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Background: All-trans-retinoic acid (ATRA) causes a number of neuroblastoma (NB) cell lines to undergo differentiation and a similar derivative is included in the final part of the treatment regime for patients with high stage disease neuroblastoma. Here, we examine the impact of ATRA treatment on promoter DNA methylation status in SK-N-BE using a whole genome profiling approach to further understand the molecular mechanism of induced differentiation.
Methods: Methylated DNA immunoprecipitation (MeDIP) was applied to microarrays representing all known promoter and/or CpG island regions of the human genome for pre and post ATRA treated SK-N-BE cells. The transcriptional activity of genes was evaluated using mRNA expression microarrays, and the methylation status of several candidate genes was further validated using bi-sulphite sequencing.
Results: Hybridization of MeDIP samples to microarrays indicated 402 gene promoters were de-methylated 7 days post ATRA treatment, 82 of which were over-expressed greater than 2-fold. One of these genes, nitric oxide synthase 1 (*NOS1*; re-expressed by 4.8 fold), has been previously shown to promote neuroblastoma cell differentiation (Ciani et al J Cell Sci 2004;117:4727). Gene ontology analysis of the genes that were de-methylated and re-expressed after ATRA treatment indicated enrichment for signal transduction pathways (p=0.0069). Mechanistically, the widespread demethylation observed following ATRA treatment might be due to the observed decrease in *DNMT1* (p = 0.004) and *DNMT3B* (p = 0.018) gene expression. In contrast, 88 genes became hypermethylated following ATRA treatment, 13 of which were under-expressed by >2 fold.
Conclusion: NB SK-N-BE cells induced to undergo differentiation by ATRA were found to have substantial alterations in DNA methylation at numerous gene promoters, potentially as a consequence of altered *DNMTs* expression. One of the genes de-methylated and transcriptionally activated in response to ATRA, *NOS1*, plays a vital role in the differentiation process, indicating that some methylation changes are functionally important in neuroblastoma cell differentiation.

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POB30

Chemokines CXCR5-CXCL13 cross-talk between malignant neuroblastoma cells and schwannian stromal cells suggests a role in the inhibition of metastatic dissemination

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Background: Neuroblastoma stroma-poor (NB-SP) is composed of small undifferentiated Neuroblastic cells (Nbc) and scarce Schwannian Stromal cells (SSc); most of NB-SP onset as metastatic disease. Ganglioneuroblastoma stroma-rich (GNB-SR) is characterized by abundant SSc and usually onsets as a localized tumor. It is suggested that SSc inhibits cell proliferation and differentiation of Nbc by secreting soluble factors. Chemokines are cytokines involved in chemotaxis and proliferation and play a crucial role in tumor growth and dissemination. Recently, using microarray gene expression analysis, we observed that *CXCL13* was more expressed in isolated SSc than in Nbc. This finding suggests to us that CXCL13 might have a functional role in the relationship between SSc and Nbc and in tumor dissemination.
Method/approach: mRNA expression of *CXCL13* and its receptor *CXCR5* was evaluated by Real-Time RT-qPCR in 14 NB-SP, 14 GNB-SR, 11 NB cell lines and in Nbc and SSc isolated by Laser Capture Microdissection. CXCR5 and CXCL13 protein expression was analysed by immunofluorescence, FACS or immunohistochemistry. Cell migration of CXCR5 positive NB cell were performed by chemotaxis assay. The effects of CXCL13 treatments on NB cell lines were investigated by MTT cell proliferation assay.
Results: We found that *CXCR5* was more expressed in NB-SP than in GNB-SR and *CXCL13* vice-versa. Nbc express *CXCR5* mRNA whereas SSc express *CXCL13*. NB cell lines show a variegated CXCR5 and CXCL13 mRNA and protein expression and several cell lines expressed both *CXCR5* and *CXCL13* suggesting an autocrine loop. In GNB-SR sections, SSc express CXCL13 protein but not CXCR5. We found that CXCR5 positive cells were able to migrate towards rhCXCL13 and that CXCL13 repressed NB cells proliferation.
Conclusion: Our findings suggest that CXCR5-CXCL13 axis could mediate a cross talk between Nbc and SSc by creating a tumor environment in which malignant neuroblasts are entrapped and inhibited to grow under the influence of CXCL13 released from stromal cells. This mechanism could affect the ability of Nbc to migrate and give distal metastasis explaining why GNB-SR disease do not show tumor dissemination.

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POB31

N-glycosylation of ALK as a potential target for disruption of prosurvival signaling pathways in neuroblastoma cell lines

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Background: The Anaplastic Lymphoma Kinase (ALK) is a 200-220 kDa N-glycosylated tyrosine kinase (TK) receptor, whose expression is restricted to the developing nervous system. The catalytic domain of ALK was originally identified in the t(2;5) translocation that produces the oncogenic fusion protein NPM-ALK and occurs in most of the Anaplastic Large Cell Lymphomas (ALCL). Recently, ALK was identified as the main predisposition gene to both familial and sporadic neuroblastoma (NB) by discovering of activating missense mutations in the TK domain. We examined the effects of tunicamycin, a specific inhibitor of N-glycosylation, on NB cell lines characterized by different ALK features.
Methods: We employed the following NB and ALCL cell lines: SH-SY5Y and LAN-5 (ALK mutation F1174L), NB1 (ALK amplified) and, as controls, LA1-5S (no ALK expression) and SU-DHL1 (NPM-ALK). Cells were treated with 500 nM tunicamycin in time course experiments for up to 60h. Total protein lysates were immunoblotted using primary antibodies for ALK, AKT, STAT3, ERK1/2 and matching phospho-proteins. Cell growth inhibition was assessed with the xCELLigence instrument, which can quantify adherent cell proliferation and viability in real-time. Cell death was performed by FACScan flow cytometry after incubation with propidium iodide and annexin-V.
Results: Tunicamycin treatment of NB cells expressing ALK resulted in the accumulation of a hypoglycosylated ALK band (180 kDa) and decreased amounts of the full size receptor (200-220 kDa). Interestingly, ALK phosphorylation was markedly decreased in the ALK driven NB cell lines (SH-SY5Y, LAN-5, NB1) after 12h and over 60h. The phosphorylation of downstream ALK effectors (AKT, STAT3, ERK1/2) was also considerably decreased. On the contrary, tunicamycin had no effects on phosphorylation of ALK effectors in LA1-5S and SU-DHL1 cells. Moreover, cell viability of ALK driven NB cells was impaired by N-glycosylation inhibition but was minimally affected in control cell lines.
Conclusion: Our results are suggestive that enzymatic steps regulating N-glycosylation are potential targets that may be exploited for novel therapeutical approaches in NB.

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POB32

Discovery of gene regulatory pathways implicated in neuroblastoma pathogenesis through integration of coding and non-coding gene expression and gene copy number data

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Background: Despite intensive multimodal therapies, survival rates for aggressive neuroblastoma (NB) remain disappointingly low, pointing at an urgent need for more effective and less toxic therapies. In this study, we identified new candidates for targeted therapy based on integrative genomic analysis.
Method: mRNA, miRNA and aCGH profiles were generated for 100 NB tumors. Correlation analysis pinpointed m(i)RNA transcripts whose expression is significantly altered as a result of copy number change. Both global analysis on the total cohort of NB tumors, as well as subset analysis of NB tumors belonging to the different genomic subgroups was performed.
Results: Pearson's correlation analysis mainly identified genes located within amplicons at 2p24 (MYCN), 2p23 (ALK), 11q13 (CCND1) and 12q15 (MDM2). In order to avoid outlier effects of the amplified genes, Spearman rank correlation analysis was used and yielded genes that were mainly located in chromosome regions 1p and 17q, in keeping with the frequent occurrence of copy number changes in these genomic regions. Within the 1p region, several genes emerged that were previously suggested as candidate tumor suppressors in other studies, e.g. CDH5, CAMTA1 and PRDM2. A similar analysis using the miRNA expression dataset revealed the most significant correlation for mir-324 (17p13), a gene that is known to be involved in the neuronal Hedgehog signaling pathway. Upon in-depth screening of the m(i)RNA lists with highest Spearman correlation using Ingenuity pathway analysis, several distinct pathways were revealed with a possible role in the pathogenesis of NB. Functional assessment of these pathways is ongoing.
Conclusion: In this study, we present a unique whole genome profiled dataset consisting of information at three different levels for a large cohort of NB tumors. This dataset enables powerful integrative genomic analysis, demonstrated by the identification of previously reported genes involved in NB pathogenesis as well as new candidate coding and non-coding genes and pathways for which further functional analysis should uncover their possible role in NB.

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POB33

The *calcium-sensing receptor* gene is inactivated by genetic and epigenetic mechanisms in neuroblastic tumors and its overexpression reduces neuroblastoma proliferation *in vitro* and *in vivo*

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Background: We have previously reported that the calcium-sensing receptor (CaR) is expressed in differentiated, favorable neuroblastic tumors (NT) and it is up-regulated upon differentiation induction, both *in vitro* and in second-look neuroblastomas (NB). However, *CaR* expression is undetectable in undifferentiated NB. The transcription of CaR is under the control of two promoters and promoter 2 (P2) is GC rich. Aims: To analyze the genetic and epigenetic mechanisms responsible for the *CaR* gene inactivation in NT. To evaluate the *in vitro* and *in vivo* effects of CaR overexpression on NB cell lines proliferation and metastatic capacities.
Methods: *CaR* mRNA was analyzed by qRTPCR in NB cell lines treated with 5-aza-2'-deoxycytidine (DAC) and/or trichostatin A (TSA). Methylation status of P2 was evaluated by bisulfite specific PCR and sequencing. A specific probe for the *CaR* locus was generated to perform fluorescence *in situ* hybridization (FISH). SK-N-LP and SK-N-JD cell lines were stably transfected with pCMV-GFP or pCMV-CaR-GFP. The proliferation rate of independent clones was assessed *in vitro* and in nude mice. Also, their metastatic capacity was evaluated in a chick embryo model.
Results: *CaR* mRNA was undetectable in 7/9 cell lines. Increased *CaR* mRNA levels were seen in LAN1, IMR32, LA1-55n, BE(2)-C, SK-N-JD, SK-N-LP, LA1-5s cell lines following DAC+/-TSA, but not in SH-SY5Y and SK-N-AS cells. Percentage of methylated cytosines in P2 was <6% in control tissues, SH-SY5Y and SK-N-AS cells, but it was 21-56% in the other cell lines. Hypermethylation of P2 among NT correlated with undifferentiated histology (P=0.002), age at diagnosis >12mo (P=0.026), high clinical risk (P=0.009), *MYCN* amplification (P=0.04) and absence of *CaR* expression (P=0.002). Allelic losses of the *CaR* locus and the entire chromosome 3 were seen among NB, GNB and GN. Stably transfected pCMV-CaR-GFP clones displayed statistically significant decreased *in vitro* and *in vivo* proliferation rates. However, the metastatic pattern of pCMV-GFP and pCMV-CaR-GFP clones was similar.
Conclusion: The *CaR* gene is silenced by genetic and epigenetic mechanisms and it exhibits tumor suppressor properties in NT.

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POB34

3D miRNA mutation screening in neuroblastoma

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Background: MicroRNAs (miRNA) are a class of small non-coding RNAs with a regulating function on gene expression. Mutations in these miRNA genes have recently been described in several malignancies. Trough large scale gene expression profiling of miRNA expression in neuroblastoma tumors we and others have shown their involvement in neuroblastoma tumor biology. Here, we propose an innovative mutation screen of all human miRNAs in a large cohort of neuroblastoma tumors.
Methods: We selected 216 primary untreated tumors and matching constitutional DNA to be screened for miRNA mutations. After phi29 based sample pre-amplification, tumors were enrolled in a 3 dimensional pooling strategy that allows for both identification of mutations in each individual sample as well as a significant reduction of the the number of sequencing reactions to be performed. All 721 currently known miRNA loci were PCR amplified from the pools of DNA and the amplicons are currently sequenced on a massively parallel short sequencing instrument. A bioinformatics variant interpretation pipeline is in place for efficient discovery of mutations.
Results: In this large scale screening study we aim for a genome wide view on the mutational landscape of miRNA genes in neuroblastoma. Our study is the first mutation screen performed on such a large scale, both regarding the number of samples as the number of miRNA genes. The amplicon sequencing strategy on pooled samples is a cost-effective and reliable way to perform large scale PCR based mutation analysis of a medium sized gene set (100-1000 amplicons) on a large sample cohort. Our screen will be followed by functional validation of the variants to discriminate potential driver from passenger mutations.
Conclusions: Results will be presented on our cost-effective large scale mutation analysis of all miRNA genes on a large cohort of neuroblastoma tumors. It is expected that this work will aid in our understanding of how miRNAs are implicated in neuroblastoma tumor biology.

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POB35

Bmi-1 promotes neuroblastoma cell proliferation by regulation of cyclin levels

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Background: Bmi-1 is a member of the Polycomb Group family of transcription repressors and has a critical role in maintaining the proliferative and tumorigenic potential of neuroblastoma cells. The molecular mechanism for the oncogenic activity of Bmi-1 in neuroblastoma pathogenesis remains poorly defined.
Methods: We have generated human neuroblastoma BE(2)-C cells with inducible knockdown of Bmi-1 expression. With this system, we examined, at both the molecular and cellular levels, the effect of Bmi-1 knockdown on neuroblastoma cell proliferation.
Results: We found that knockdown of Bmi-1 arrested BE(2)-C cells in the S phase. This action of Bmi-1 is independent of p14ARF and p16INK4a, as downregulation of Bmi-1 had no effect on p16INK4a expression and overexpression of p14ARF showed no significant impact on the survival or proliferation of neuroblastoma cells. By contrast, Bmi-1 knockdown resulted in a marked decrease in the protein levels of cyclins, which complex with cyclin-dependent kinases to drive cell cycle progression. Microarray analysis of gene expression profiles and quantitative real-time PCR revealed no changes in mRNA levels of the cyclins between control and Bmi-1-knockdown BE(2)-C cells. Interestingly, Bmi-1 knockdown is associated with upregulation of several F-box proteins that function as substrate adapters for the Skp1-Cul1-F box protein-Rbx1 (SCF) ubiquitin ligase complexes and are responsible for the specific ubiquitination of many cell cycle regulators including cyclins.
Conclusion: Our findings suggest a critical role of Bmi-1 in the control of cyclin degradation in neuroblastoma cells by regulating expression of F-box proteins.

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POB36

Prickle1: Possible tumour suppressive role in neuroblastoma

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Background: Wnts are a family of intercellular signalling factors that regulate a wide range of functions during embryonic development including cell proliferation, cell fate determination, differentiation, cell polarity and cell migration. They bind to Frizzled receptors and signalling thereafter proceeds via Dishevelled (Dvl) proteins to several intracellular pathways known as the canonical and non-canonical signalling pathways. Activation of the canonical pathway results in build up of beta-catenin levels and targets gene transcription. Increased Wnt/beta-catenin signalling is associated with many forms of cancer, including neuroblastoma. For long it has been suspected that the non-canonical Wnt signaling pathways could interfere with canonical signalling. This is now genetically confirmed, although the mechanism is still unclear. In this study we investigated the function of Prickle1, a planar cell polarity (PCP) protein, in regulation of the cytoskeleton and Wnt/ beta-catenin activity in neuroblastoma.
Method/approach: The expression of human Prickle1 was examined in SH-SY5Y neuroblastoma cells and the interactions with other PCP proteins were evaluated using immunoprecipitation. Prickle1 and Prickle1 mutants were overexpressed through transfection in SH-SY5Y and the effects on Rac1 and Actin were analyzed by immunocytochemistry. Prickle1 and beta-catenin levels were quantified by Western blotting.
Results: We found that Prickle1 is expressed in neuroblastoma (SH-SY5Y) cells. Preliminary, increased levels of Prickle1 seem to affect the cytoskeleton of SH-SY5Y cells, as assayed by Rac1 and actin immunoreactivity. Immunoprecipitation demonstrated that Prickle1 is present in complex with Vangl2, another planar cell polarity (PCP) protein known to interfere with the actin cytoskeleton.
Conclusion: The planar cell polarity protein Prickle1 is expressed in neuroblastoma with a potential tumour suppressive role with significance for tumour cell behavior by affecting the cytoskeleton. However, the exact mode of interaction between Prickle1 and Vangl2 remains to be elucidated.

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POB37

X-linked Inhibitor of Apoptosis (XIAP) as a new target for NB therapy

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XIAP (x-linked inhibitor of apoptosis) is the best characterised member of the inhibitor of apoptosis family of proteins (IAP's) able to block cell death initiated via the mitochondrial or death-receptor pathway by binding directly caspases. Overexpression of XIAP is found in a broad range of malignant diseases and is usually associated with an unfavorable outcome. Neuroblastoma (NB) is the most common solid cancer during childhood and effective treatment strategies are missing so far. The search and development of novel therapy approaches therefore is one of the main objectives in pediatric neuroblastoma research. Novel data indicate that XIAP might be a promising target for NB treatment like its IAP family member survivin which requirement for NB tumor growth was shown before and which is an effective target in immunotherapy. We demonstrate that XIAP protein is highly upregulated in NB cell lines and primary tumor samples in comparison to healthy murine adrenal glands serving as controls. The mRNA expression levels in the analyzed samples were on the contrary at equal levels speaking for a posttranscriptional regulation. Treatment of NB cell lines in an in-vitro cytotoxicity assay with a specific XIAP inhibitor showed a strong antiproliferative effect and an increase in sensitivity for chemotherapeutics like etoposide and vincristine used in common NB therapy. The effects observed after blocking of XIAP protein with the inhibitor used are consistent with a distinct increase in the rate of apoptosis shown by flow cytometry. XIAP expression is necessary for survival of NB tumor cells by inhibiting the apoptotic stimuli usually affecting cancer cells. In a second immunotherapeutic approach, XIAP DNA minigene vaccines expressing epitopes of either the murine or human XIAP protein sequence and a vaccine expressing the full length human cDNA sequence were successfully cloned and tested in-vitro and in-vivo. In summary, targeting of XIAP with inhibitor or immunotherapy may provide an interesting venue for NB therapy.

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POB38

Focal amplifications and deletions at miRNA loci in neuroblastoma

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Background: Recent studies have advanced insights into the genetic basis of neuroblastoma (NB) but more in depth knowledge is required in order to define new molecular targets for therapeutic agents. As part of our ongoing investigation for the role of miRNAs involved in NB, we performed a genome wide screening for focal copy number alterations at miRNA loci.
Method/approach: Array CGH data were obtained from 188 NB samples and 33 cell lines using a 44K/60K custom oligonucleotide array platform, specifically enriched with probes for miRNAs, transcribed ultra conserved regions (T-UCRs) and genes or genomic regions recurrently implicated in NB. Circular binary segmentation values were wave corrected and extracted from our in-house developed database arrayCGHbase. The R environment was used to identify genes residing in focal (defined as smaller than 1 Mb) aberrations.
Results: Array CGH screening detected focal copy number alterations affecting 64 different miRNA loci. Of particular interest was a high level gain of the miR-17-92 locus in cell line NLF, a miRNA cluster known to be directly regulated by MYCN and shown to be amplified in other tumor types. Focal deletions were detected at 33 miRNA loci, 16 of which were implicated in at least two tumors. Several of these miRNAs are known or presumed to be involved in neuronal development or tumorigenesis. For example, we provide for the first time evidence that miR-15a/16, a known tumor suppressor miRNA, might be implicated in NB as it is deleted in several cases. In addition to these findings, our screening also revealed new information regarding protein coding genes such as a rare amplification of two tyrosine kinase receptors and the telomerase reverse transcriptase hTERT gene as well as a focal deletion of TCF4 and a new recurrent distal 1q deletion.
Conclusion: This study revealed focal copy number alterations targeting miRNAs in NB, hence underscoring a role of miRNAs in NB pathogenesis. Further functional analyses are ongoing in order to determine their contribution to the NB phenotype. Previously unnoticed copy number alterations were also detected for important protein coding genes, such as hTERT and TCF4.

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POB39

Involvement of delta-like 1 homolog (drosophila) in the development of chemoresistance in neuroblastoma

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Background: As in other cancers, the development of chemoresistance represents a major obstacle in the successful treatment of high grade neuroblastoma (NB). In addressing the mechanisms underlying the chemoresistant phenotype in NB, we previously reported overexpression and activation of FZD1, and hence the association of pathological activation of the wnt/β-catenin pathway to the resistant phenotype of NB.
Method/approach: In this study, a closer analysis of the gene expression profile of doxorubicin-resistant cells (LAN-1-R) was performed.
Results: This analysis allowed us to identify Delta-like 1 homolog (drosophila) or DLK1 as another, moderately but significantly, overexpressed gene in the resistant variants. DLK1, a member of the Notch/delta/serrate family of proteins, is expressed in several embryonic tissues and in adult adrenal glands. DLK1 is also highly expressed in neuroendocrine tumors such as NB, suggesting a possible involvement in the development of the disease. We confirmed the increase in DLK1 expression by real-time quantitative PCR in LAN-1-R vs the non resistant LAN-1 cells with a 5.2-fold stimulation. Higher amounts of DLK1 protein were detectable on the cell membrane of the LAN-1-R by Western blot, as well as released in resistant LAN-1-R cells culture fluid as compared to non resistant LAN-1 cells. To further explore the contribution of DLK1 to the multidrug resistant and malignant phenotype of NB cells, DLK1 was overexpressed in different NB cell lines with variable endogenous DLK1 expression, or silenced by lentiviral-mediated micro-adapted shRNA, in LAN-1-R or NB8 cell lines. The resulting resistance to drugs, tumorigenic and tumor-initiating properties are presented.
Conclusion: Our data which fully support a recent report, convincingly implicating DLK1 in enhanced tumorigenic and undifferentiated characteristics of NB cells, further propose a role for DLK1 in their multi-drug resistant phenotype. These observations which associate DLK1 to multiple mechanisms leading to the particularly malignant behaviour of NB, deserve further investigation.

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POB40

Mechanisms of bHLH mediated neuronal differentiation

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Background: Neuroblastomas resemble undifferentiated neuronal precursors. bHLH transcription factors are 'master regulators' of differentiation, and NeuroD2 is a potent neurogenic bHLH protein. However, its neurogenic capability is limited by cell type and state, and neuroblastoma cells are unresponsive to NeuroD2. Using this model, our goal is to identify the critical components modulating the neurogenic program.
Methods: NeuroD2 lentivirus was introduced into P19 mouse embryonocarcinoma cells to induce neurogenesis, and mRNA was isolated from control and NeuroD2-expressing cells and analyzed on Affymetrix Expression Arrays. NeuroD2 expressing mouse embryonic fibroblasts (MEF) were used as comparison. For chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-Seq) studies, DNA from both P19s and MEFs expressing NeuroD2 was crosslinked, sonicated, immunoprecipitated with NeuroD2 antibody, then decrosslinked and sequenced with an Illumina Genome Analyzer II.
Results: We observed potent neuronal differentiation in P19 cells expressing NeuroD2, but no differentiation in neuroblastoma cell lines or MEFs. Subsequent studies were performed with MEFs to avoid interspecies variability. Expression arrays demonstrated over 500 induced genes in both P19s and MEFs. 529 genes were upregulated solely in P19s, and were involved in neuronal development by gene ontology analysis. Surprisingly, ChIP-Seq analysis demonstrated over 60,000 sites in both P19s and MEFs. As expected, the majority of binding occurred at E-boxes (CANNTG), although there was enrichment for the sequence CAGATGG in P19s. Additionally, areas adjacent to NeuroD2 binding sites in P19s showed enrichment for binding site motifs to Pbx and homeodomains.
Conclusion: Our results suggest how the neuronal program may be controlled through the use of cofactors to limit the function of transcription factors, and also demonstrate thousands of NeuroD2 binding sites in both differentiated and resistant cells. The latter may be involved in epigenetic modification of the genome, affecting DNA accessibility. We hope to apply these findings to understand how cells like neuroblastomas avoid neurogenesis.

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POB41

Nf-kB and IRF1, but not MYCN, control the expression of MHC class I and endoplasmic reticulum aminopeptidases in human neuroblastoma cells

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Amplification and overexpression of the MYCN oncogene characterize the most aggressive forms of neuroblastoma and are believed to severely down-regulate MHC class I expression by transcriptional inhibition of the p50 NF-kB subunit. We found that in human neuroblastoma cell lines, high MYCN expression is not responsible for low MHC class I expression, since neither transfection-mediated overexpression nor siRNA suppression of MYCN affects MHC class I and NF-kB p50 protein levels. Furthermore, we identified NF-kB as the main regulator of MHC class I, since the p65 NF-kB subunit binds MHC class I promoter in chromatin immunoprecipitation experiments, and MHC class I expression is enhanced by p65 transfection and reduced by inhibition of p65 activity. Interestingly, IRF1 is also involved in the regulation of MHC class I expression, since its overexpression particularly when combined with the overexpression of NF-kB p65, reverses the MHC class I-low phenotype in the most aggressive neuroblastoma cell lines. Like MHC class I, the endoplasmic reticulum aminopeptidases ERAP1 and ERAP2, known to generate MHC class I binding peptides, are regulated by NF-kB and IRF1 and contain functional NF-kB and IRF1-binding elements in their promoters. Consistent with these findings, nuclear p65 and IRF1 could only be detected in the maturating neuroblastic cells that express higher levels of MHC class I molecules in tumor speciments. These findings provide molecular insight into defective MHC class I expression in neuroblastoma, and indicate that activating NF-kB and IRF1 in MHC class I-low, aggressive neuroblastoma cells could be instrumental for successful application of T cell-based immunotherapy.

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POB42

Differential expression of PI3K-Akt pathway genes in neuroblastoma.

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Background: The phosphatidylinositol 3-kinase (PI3K)-Akt pathway transmits intracellular signals that regulate cell growth, proliferation and survival and is frequently affected in human cancer. Neuroblastoma of different stages are known to be biologically diverse and in this study we investigated whether expression of PI3K-Akt pathway genes differ between favourable and aggressive neuroblastomas.
Methods: The level of mRNA expression of 88 PI3K pathway genes in 24 primary neuroblastomas of different stages was investigated by whole genome GeneChips (HU133A and HU133plus2.0, Affymetrix). The 12 genes that showed significant difference of expression between either INRG localized versus metastasizing tumours, or between INSS Stage 1-2 versus Stage 4 neuroblastomas in the microarray study were confirmed by real-time PCR with TaqMan in a larger set of tumours (52 samples). In another set of 24 neuroblastomas the protein expression was examined by Western Blot.
Results: The PI3K pathway genes *PDGFRA* and *ITGB1* were significantly up-regulated, and *PIK3R1, EIF4EBP1, TSC2, CDC42, AKT1, PIK3CD, BAD, PRKCB1, PRKCZ* and *FOXO3* were significantly down-regulated in aggressive compared to favourable neuroblastoma tumours in the microarray analysis. Three of the down-regulated genes (*CDC42, PIK3CD, PRKCZ)* are located in the 1p36 chromosomal region, and could partly be due to 1p-deletion in the aggressive neuroblastomas.
Conclusion: Some of these genes are also involved in other cell signalling pathways, like MAPK signalling (*PDGFRA, BAD, PRKCB1*). Taken this into account, our conclusion is that the PI3K-Akt pathway is up-regulated in favourable neuroblastoma tumors, and other pathways, like the MAPK signalling pathway, are more important in the aggressive tumours.

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POB43

The impact of MYCN on the response to MDM2-p53 antagonists in neuroblastoma

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Background: *MYCN* amplification is a major negative prognostic marker occurring in 25-30% of neuroblastomas. MYC proteins are transcription factors with roles in both cell proliferation and apoptosis. We have recently published evidence showing that p53 is a direct transcriptional target of MYCN in neuroblastoma and p53 mediated apoptosis is an important mechanism of *MYCN* dependent apoptosis (Chen et al 2010, Cancer Res). Co-amplification of *MDM2* with *MYCN* provides a contributory mechanism by which *MYCN* amplified neuroblastoma evades p53 mediated apoptosis. MDM2-p53 antagonists activate wild-type p53 that is suppressed by MDM2 and have been found to have antitumour activity in neuroblastoma preclinical models. Hypothesis: The activity of MDM2-p53 antagonists is increased in the presence of MYCN.
Method/approach: Growth inhibition assays for the MDM2-p53 antagonists Nutlin-3 and MI-63 were carried out in the tetracycline regulatable MYCN SHEP Tet21N cell line. Knockdown of MYCN was achieved using siRNA in two *MYCN* and *MDM2* co-amplified neuroblastoma cell lines (NGP and LS) followed by treatment with Nutlin-3 and MI-63. Induction of p53 responsive genes and apoptotic markers were observed using Western blot and changes in cell cycle by flow cytometry. Apoptosis was determined by caspase activity and annexin V staining.
Results: The GI50s for Nutlin-3 and MI-63 increased 4 fold in MYCN- compared to MYCN+ Tet21N cells. Following treatment with MDM2-p53 antagonists alone, an increased p53 response was observed with subsequent G_1 arrest in NGP cells and apoptosis in both NGP and LS cells. After MYCN knockdown and treatment with Nutlin-3 and MI-63, no further effect was seen on the proportion of cells in G_1. However, a reduction in levels of apoptotic markers was observed by Western blot in both cell lines and a significant reduction in caspase activity was observed in NGP cells (50% reduction compared to control after MYCN knockdown, $p<0.0001$).
Conclusion: The reduction of MYCN expression decreases the apoptotic response to MDM2-p53 antagonists. MDM2-p53 antagonists may therefore be particularly effective in high risk *MYCN* amplified neuroblastoma.

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POB44

The role of MDMX on the response to MDM2-p53 antagonists in neuroblastoma

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Background: The MYCN protein plays a dual role in driving proliferation and sensitising cells to apoptosis. MDM2 is the major negative regulator of the p53 tumour suppressor and in neuroblastoma cell lines it is often co-amplified with *MYCN* and in these circumstances may be a mechanism by which *MYCN* amplified neuroblastoma circumvent MYCN mediated apoptosis. Most neuroblastomas have wildtype p53 and MDM2-p53 antagonists are being investigated to reactivate p53 through inhibition of MDM2. MDMX is also a negative regulator of p53 and is similar in structure to MDM2, but whereas MDM2 regulates p53 stability and activity, MDMX regulates activity only. MDMX removal or inhibition may be necessary to fully activate the p53 response.
Hypothesis: Knockdown of MDMX results in an enhanced response of *MYCN* and *MDM2* co-amplified neuroblastoma cell lines to MDM2-p53 antagonists.
Method/approach: Knockdown of MDMX was achieved using siRNA in two *MYCN* and *MDM2* co-amplified neuroblastoma cell lines (NGP and LS), followed by treatment with the MDM2-p53 antagonists, Nutlin-3 and MI-63. Induction of p53 responsive genes and apoptotic markers were observed using Western blot and changes in cell cycle investigated by flow cytometry. Apoptosis was assessed by caspase activity and annexin V staining.
Results: An increased p53 response was observed following MDM2-p53 antagonist treatment alone, shown by a G_1 arrest in NGP cells and induction of apoptosis in both cell lines. Following MDMX knockdown alone, an increase in $p21^{WAF1}$ was observed indicating that the p53 response is activated. However, upon addition of MDM2-p53 antagonists following MDMX knockdown the NGP cells still arrested in G_1 but in both cell lines the proportion of cells undergoing apoptosis decreased.
Conclusion: This data indicates that cells are more resistant to MDM2-p53 antagonists following MDMX knockdown, suggesting that MDMX expression may be important in determining the response to MDM2-p53 antagonists in neuroblastoma.

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POB45

Distinctive expression patterns of MicroRNA in neuroblastoma tumors of opposite outcomes

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Background: High risk neuroblastoma (HRNB) includes i) tumors with MYCN amplification (MNA or type C) independently of stage and ii) non-MNA (NMNA) tumors of stage 4 from patients over 18 months of age. Among these patients, a very small group (6%) exhibits an unexpected long survival rate. MicroRNAs (miRNAs) through their target genes play a major role in developmental processes and cancer initiation and progression. We hypothesized expression of a specific miRNA pattern in neuroblastoma specimens from high risk patients with unexpected favorable outcome.
Material & Methods: Tumor specimens were obtained from the Institut Gustave Roussy bank. Structural gene alterations especially MYCN amplifications were identified by CGH-on 244K Agilent arrays. Total RNA was hybridized to Agilent Human miRNA Microarrays v2.0 (about 800 microRNAs probed). The expression profile was analyzed using the Resolver software. Candidate alterations were validated using quantitative real-time PCR (qRT-PCR).
Results: The miRNA profile was compared in two NB subgroups with opposite outcome: good outcome (GO: > 5 years survival) and bad outcome (BO: < 20 months' survival). Out of fifteen tumor specimens, 6 were from localized stage patients (4 GO & 2 BO), 1 from stage 4S (GO) and 9 from HRNB (3 GO and 6 BO). Among HRNB, comparison of BO to GO cases led to several differential miRNAs, many of them not previously annotated. The most abundant were miR-575 (p-value 0.016) in BO-NB and miR-15a, miR-15b, miR-16, miR-214 and miR-451 in GO-NB. As recently reported by other groups, we found that miR-92a, miR-181a and miR-181b were upregulated in MNA regardless of patient outcome. Interestingly, miR-451 overexpression was found to be independent of MYCN status as assessed by qRT-PCR. Well worthy, the miR-451 expression was inversely correlated with MDR1 expression.
Conclusions:
- A second cohort of NB tumors is currently studied to validate these preliminary results.
- Cell lines derived from MNA patients with opposite outcome are currently used for functional studies.

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POB46

Neuroblastoma differentiation signalling pathways

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Background/Aims: The differentiation potential of neuroblastoma, including the (trans-) differentiation of neuroblastoma into ganglioneuroblastoma/ganglioneuroma or vice versa, is poorly understood. Elucidation of he signal transduction pathways involved in this phenomenon can identify basic aspects of neuroblastoma pathogenesis. We therefore analyzed a neuroblastic tumor series for expression of transcription factor genes involved in neural crest development and tested their function in neuroblastoma cell lines.
Methods: Genome-wide mRNA profiles (Affymetrix HU133 Plus 2.0) of 110 neuroblastic tumors and 24 neuroblastoma cell lines were generated. In addition, we manipulated the expression of the transcription factor genes MEIS1, MSX1, MYCN, NOTCH3, and PHOX2B in neuroblastoma cell lines by shRNA-mediated knockdown and inducible over-expression. Time-course experiments after expression manipulation were profiled to identify the downstream pathways of these genes.
Results: Expression profiling showed that MEIS1, MYCN, and PHOX2B were highly expressed in most neuroblastomas, but only weakly in ganglioneuroma. MSX1 showed the opposite expression pattern. Indeed, a mutual negative regulation between MSX1 and PHOX2B was identified in IMR32 and SJNB8 cell lines with inducible PHOX2B and MSX1 expression. In agreement with a role in differentiation, MSX1 induced cell cycle arrest in neuroblastoma cell lines. MSX1 activated central genes in neural differentiation routes, like DLK1, HEY1, and NOTCH3 in the Delta-Notch, and DKK1-3 and SFRP1 in the Wnt pathway. These target genes showed the same expression patterns in neuroblastoma versus ganglioneuroma tumors as MSX1.Together, these data suggest that MSX1 is a key transcription factor activating neural differentiation pathways in neuroblastoma, while MEIS1, MYCN, and PHOX2B repress these signal transduction routes.
Conclusion: We have identified a core signaling network governing neural differentiation in neuroblastoma. PHOX2B, mutated in 3% of neuroblastomas, and MYCN are part of this network. We propose that this network is instrumental in the balance between differentiation and progressive disease in neuroblastoma.

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POB47

The polyamine metabolism genes ornithine decarboxylase and antizyme 2 predict aggressive behavior in neuroblastomas with and without MYCN amplification

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Background: High polyamine (PA) levels and ornithine decarboxylase (ODC) over-expression are well-known phenomena in many aggressive cancer types. We analyzed the expression of ODC and ODC-activity regulating genes antizymes 1-3 (OAZ1-3) and AZ inhibitors 1-2 (AZ-IN1-2) in human neuroblastoma (NB) tumors and correlated these with genetic and clinical features of NB. Since ODC is a known target gene of MYCN, the correlation between ODC and MYCN was of special interest.
Methods: Data were obtained from Affymetrix micro-array analysis of 88 NB tumor samples. In addition, mRNA expression levels of ODC, OAZ2, and MYCN in a MYCN-inducible NB cell line were determined by quantitative real-time RT-PCR.
Results: ODC mRNA expression in NB tumors was significantly predictive of decreased overall survival probability and correlated with several unfavorable clinical NB characteristics (all $P < 0.005$). Interestingly, high ODC mRNA expression also showed significant correlation with poor survival prognosis in Kaplan-Meier analyses stratified for patients without MYCN amplification, suggesting an additional role for ODC independent of MYCN. Conversely, high OAZ2 mRNA expression correlated with increased survival and with several favorable clinical NB characteristics (all $P < 0.003$). In addition, we provide first evidence of a role for MYCN-associated transcription factors MAD2 and MAD7 in ODC regulation. In NB cell cultures, ectopic over-expression of MYCN altered ODC, but not OAZ2 mRNA levels.
Conclusion: Our data suggest that elevated ODC and low OAZ2 mRNA expression levels correlate with several unfavorable genetic and clinical features in NB, offering new insights into PA pathways and PA metabolism-targeting therapy in NB.

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POB48

Functional pRB loss is involved in impaired drug induced DNA damage response in *MYCN* amplified neuroblastoma cells

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Background: Neuroblastoma tumors with amplified *MYCN* initially respond to chemotherapy but show pronounced drug resistance after relapse. To date specific mechanisms of relapsed tumors to escape therapy are poorly understood. A better mechanistic understanding of the drug induced DNA damage response is urgently needed to develop new therapeutic concepts for relapsed tumors. Protein analyses showed functional abrogation of pRB and p53 in *MYCN* amplified cells. The aim of this work is to define whether functional restoration of pRB and/or p53 sensitizes *MYCN* amplified cells to doxorubicin-induced DNA damage.
Method/approach: Flow cytometry was used to determine the cellular phenotype of different neuroblastoma cells with distinct genomic alterations (amplified *MYCN*, *CDK4*, *MDM2*, *mutant TP53*) upon doxorubicin treatment. Furthermore, genetically modified neuroblastoma cells allowing conditional expression of INK4 proteins ARF (p14), CDKN2A (p16) and CDKN2D (p19) were analyzed. In addition, selective small molecules and/or shRNAs that target CDK1, CDK2, CDK4 and the MDM2-p53 interaction were used to sensitize neuroblastoma cells to doxorubicin.
Results: Doxorubicin treatment resulted in impaired DNA damage response characterized by reduced number of G1 phase cells and high enrichment of cells in G2/M phase most pronounced in *MYCN* amplified cells. Enrichment of G2/M phase cells was inversely correlated with apoptosis (Pearson's correlation -0.58, CI -0.85-0.08, P=0.03). Disruption of the MDM2-p53 interaction at least partly restored apoptosis induction but not cell cycle arrest. Inhibition of CDK4 by siRNAs, small compounds or p19 induction as well as silencing of CDK2 restored G1 phase arrest but poorly affected apoptosis. Inhibition of CDK1 favored both cell cycle arrest and apoptosis. In contrast, induction of p16 neither seemed to affect cell cycle distribution nor apoptosis.
Conclusion: Our results show that the wild type functions of pRB and p53 are disabled in *MYCN* amplified cells. Functional pRB also contributes to drug induced DNA damage response as p53. Functional restoration of pRB and p53 are needed for improved therapeutic approaches in neuroblastoma.

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POB49

Modulation of neuroblastoma cell sensitivity towards anticancer drugs by MycN expression

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Background: Neuroblastoma (NB) is the most common and deadly tumor of childhood. Amplification of the MycN oncogene characterizes the subset of aggressive NBs resistant to various therapeutics. However, the data on the role and significance of MycN amplification for cell survival are controversial. The involvement of mitochondria in the regulation of cell death pathways makes targeting of these organelles a promising strategy for tumor cell elimination. A redox-silent analogue of vitamin E, α-tocopheryl succinate (α-TOS), was shown to cause apoptosis via production of reactive oxygen species (ROS) and Bax-mediated release of cytochrome c from mitochondria.
Methods: MycN expression in Tet21N cells was regulated by doxycyclin. Analysis of ROS production was performed using mitochondria-incorporated sensor. Calcium fluxes were monitored by confocal microscopy.
Results: α-TOS facilitates induction of mitochondrial permeability transition (MPT) in NB Tet21N cells, Jurkat T-lymphocytes, and isolated rat liver mitochondria. α-TOS stimulates a rapid entry of Calcium into the cell with subsequent accumulation of these ions in mitochondria - a prerequisite step for MPT induction. ROS produced by α-TOS can contribute to MPT via oxidation of thiol groups in the adenine nucleotide translocase and alteration of redox state of glutathione or pyridine nucleotides. α-TOS also facilitates MPT induction via moderate uncoupling of mitochondria, since MPT is stimulated by low $\Delta\Psi$. Calcium accumulation in mitochondria is important for apoptosis progression, since inhibition of mitochondrial Calcium uptake significantly mitigated apoptotic response. Interestingly, downregulation of MycN markedly decreased the rate of Calcium accumulation by mitochondria. As a result, the level of Ca2+ in cytosol remained elevated that caused processing and release of another pro-apoptotic mitochondrial protein, AIF.
Conclusions: Downregulation of MycN made cells resistant towards cisplatin, but sensitized them towards α-TOS. Thus, different mechanisms are involved in cell death induced by these drugs, and involvement of MycN has a pathway-specific character

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POB50

Epigenetic alterations in disseminated neuroblastoma: influence of *TMS1* gene hypermethylation in relapse risk

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Background: Most neuroblastoma patients over 18 months of age at diagnosis show disseminated disease in bone marrow (BM), which is quantified to evaluate treatment response. There are different genetic and epigenetic alterations between tumour cells from primary tumours and those tumour cells from BM metastasis. Epigenetic alterations in certain tumour cells may confer a selective advantage over tumour dissemination process, and be helpful in the clonal selection of tumour-specific cells that could originate metastasis. AIMS: evaluate the presence of specific hypermethylation in disseminated neuroblastoma disease, and its putative association with patients' survival.
Methods: We performed real-time quantitative PCR to identify the presence of high expression biomarkers of disseminated tumour cells in bone marrow samples from stage 4 patients at diagnosis. We selected BM samples with high level of disseminated tumoral cells. We used MSP to analyse the methylation profile of 20 genes putatively implied in dissemination.
Results: The analysis of hypermethylation rate in disseminated tumour cells opposite to age at diagnosis and *MYCN* amplification showed no significant results. In contrast, the high rate of hypermethylation in disseminated tumour cells was associated with relapse risk (P=0.0042). In a survival analysis using Cox regression method we considered the methylation rate in disseminated tumour cells, as well as other established prognostic factors such as age at diagnosis and *MYCN* amplification. The results pointed out the high methylation rate in disseminated tumour cells as a variable with a high influence in relapse risk (P=0.02). Respect to the influence of the hypermethylated status for each gene on survival, we obtained significant association between hypermethylation in disseminated tumour cells and a high relapse risk in *TMS1* (P=0.0046), *MGMT* (P=0.0317) and *RARβ2* gene (P=0.0414).
Conclusions: We could not confirm the presence of a specific methylation profile in disseminated neuroblastoma tumour cells, but a high accumulation of epigenetic events in those disseminated cells is associated with a high risk of relapse, independently of *MYCN* amplification.

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POB51

Combining peptide vaccination with immunostimulatory monoclonal antibodies provides potent immunotherapy in neuroblastoma

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Immunotherapy is an attractive therapeutic approach for children with high risk neruoblastoma, holding the possibility of a more specific and less toxic treatment than conventional therapies. Spontaneous immune reponses in neruoblastoma are well-documented and a number of tumour antigens have been identified. Survivin is particularly promising as an immunotherapy target as it is expressed in 80-100% of high risk tumours and only minimally in normal tissue. Spontaneous anti-survivin T cell responses have been reported, with one study demonstrating responses in 8 of 9 children with neuroblastoma. Immunostimulatory monoclonal antibodies (mAbs) potentially offer a practical and potent means of boosting these weak endogenous responses to achieve therapeutic immunity. Such mAbs target co-stimulatory molecules (e.g. 4-1BB, OX40, CD40, CTLA-4), providing agonistic or counter-regulatory signals. They have already shown promise in adult clinical trial in a number of different malignancies. There is, as yet, no clinical experience in paediatric patients Here we demonstrate the efficacy of immunostimulatory mAbs in murine syngeneic neuroblastoma models. In the weakly immunogenic Neuro2a neuroblastoma model, treatment of established subcutaneous tumour with either anti-4-1BB, anti-CD40 or anti-CTLA-4 mAb results in resolution of tumour and long-term survival in 40-60% of mice. This is dependent on NK and CD8+ T cells and a tumour CD8+ lymphocyte infiltrate is observed. Therapy is only achieved if mAb is given to mice once tumours are established, suggesting that it is dependent on the presence of antigen from the tumour. In the aggressive, poorly immunogenic AgN2a and NXS2 neuroblastoma models, single agent immunostimulatory mAb therapy results in only marginal slowing of tumour growth. However if mAb (anti-CTLA-4) is given in conjunction with survivin peptide vaccination then 60% long term survival is achieved. Surviving mice have persisting (> 90 days) T-cell immunity to survivin as demonstrated by intracellular interferon-γ staining. This data suggests the combination of antigen and co-stimulatory mAb may provide effective immunotherapy against neuroblastoma.

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POB52

Expression QTL analysis of tumor susceptibility in a mouse model of neuroblastoma

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We created a transgenic mouse model for neuroblastoma by targeting expression of the MYCN oncogene to the neural crest under the control of the tyrosine hydroxylase promoter. Tumor penetrance in this model is highly dependent on mouse strain genetic background, providing the opportunity to explore the molecular development of tumors using genetic perturbations. Transgenic mice on an FVB/N genetic background are completely resistant to tumors, while transgenic mice on a 129/SvJ background are almost completely penetrant. We generated a backcross population of (TH-MYCN-FVB/N x 129/SVj) x 129SvJ mice that displayed a 33% tumor incidence. We genotyped 200 backcross mice with 348 markers and identified a locus on chromosome 10 linked to tumor susceptibility with a lod score of 4.5. Using a 2-QTL model, we found several other significant interacting loci. To identify candidate genes at these loci, we analyzed RNA from 46 tumors and 116 superior cervical ganglia from backcross mice using Affymetrix mouse exon arrays, and tested for genetic control of gene expression (expression QTLs). We have identified several dozen highly-significant expression QTLs, many mapping to tumor susceptibility loci and sharing common molecular signaling and metabolic pathways. We have also identified several more QTLs at an exon-level resolution, suggesting possible differential splicing of transcripts between strains that may play a role in tumor development. Further analysis of gene correlation networks and expression QTLs should illuminate novel molecular pathways and potential drug targets in neuroblastoma.

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POB53

Age-dependent genotypes in aggressive neuroblastoma: MYCN amplification represents a few-hit/early-age form

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Background: Presence of MYCN amplification (MNA) or of 11q loss represent two major tumor genotypes in high-risk neuroblastoma (HR-NB). Most HR-NBs are diagnosed after 18 months of age but can occur in younger children in association with MNA. The aim of the present investigation was to further explore the age-dependence of the tumor genetics of aggressive neuroblastoma.
Method/approach: A consecutive series of Swedish HR-/poor outcome NB, comprising 29 HR and 4 Intermediate Risk tumors, were analyzed for DNA copy number alterations, assessed on a 32K clone-based array platform. Affymetrix 250K and Illumina 610Q SNP array chips were used for validation. The age-dependence of MNA status was compared to data from 111 HR-NBs annotated in the Swedish NB registry over a 25-year period.
Results: MNA was associated with young age at diagnosis, mean age 30.6 months (n=15) - found in 10/11 tumors of the youngest (range 4-30 months) but absent in 10/11 tumors of the oldest children (range 57-169 months) of our series. On the opposite, mean age at diagnosis of patients with non-MNA tumors was significantly higher, 65.6 months (n=18), and 12 of these tumors displayed 11q loss (mean age at diagnosis 69.5 months). Furthermore, we observed that while MNA tumors harbored few additional segmental chromosomal aberrations (mean=5.0), in tumors with 11q loss these were more numerous (mean=11.5) and showed increasing tendency by age at diagnosis (p=.037). The age-dependence of MNA status in HR-NB was verified in the Swedish NB registry, with mean ages at diagnosis of 29.4 and 54.8 months for MNA (n=65) and non-MNA (n=46) tumors, respectively.
Conclusion: The data suggests two major pathways in the genesis of aggressive NB: MNA represents a fast track, largely occurring during an early time window when progenitor cells are still abundant, requiring few additional genetic events. On the other hand, presence of 11q loss implies a slower, multi-step process facilitated by genomic destabilization, as indicated here by older ages at diagnosis and more numerous segmental chromosomal aberrations in an age-dependent manner.

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POB54

Modeling the neuroblastoma tumor initiating cell microenvironment in 3D culture

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Background: Tumor initiating cells (TICs) are highly tumorigenic cells having the ability to self-renew and differentiate into the cell lineages in the tumor. TICs are being identified in a growing list of tumor types. Neuroblastoma (NB) TICs have been isolated from NB bone marrow metastases by growing tumor cells as a sphere culture in conditions normally used for neural crest stem cells (Hansford et al., 2007). Normal tissue specific stem cells are precisely controlled, regarding self-renewal and differentiation, by the chemical and physical signals of the microenvironment. Chemical cues include growth factors, extracellular matrix and ligands/receptors that engage in cell-cell communication. Physical variables include geometrical constraints, the 3D architecture and surrounding mechanical properties. Accumulating evidence suggests that corresponding microenvironment dependence exists for tumor initiating cells. Indeed, NB cell lines grown in a 3D collagen scaffold showed considerable differences in morphology and gene expression compared to cells grown in 2D (Li et al., 2007).
Aims: We aim at developing a 3D culture system, more closely resembling the in vivo microenvironment, and thus being of general interest in the investigation of NB TIC biology. Support cells, mimicking the tumor stroma as well growth factors and extracellular matrix components will be incorporated in the scaffold to mimic the TIC/niche communication. Furthermore we aim at varying the elasticity of the scaffold, resembling the elasticity of the sites of NB metastases.
Methods/Results: We have developed a porous polymer 3D scaffold to support NB TIC growth. Out of several synthetic polymers, a commercial diblock polymer of poly-d,l-lactide-co-glycolide (PLGA) and poly-ethylene glycol (PEG) showed the most suitable properties for sustained TIC growth. TIC proliferation and maintenance of undifferentiated state in the 3D cultures resembled those in sphere cultures.
Conclusion: NB TICs can be cultured in vitro in synthetic 3D scaffolds. The 3D scaffolds constitute a technology platform that can be developed with increasing degree of complexity to mimic the NB TIC microenvironment.

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POB55

Epigenetically silenced microRNAs contribute to neuroblastoma pathogenesis

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Background: Neuroblastoma (NB) is one of the most frequent and deadly solid tumors in children. However, the molecular basis of neuroblastoma development and its progression is poorly understood. MicroRNA (miRNA) deregulation was recently identified as a major contributor to cancer initiation and progression. As it has been shown that abnormal DNA methylation at miRNA CpG islands contributes to tumor formation, we aimed to investigate the potential role of epigenetically silenced miRNAs in NB pathogenesis.
Method/approach: Using quantitative PCR preceded by stem-loop megaplex reverse transcription, we measured the expression level of 384 miRNAs in a panel of 7 NB cell lines before and after treatment with a demethylating agent (5-aza-2'-deoxycytidine, DAC), a histone deacetylase inhibitor (trichostatine A, TSA), or a combination of both. miRNA profiles were also determined for 100 primary NB tumors, and normal NB progenitor cells that have been isolated by laser capture microdissection from fetal adrenal glands.
Results: Based upon the association with a CpG island and the absence of expression in a subset of NB tumors, miR-34b and miR-34c were identified as strong candidate suppressor miRNAs. Those miRNAs belong to the same cluster located on 11q23.1, a region critically deleted in a subset of aggressive NB. Reconstitution of expression of the down regulated miRNAs resulted in decreased cell viability. Interestingly, miR-449a, a member of the same family of conserved miRNAs, was reactivated by epigenetic treatment in all investigated cell lines, thus also pointing at a putative tumor suppressor activity. Using miR-449a mimics forced overexpression indeed resulted in cellular differentiation and cell cycle arrest and demonstrated significant cell viability reduction in wild type *TP53* NB cells. Identification of critical target genes is ongoing.
Conclusion: miRNA profiling successfully identified specific miRNAs silenced by epigenetic mechanisms in NB. Hence, this analysis may enable the identification of epigenetically regulated miRNAs that contribute to NB pathogenesis and which represent new targets for therapy.

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POB56

The interaction between GRP75 and retinoic acid receptor-α/ retinoid X receptor-α is essential for retinoic acid-induced neuronal differentiation of neuroblastoma cells

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Background/Aims: The tumorigenesis of neuroblastoma (NB), the most common extracranial solid tumor in children, is thought to be caused by defects in the neural development of neuroblasts, resulting in aberrant cell-cycle exit and cell differentiation. Retinoic acid (RA) has been used in clinic practice as a differentiation therapy for NB. Given that GRP75 is a favorable prognostic factor and is essential for RA-induced neuronal differentiation of NB cells. The aim of this study is to determine the molecular mechanism underlying GRP75-mediated regulation of RA-elicited neuronal differentiation.
Methods: Co-immunoprecipitation was employed to determine whether GRP75 can interact with RA receptors, retinoic acid receptor (RAR)α and retinoid X receptor (RXR)α. The functional role of GRP75-bound RARα / RXRα heterodimers in gene expression was determined by real-time PCR and chromatin immunoprecipitation.
Results: We demonstrated that GRP75 can be translocated into nucleus and physically interact with RARα and RXRα and that the nuclear GRP75-bound RARα /RXRα complexes can augment RA-elicited regulation of gene expression. Furthermore, GRP75 can govern the recruitment of RA-bound RARα/RXRα complexes to RARE in the regulatory regions of RA target genes, resulting in the transcriptional modulation downstream of RA signaling. Our data also showed that GRP75 is essential for stabilizing RARα/RXRα complexes in the presence of RA by reducing the proteasome-mediated degradation of RARα and RXRα.
Conclusions: Our present findings delineate a novel mechanism underlying the GRP75-dependent regulation of RARα/RXRα-mediated gene expression in the RA-induced neuronal differentiation, providing the basis for the development of novel therapeutics for NB.

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POB57

Neurod1 is involved in the development of neuroblastoma expecially at initiation stage

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Background: MYCN transgenic (Tg) mouse is a model for neuroblastoma(NB). Because the celiac ganglion is the tumor origin in most cases, and the period of tumor onset is restricted (around 2-week old in homozygous mice), these mice are profitable to address gene expression profiles in the precancerous and initial stages of NB. We seek for candidate molecule targets for NB therapy through the analysis of these expression profiles.
Method/approach: To examine gene expression profiles, we dissected following tissues: 1) normal celiac ganglion (wild-type mice, 2-week old), 2) celiac ganglion at hyperplasia stage (homozygous mice, 2-week old), 3) NB at initial stage (homozygous mice, 3-week old), 4) NB at initial stage (hemizygous mice, 9-10-week old). cDNAs were synthesized and hybridized to MG 430 2.0 Array (Affymetrix). From the interested candidates we pick out neurod1 as our first priority to focus on. We developed autograft tumor from MYCN hemizygous mice. We also developed tumor sphere formation to manipulate neurod1 expression in vitro. For knocking down neurod1 expression we use shRNA against neurod1. We inoculated autograft tumor sphere treated or not by neruod1 shRNA to nude mice to check tumorigenesis.
Results: 626 genes were upregurated in hyperplasia ganglia comparing to normal ones. Among them, 15 genes, containing 6 transcription factors, were upregulated more than 10 times. Among them neurod1 was an important transcription factor which had already been reported to be essential for the survival and maturation of adult-born neurons. We used human NB cell lines to evaluate its role in tumor growth. Data showed that neurod1 could enhance NB cell survival and increase the proportion of S and G2/M phase in the cell cycle. Compared to control group autograft tumor spheres whose neurod1 expression knocked down by shRNA showed lower growth speed and sphere formation speed. Data from in vivo tumorigenesis experiment also shows neurod1 is essential for tumor development.
Conclusion: Neurod1 is involved in the tumorigenesis of neuroblastoma at an initial stage. Targeting neurod1's expression may be helpful to cure this diease.

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POB58

An observation of chromosomal abnormalities and MYCN and AURKA gene changes in neuroblastoma patients

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Background: Neuroblastoma is an embryonal tumor of the sympathetic nervous system. Numerous gene abnormalities and the MYCN gene are known to be the causative effects in the formation of neuroblastoma. The AURKA gene is claimed to have an influence on the MYCN gene. We aimed to investigate these genetic changes considered to play a role in the etiology of neuroblastoma.
Methods: 25 patients diagnosed neuroblastoma were investigated in University of Cukurova Medical Faculty, Pediatric Hemathology/Oncology Department. The control group consisting of 25 children with the same age and gender and without a family history of cancer were investigated in cytogenetic terms.
Results: The MYCN and AURKA genes in the paraffine tissue of 9 (36%) patients were determined through the FISH technique. The chromosomal abnormalities in both patients and the control group were investigated in blood samples through the utilization of standard cytogenetic procedures. Of 21 (%84) patients had chromosomal abnormalities. 18,4% of the cells of the patient group and 2,6% of the control group showed chromosomal defects. The difference between the patient and the control group was considered to be statistically significant (p<0,0001). It was reported that 72% of these abnormalities were structural while that of 28% were quantitative. Of these abnormalities, 1q21, 1q32, 2p24, 2q21, 2q31, 4q31, 9q11, 9q22, 13q14, 14q11, 14q24 and 15q22 were identified to be critical regions in the formation of NB. These areas were also reported to be the hot regions where oncogenes and protooncogenes are present and are involved in the etiology of neuroblastoma. Paraffine tissue was studied in 6 (66,7%) of the 9 patients, MYCN and AURKA gene amplifications were identified in 85.7% of stage IV patients. The percentages obtained in the present study were found to be related with literatures.
Conclusion: 1q21, 1q32, 2p24, 2q21, 2q31, 4q31, 9q11, 9q22, 13q14, 14q11, 14q24, 15q22 and the increase in MYCN and AURKA gene amplification could be regarded as important criteria in the diagnosis and prognosis of neuroblastoma.

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POB59

Chromosomal instabilities in a neuroblastoma patient

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Aim: To investigate chromosomal aberrations in blood samples of 2 years-old boy diagnosed with grade IV neuroblastoma.
Introduction: Neuroblastoma is an embryonal tumor and originates in precursor cells of the sympathetic neural tissue, accounting for eight percent of all neoplasia in childhood. The biology underlying the clinical heterogeneity of neuroblastoma is directed to histopathologic and genetic abnormalities. Many recurrent chromosome abnormalities associated with poor clinical outcomes have been identified in neuroblastoma. Several genetic abnormalities have been reported as prognostic markers. (1).
Method: GTG-banding in blood samples was performed.
Results: Hidden recurrent deletions and translocations in various chromosomes were detected. All deletions were unbalanced. The most prevalent recurrent unbalanced deletions resulted in del(1)(p32-pter) del(3)(cen-qter)(1/50), del(2)(q31-qter)(1/50), del(3)(q11-qter)(1/50), del(7)(q11.1-qter)(1/50), del(9)(q11.1-qter)2/50)X2, del(9)(q13-qter)(1/50), del(10)(q24-qter)(1/50), del(12)(p11-qter)[2](1/50), 46,XY,del(13)(q14.3-p13)(1/50), del(15)(q25-qter)(1/50), del(18)(q21-qter)(1/50), del(19)(q13.3-qter)(1/50). Translocations [46,XY,der(3)t(3;12)(p26;q15-qter)(1/50), 46,XY,der(11)t(11;18)(q25;pter-q21)(1/50),] and marker [47,XY,+ace,del(2)(q31-qter)(1/50)] were found the most frequent chromosomal abnormalities. Furthermore, sex chromosome aneuploidies were the other significant result [47,XYY(1/50)] in this case.
Conclusion: More than one chromosomal abnormalities in the same cell and del(9)(q11.1-qter) in two different cells were remarkable. These findings may be corresponded that why resistance to the treatment and distance metastasis were seen in our case. Therefore, recurrent deletions and translocations in various chromosomes should be taken into account among poor prognostic factors in neuroblastoma patients.

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POB60

Constitutive activated *Hen2* expression in neural crest cells could be a trigger of neuroblastoma development

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Background: *Mash1* is one of the cell fate determination genes of the sympathoadrenal lineage. Its expression is abnormally upregulated especially in aggressive neuroblastomas. However, the molecular mechanism remains unclear. We have reported that LIM-only protein, LMO3 and basic helix-loop-helix protein, HEN2 act as oncogenes by upregulating *Mash1* expression in neuroblastoma cells by suppressing the inhibitory function of HES1 against *Mash1* transcription (ANR2006). To confirm these previous results *in vivo*, we made transgenic mice of *Hen2* and *Lmo3* and examined the phenotypes.
Methods: Transgenes mouse *Hen2* and mouse *Lmo3* were expressed under the control of mouse *Wnt1* promoter. Neural crest cells (NCCs) were prepared from intestine of transgenic mice and the littermate control embryos because the preparation and culture are easier than that from neural tube. Differentiation of NCCs was induced by culturing in low mitogen and growth factor culture medium. Differentiation, proliferation and cell death were examined by immunostaining, BrdU incorporation and TUNEL assay.
Results: When differentiation was induced, proliferation and survival rates of NCCs prepared from transgenic mice embryos were increased as compared with those prepared from littermate controls. Differentiation rates of these transgenic NCCs were decreased and maturely differentiated less than wild-type cells. The Mash1-positive cells were slightly increased in transgenic NCCs. Because continued expression of Mash1 is recently reported to be involved in proliferation of sympathetic neuroblasts, constitutive expression of *Hen2* could be a cause of promoted proliferation of NCCs. Since hydrocephaly was developed in *Hen2* and *Lmo3* transgenic mice, similar effects of *Lmo3* on proliferation, survival and differentiation of NCCs were expected.
Conclusions: We previously indicated HEN2 upregulates expression of *Mash1* by suppressing the inhibitory function of HES1 against *Mash1* transcription in neuroblastoma cell lines. The present study also suggested that this activity of mouse *Hen2* may exist in NCCs and that HEN2 could act as a trigger of neuroblastoma in NCCs.

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Posters

POB61

A novel orphan receptor, NLRR3, induces neuronal differentiation and is negatively regulated by MYCN and Miz-1 in neuroblastoma

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Background: The human *NLRR* family genes were originally identified from the cDNA project of neuroblastoma (NB) we generated as the genes whose expression is differential between favorable and unfavorable NBs. The *NLRR* family genes encode orphan receptors with unknown function. Our preliminary data showed that expression of *NLRR1* is high in unfavorable NBs, whereas that of *NLRR3* is high in favorable NBs. However, their precise molecular mechanisms remain elusive.
Method/approach: Luciferase reporter, transcriptional profiling, immunoprecipitation and chromatin immunoprecipitation (ChIP) assays were performed to investigate the transcriptional regulation of *NLRR3* by MYCN. Quantitative real time PCR was applied to examine the mRNA expression.
Results: Expression of *NLRR3* mRNA was significantly high in favorable NBs as compared to unfavorable ones (n=36 vs 50, p<0.001), whereas that of *NLRR1* was high in unfavorable NBs (p=0.028). The multivariate analysis showed that expression of *NLRR3* was an independent prognostic factor. The immunohistochemical study showed that NLRR3 is strongly positive in favorable NB cells, though it mainly localized in the cell nuclei. NLRR3 was up-regulated during retinoic acid-induced differentiation of RTBM1 NB cells accompanying with down-regulation of endogenous MYCN, whereas it was decreased after induction of MYCN expression in SH-SY5Y NB cells. In addition, expression of NLRR3 was down-regulated by overexpression of MYCN and was up-regulated by knockdown of MYCN at both mRNA and protein levels. Furthermore, like *NLRR3,Miz-1*, which is a co-repressor of MYCN, is highly expressed in favorable NBs (p<0.0004), suggesting that *NLRR3* expression may be regulated by MYCN and Miz-1. Indeed, MYCN repressed transcription of *NLRR3* in cooperation with Max and Miz-1. The chromatin immunoprecipitation (ChIP) analysis also demonstrated that MYCN, Max and Miz -1 are recruited on to the promoter regions of *NLRR3* gene and act as a transcriptional repressor complex for NLRR3 expression.
Conclusion: NLRR3 regulates neuronal differentiation and survival and its expression is negatively regulated by MYCN in association with Miz-1 in aggressive NBs.

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POB62

Comprehensive screen for genes involved in tumorigenesis and tumor-initiating cell formation in MYCN Tg mice

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Background: MYCN transgenic (Tg) mouse is a model for neuroblastoma. These mice spontaneously develop neuroblastoma whose origin is superior mesenteric ganglion (SMG). Based on the gene expression and chromosomal aberration patterns in MYCN Tg mice, we are trying to identify the genes involved in tumorigenesis and tumor-initiating cell (TIC) formation. Those genes must be potent targets for molecular therapy and prognosis prediction.
Method/approach: In order to examine the chromosomal aberration, we carried out CGH array analysis (Agilent, 44K) with 12 tumors from MYCN Tg mice. Spleen from each mouse was supplied for the comparative normal tissue. On the other hand, we prepared tumors from MYCN Tg mice, and tumor spheres cultured from those tumors. Tumor sphere contains putative TICs. We carried out gene expression array analysis (Affymetrix, MG430 2.0 Array) with those samples to identify the TIC-specific genes.
Results: CGH array analysis showed that the gene C was deleted in all 12 tumors. Although there is no report about the relationship between the C and neuroblastoma, its function is implicative enough. C is essential for the normal differentiation of neural crest-derived cells. We also picked up 43 genes whose expressions are up-regulated in tumor spheres, and 48 genes down-regulated. ALK expression was remarkably increased from normal SMG to original tumor, and also from original tumor to tumor spheres. Kitl (also known as stem cell factor: SCF) was expressed in original tumor, but its expression was totally absent in tumor spheres.
Conclusion: As to gene C, we hypothesize that its deletion is required for neuroblastoma cells to keep undifferentiated state. ALK expression is markedly up-regulated in MYCN-driven neuroblastoma and increased further in TICs. ALK might be a downstream factor of MYCN, and has some function in TICs. Although Kitl has been reported to be expressed in neuroblastoma and be a potent target for molecular therapy, our results suggest that Kitl is a marker for mature tumor cells. Taken together, through our screen targeting TIC, we have obtained many candidates which could be targets for molecular therapy.

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POB63

Common pathways in neuroblastoma and early-onset breast cancer

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Background: Recent experience of families with cases of early-onset breast cancer (BRC) and neuroblastoma (NBL) has suggested that mutations or copy number changes in the same loci may predispose both diseases. While it is likely that interactions between higher frequency alleles factor into the development of NBL and BRC, it is difficult to predict such interactions without the use of novel methods. To test the hypothesis that BRC and NBL carry mutations in the same pathways, we have begun a systematic reanalysis of genome wide association study (GWAS) results from NBL and BRC.
Method/approach: We combined p-values from both GWAS using Fisher's method, and used empirical distribution-free statistical testing to search for enriched Gene Ontology categories in each GWAS. Over 2,000 Gene Ontology categories were tested for enrichment of significant associations, and enrichment testing results were adjusted for false discovery rate (FDR).
Results: While there appeared to be relatively little overlap of significant associations between the two studies at the individual SNP level, gene ontology analysis indicated a strong over-representation of highly significant SNPs near genes encoding proteins required for cell adhesion, ion transport, and transmembrane receptor tyrosine kinase signaling in both BRC and NBL (p_{FDR}<0.015).
Conclusion: Gene Ontology analysis of GWAS results from BRC and NBL suggests that the activation of different genes in many of the same pathways affects cancer progression similarly in both diseases. Because exclusive mutations may lead to common outcomes, identification of the interactions within members of these pathways could provide a molecular basis for shared predisposition.

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POB64

Neuropeptide Y in neuroblastoma - Interactions with BDNF and effect on cell survival

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Background: Neuroblastomas (NBs) release the sympathetic neurotransmitter neuropeptide Y (NPY), of which elevated plasma levels in NB patients were associated with a poor clinical outcome. In agreement with this, we have shown that endogenous NPY stimulates proliferation of NB cells and tumor angiogenesis via its Y2 receptors (Rs), while blocking Y2Rs inhibits tumor growth. The goal of this study was to determine the interactions of the NPY system with another known growth-promoting factor in NB - BDNF.
Methods: Gene expression was measured by real-time RT-PCR, cell viability by MTS assay and apoptosis by caspase 3/7 activity.
Results: The study was performed on NB cells derived from primary (CHLA-15, SMS-KAN) and post-therapy tumors (SK-N-BE(2), CHLA-20, , SMS-KANR) expressing the functional BDNF R, TrkB, as determined by RT-PCR and MAPK activation upon BDNF treatment. We have shown that BDNF up-regulates the expression of NPY and its Y2Rs in NB cells and, more dramatically, induces expression of Y5Rs, which are not detectable in most NB cell lines under basal conditions. In agreement with this, expression of Y5Rs correlated with TrkB expression in human NB samples. Since BDNF is a known survival factor for NB cells, implicated in its resistance to chemotherapy, we sought to determine if pro-survival activity of BDNF is mediated by NPY. Y2R antagonist, and even more dramatically Y5R antagonist, reduced the anti-apoptotic effect of BDNF in chemotherapy-treated NB cells, while NPY mimicked the effect of BDNF. Moreover, treatment with chemotherapy up-regulated the expression of both systems - BDNF and TrkB, as well as NPY and its Rs. This was further supported by elevated expression of NPY and Y5Rs in cells derived from chemotherapy-treated patient (CHLA-20) as compared to cells derived from primary tumors of the same patient (CHLA-15).
Conclusion: While Y2Rs are the main NPY Rs constitutively expressed in NBs and responsible for its proliferative effect, expression of Y5Rs is induced by BDNF and chemotherapy and enhances the pro-survival functions of the peptide. These anti-apoptotic actions of NPY can additionally augment the known direct survival effects of BDNF.

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POB65

Involvement of the CXCL12/CXCR4/CXCR7 axis in the malignant progression of human neuroblastoma

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Background: Chemokines and their receptors, particularly the CXCR4/CXCL12 axis, have been involved in tumour progression in several cancers including neuroblastoma. We previously reported a tumour type-specific and microenvironment-related growth-promoting role for the CXCR4 receptor. Growth-promoting effects were highly significant only when NB cells were orthotopically implanted in adrenal glands of nude mice, thus highlighting the impact of the tumour microenvironment on the CXCR4-mediated NB growth. We further explored the participation of tumour microenvironment in the behaviour of CXCR4-expressing cells, by 2D co-culture strategy. We also addressed the role of CXCR7, the recently identified second CXCL12 receptor. Although reported to confer atypical properties to cancer cells, the role of CXCR7 in the cross-talk with the microenvironment is still unknown.
Method/approach: A 2D co-culture of CXCR4 expressing NB cells and fibroblasts was developed. CXCR7 participation in the CXCR4/CXCL12/CXCR7 axis was analysed by gain of function strategies and in vitro differentiation assays.
Results: When co-cultured on a layer of fibroblasts prepared as a human immortalized and irradiated cell line, CXCR4 overexpressing cells displayed a significantly faster but ligand-independent growth as compared with controls. In parallel, a screening of NB tumour samples revealed a CXCR7 staining pattern as specifically associated to differentiated and/or mature cells of the tumour. NB cell lines showed a specific CXCR7 expression, which increased upon exposition of cells to differentiation agents. Moreover, overexpression of CXCR7 was shown to support a ligand-independent in vitro survival and growth of NB cells. In contrast, when co-expressed with CXCR4, CXCR7 led to a decrease of NB growth which was only observed in presence of CXCL12.
Conclusion: This study confirms the microenvironment-related growth-promoting role for CXCR4. Our preliminary data also indicate that CXCR7 expression may reflect the tumour differentiation pattern, and suggest that CXCR7 may play a regulatory role in the CXCR4/CXCL12 axis activation.

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POB66

Mycn metabolic programs enforce glucose more than glutamine addiction in neuroblastoma cells

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Background: MYC reprograms cells toward glycolysis and glutaminolysis to support energetics and macromolecular synthesis. We investigated MYCN dependencies in NB as addiction to these substrates has been theorized.
Methods: RNA expression from 101 NBs of Low-risk (LR), High-risk without (HR) and with MYCN amplification (HR+A) were assessed. MYCN-inducible RPE1-MYCN (neural control), SHEP-MYCN, SKNAS-MYCN, and 10 NB cell lines (7 MYCN Amp) were studied under conditions of +/- glucose (Glu), +/- glutamine (Gln), and with rescue with Gln or α-ketoglutarate (aKG). Phenotype was by morphology and cell kinetics (RT-CES and MTT). LC-MSMS (LTQ-Orbitrap) was used for 10 NB cell lines against a SILAC-labeled reference pool for quantitative protein expression.
Results: >90% of Glu/Gln/TCA enzymes were quantifiable at the mRNA and protein level. Glu transporters are not differentially expressed among LR, HR or HR+A NBs, yet HR+A NBs overexpress 7 of 10 glycolytic enzymes including all 3 regulated enzymes (HK2, PFKM, PKM2; $p<0.001$). HR+A NBs also have altered LDHA/B, PDHA1 and PDK increasing pyruvate flux to both lactate and the TCA cycle. Glu deprivation of cells led to florid apoptosis enhanced by MYCN, but also seen in all NB cell lines. In contrast, Gln transporters (SLC1A5, SLC7A5), GLS2 and GOT2 are upregulated in HR+A NBs ($p<0.001$) preferentially shunting Gln to the TCA cycle for biosynthesis (anaplerosis). Gln deprivation growth inhibited only a subset of NB cells (also MYCN-dependent). All cells sensitive to Gln deprivation could be rescued fully by Gln (4mM) but not by aKG (7mM), suggesting Gln was not utilized solely for TCA substrates. Asparaginase has glutaminase activity, and all NB cells were L-Asp sensitive (<5 IU/ml), even those resistant to Gln deprivation, suggesting asparaginase is essential to NB.
Conclusion: NBs with MYCN amplification have co-ordinate high flux through Glu and Gln metabolism. NBs are better able to compensate for reduced Gln relative to Glu, contrary to emerging predictions from c-Myc. Glu metabolism can sustain TCA intermediaries in the absence of Gln, whereas Gln cannot provide the energetic needs of all NBs in the absence of Glu.

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POB67

Haploinsufficiency of candidate tumor suppressor gene CASZ1 blocks embryonic stem cell neurogenesis

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The human homolog of the Drosophila neural fate-determination gene CASZ1 is a zinc-finger (ZF) transcription factor and maps to chromosome 1p36.22, a region implicated in neuroblastoma (NB) tumorigenesis. CASZ1 is highly expressed in good prognosis NB tumors with a differentiated histopathology. Retinoic acid and epigenetic modulators induce CASZ1 expression during NB cell differentiation. CASZ1 has two major protein isoforms, hcasz5 (CASZ1b) and hcasz11 (CASZ1a), which contain 5 and 11 zinc fingers, respectively. Restoration either hcasz5 or hcasz11 in NB cells enhanced cell adhesion, inhibited migration and suppressed tumor growth in in vitro soft agar colony formation assays and in vivo tumor growth in mouse xenografts. Expression profiling revealed that >95% of hcasz5 target genes overlap with hcasz11 targets and are involved in cell proliferation and developmental processes. Consistent with their function at suppressing tumor growth, both hcasz5 and hcasz11 induced NB tumor suppressor genes NGFR (~15-fold), TrkA (~2-fold) and clusterin (~10-fold). To test the impact of loss of CASZ1 isoforms on neural differentiation, we utilized in vitro Embryonic Stem (ES) cell differentiation models. Wild type ES cells (CASZ1+/+) formed Embryoid Bodies (EBs) that can be induced to differentiate to neuronal cells that express beta III tubulin. However, ES cells hemizygous for CASZ1 (CASZ1+/-) expressed 50% less CASZ1 mRNA and formed ½ the EBs compared to wild type ES cells. Moreover, CASZ1+/- ES cells failed to differentiate into neuronal cells. CASZ1+/- and CASZ1+/+ ES cells had similar alkaline phosphatase activity (a marker of ES cell self-renewal) and the loss of CASZ1 did not affect the expression of critical self-renewal genes c-Myc, Oct3 and Nanog. Thus CASZ1+/- ES cells have self-renewal properties similar to wild-type cells but have a block in their ability to form EBs and differentiate into neural cells. This study demonstrates that CASZ1 levels are critical for neuronal differentiation and supports the hypothesis that haploinsufficiency of CASZ1 contributes to neuroblastoma tumorigenesis.

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POB68

Screening of ALK mtations and abnormalities in neuroblastoma cell lines and Italian neuroblastoma cases

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Background: The Anaplastic Lymphoma Kinase (ALK) has emerged as a gene playing a pivotal role in at least a subset of neuroblastomas (NB). We screened for ALK tyrosine kinase (TK) mutations additional 71 Italian sporadic NB cases to those already reported [1]. Furthermore, we screened individuals from 6 novel Italian families with recurrent NB and a panel of 30 NB cell lines, which were also partially investigated for alk expression. Moreover, we sought for other mechanisms of ALK activation such as gain/amplification and translocations in sporadic NB cases and cell lines.
Methods: Direct sequencing for ALK exons 20-28 was performed by BigDye Terminator v1.1 kit on the ABI-Prism 3130 genetic analyzer. Immunoblots for ALK and pALK were performed on total protein lysates. ALK and MYCN gain was investigated by Multiplex Ligation-dependent Probe Amplification (MLPA). ALK rearrangements were studied by FISH analysis using the Vysis LSI ALK Dual Color, Break Apart Rearrangement Probe.
Results: Overall, we detected 6/114 (5.3%) ALK mutations in Italian NB population and 5 of these alterations occur at amino acids F1174 and R1275, which are the two most frequently impaired. We also found a novel missense mutation, 3509T>G (I1170S), which we already reported [1]. Moreover, we detected the R1192P ALK mutation in a recently collected NB family. So far, the latter mutation has only been reported for familial cases. Eight out of 30 (26.7%) NB cell lines showed F1174L and 1/30 (3.3%) had F1275Q. All mutated NB cell lines but LA1-5S (F1174L) had detectable levels of ALK and pALK. MLPA analysis carried out on 112 NB cases without MYCN gain revealed no ALK gain too. On the contrary, 31 out of 33 (93.9%) MYCN gain NB cases showed also ALK gain. Finally, FISH analysis performed on 8 congenital and familial NB revealed no ALK translocation. However, Kelly NB cells were confirmed to carry a deletion of the ALK TK telomeric region.
Conclusion: In summary, our study adds information about the involvement of ALK in NB, although further efforts should be made to define all the possible mechanisms that impair ALK activity.
[1] Passoni L et al. Cancer Res. 2009. 69(18):7338-46.

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Posters

POB69

Ephrin/Eph signaling in neuroblastoma tumor initiating cells

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Background: Cell-cell communication via the ephrin/Eph pathway plays a central role in development, mediating guidance and stabilizing tissue borders. Ephrin ligands and Eph receptors are misexpressed in most cancers. In neuroblastoma, high expression levels of EphB6, ephrinB2, and ephrinB3 predict a favorable outcome. Tumor initiating cells (TICs) are highly tumorigenic cells that have the ability to self-renew and differentiate and are thought to be involved in treatment resistance and disease relapse. TICs have been isolated from neuroblastoma bone marrow metastasis. Our guiding hypothesis is that Eph/ephrin signaling modulates the neuroblastoma TIC state. The knowledge gained has the potential to aid in the development of strategies that target ephrin/Eph in the treatment of neuroblastoma. We aim to investigate the role of ephrin/Eph signaling on the proliferation, differentiation and migration of neuroblastoma tumor initiating cells (NB TICs) as well as the cross talk between growth factors and ephrin/Eph signaling in NB TICs.
Methods: NB TICs have been cultured in vitro and the ephrin/Eph expression was analyzed by quantitative PCR. A novel assay is being developed that allows for systematic and accurate analysis of the roles of ephrin/Eph in NB TIC biology, based on the formation of microislands of NB TICs surrounded by substrate immobilized ephrin ligands and Eph receptors.
Results: We have mapped the expression of ephrin ligands and Eph receptors in NB TICs by quantitative PCR, which shows significant overlap with the previously reported expression profile of ephrin/Eph of neural crest stem cells. Furthermore, we have implemented a method to immobilize patterns and gradients of proteins. This is the corner stone for the development of an assay to systematically investigate NB TIC responses to ephrin/Eph presentation.
Conclusion: NB TICs show an ephrin/Eph expression profile similar to that of neural crest stem cells. Ephrin/Eph family of membrane receptors tyrosine kinases presents potential therapeutic targets for neuroblastoma and therefore it is important to understand NB TIC responses to ephrin/Eph signaling.

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POB70

TRIM16 acts as a tumour suppressor via inhibitory effects on cytoplasmic vimentin and nuclear E2F1 in neuroblastoma cells

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The family of tripartite-motif (TRIM) proteins are involved in diverse cellular processes, but are often characterised by critical protein-protein interactions which are necessary for their function. TRIM16 expression levels are increased in several cancer types, particularly when the cancer cell is forced to proceed down a differentiation pathway. We have identified TRIM16 as a DNA binding protein with histone acetyl transferase activity, which is required for the retinoic acid receptor β_2 transcriptional response in retinoid-treated cancer cells. Importantly, TRIM16 overexpression can confer retinoid sensitivity upon retinoid resistant cancer cells. Here we examined the potential role of TRIM16 as a tumor suppressor. We found that overexpressed TRIM16 in BE(2)-C neuroblastoma tumor cells reduced cell viability and anchorage-independent growth. Moreover, TRIM16 overexpression substantially decreased neuroblastoma tumour growth, compared to controls ($P < 0.01$). Expression of TRIM16 expression enhanced retinoid-induced neuritic differentiation of neuroblastoma cells in vitro, and, was significantly stronger in the more differentiated component of human neuroblastoma tissues, compared to poorly differentiated regions ($P < 0.001$), across all clinical stages. We used a TRIM16-specific antibody in immunoprecipitation and liquid chromatography-tandem mass spectrometry analyses, to identify vimentin and E2F1 as candidate TRIM16-binding partners. TRIM16 bound directly to cytoplasmic vimentin and nuclear E2F1 in neuroblastoma cells. TRIM16 expression reduced neuroblastoma cell motility, and, this was dependent on down-regulation of vimentin expression. Retinoid treatment and enforced overexpression, caused TRIM16 to translocate to the nucleus, and there bind to and down-regulate nuclear E2F1 levels, which was required for cell replication. This study demonstrates that TRIM16 acts as a tumour suppressor, affecting neuritic differentiation, cell migration and replication through simultaneous interactions with cytoplasmic vimentin and nuclear E2F1 in neuroblastoma cells.

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POB71

High-risk neuroblastoma without MYCN amplification - 11q-deletion tumors reveal a poor prognostic chromosome instability phenotype with later onset

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Analysis of chromosomal aberrations is used to predict clinical prognosis of children with neuroblastoma and to stratify risk-based therapy. MYCN amplification (MNA) alone is incomplete as a poor prognostic factor and 11q status has recently been included in risk classification. We analyzed 170 neuroblastomas using high-density SNP microarrays and describe and compare the high-risk groups defined by MNA (n=37) and 11q-deletion (n=21). Median age at diagnosis was 21 months for the MNA group and 42 months for 11q-deleted, while median survival from diagnosis was 16 months for MNA and 40 months for 11q-deletion. Overall survival was similarly poor, 35% at eight years for both groups. MNA and 11q-deletion are almost mutually exclusive; only one tumor harbored both aberrations. The numbers of segmental aberrations differed significantly; the MNA group had a median of four aberrations, while the 11q-deleted group had 12. The high frequency of chromosomal breaks in the 11q-deletion group is suggestive of a chromosomal instability phenotype gene located in 11q, and one such gene, H2AFX, is located in 11q23.3 (within the 11q-deleted region in all tumors). Furthermore, in the groups with segmental aberrations without MNA or 11q-deletion, children with tumors with 17q gain had worse prognosis than those with segmental aberrations without 17q gain, who had a favorable outcome in our material. This study has implications for understanding of neuroblastoma tumor genetics, prognostic assessment and choice of therapy for different risk groups and stresses the use of genome wide microarray analyses in clinical management to evaluate patient diagnosis, risk and treatment.

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POB72

Appearance of the novel activating F1174S ALK mutation in neuroblastoma correlates with disease progression, aggressive tumour behavior and unresponsiveness to therapy

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Background: Mutations in the kinase domain of Anaplastic Lymphoma Kinase (ALK) have recently emerged as important players in neuroblastoma genetics, both in familiar and sporadic subsets. Here we report the appearance of a novel ALK mutation in neuroblastoma, correlating with disease progression and aggressive tumour behaviour.
Methods: A sporadic MYCN-nonamplified infant INSS stage 4 neuroblastoma assessed as INRG intermediate risk was treated with chemotherapy according to SIOPEN infant protocol 99.3. At disease progression 9 months from diagnosis resistant disease emerged and the tumour genetics was reevaluated.
Results: Analyses of genomic DNA from biopsy samples initially showed ALK sequence to be wild-type (homozygous 3521T/3521T i.e. F2274/F1174). However, during disease progression mutation of amino acid F1174 to a serine within the ALK kinase domain was observed, which correlated with metastatic progression and resitance to chemotherapy. Thus, the DNA from tumor 9 months after diagnosis was homozygous for mutation 3521T>C, i.e. F1174S/F1174S. We show here that mutation of F1174 to serine generates a potent gain-of-function mutant, as observed in two independent systems.
Firstly, PC12 cell lines expressing ALKF1174S displayed ligand independent activation of ALK and further downstream signaling activation. Secondly, analysis of ALKF1174S in Drosophila models confirms that this mutation mediates a strong rough eye phenotype upon expression in the developing eye.
Conclusion: We report a novel neuroblastoma ALKF1174S mutation, which displays ligand independent activity in vivo, correlating with aggressive clinical disease progression resistant to further therapy and oncogenic gain of function in different models.

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POB73

An integrative genomics screen uncovers ncRNA T-UCR functions in neuroblastoma tumours

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Background: Different classes of non-coding RNAs, including T-UCRs, have recently been implicated in the process of tumourigenesis.
Method/approach: In this study, we designed an RT-qPCR based T-UCR profiling platform to examine the expression and putative function of a novel class of non-coding RNAs known as transcribed ultraconserved regions (T-UCRs) in neuroblastoma.
Results: Genome wide expression profiling of 481 T-UCRs revealed correlations between specific T-UCR expression levels and important clinicogenetic parameters such as MYCN amplification status. A functional genomics approach based on the integration of multi-level transcriptome data was adapted to gain insights into T-UCR functions. Assignments of T-UCRs to cellular processes such as TP53 response, differentiation and proliferation were verified using various cellular model systems.
Conclusion: For the first time, our results define a T-UCR expression landscape in neuroblastoma and suggest widespread T-UCR involvement in diverse cellular processes that are deregulated in the process of tumourigenesis.

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POB74

Mir-17-92 is a master regulator of TGFβ-pathway activity in neuroblastoma

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Background: The miR-17-92 gene cluster is often activated in cancer cells, including neuroblastoma (Mestdagh et al., Oncogene, 2010). Thus far, the number of known miR-17-92 coding gene targets is relatively small and efforts to identify the downstream effectors have been restricted to the study of only one or two miRNAs from this cluster.
Method/approach: Here, we examined the effects of entire cluster miR-17-92 activation on global protein expression in neuroblastoma. Using quantitative mass spectrometry, we analyzed the response of thousands of proteins upon miR-17-92 activation.
Results: Analysis of the responsive proteins revealed cooperation between individual miR-17-92 miRNAs and implicates miR-17-92 in multiple hallmarks of the tumorigenic program including proliferation and cell adhesion. In addition, we show that miR-17-92 is a potent inhibitor of TGFβ-signaling. By acting both upstream and downstream of pSMAD2, miR-17-92 dampens TGFβ-response in a multifaceted way.
Conclusion: Our results associate impaired TGFβ-signaling to poor outcome of neuroblastoma patients and further elucidate the miR-17-92 – TGFβ connection. In vivo assessment of miR-17-92 activation in mice xenografts is currently ongoing, and results will be presented.

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POB75

Phosphoproteomic and expression analyses of a *MYCN*-amplified neuroblastoma cell line

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Background: When faced with oncogenic signals, cells activate tumor suppressive responses leading to irreversible cell cycle arrest or apoptosis. The *ARF-MDM2-p53* signaling pathway is crucial for this response is subverted in essentially all human cancers. Surprisingly, neuroblastomas rarely demonstrate *ARF* or *p53* inactivation or *MDM2* amplification at diagnosis. We are using genome-wide and proteomic approaches to probe how *MYCN*-amplified neuroblastoma cells tolerate oncogene expression. Methods:
Methods: neuroblastoma cell line, with *MYCN* constitutively over-expressed but silenced under tetracycline control. After incorporation of stable isotope labeled amino acids for quantitation, cells were harvested, phosphoproteins isolated and GeLC-MS/MS performed. Gene expression analysis was performed at 4, 24, or 48 hours using the Hu1.0ST Affymetrix gene array. Results:
Results: proteins, and a subset was analyzed by Western blotting to validate increased amounts of nucleophosmin (*NPM1*), matrin-3 (*MATR3*), and SET protein (*SET*) and decreased amounts of aldehyde dehydrogenase (*ALDH1A1*). Gene expression analysis of revealed a unique profile for *MYCN*-amplified cells, including increased *TAF4B* and decreased *MGP* and *RARRES3*. There was no correlation between gene expression and phosphoproteomic hits, suggesting post-transcriptional regulatory mechanisms. As *NPM* is known to interfere with the *ARF-p53* pathway, we are exploring studies of *NPM* induction. siRNA-*NPM* knockdown in *MYCN*-amplified SKNBE2 cells revealed no differences in proliferation or viability, indicating that suppression of *NPM* expression does not restore *p53*-independent functions.
Conclusion: Our proteomic and gene expression analyses suggest several other interesting targets for study, which are being validated by further proteomic analyses as well as in tumor samples obtained from COG. Modeling of cellular pathways important in *MYCN*-mediated tumorigenesis may identify interesting proteins for further signaling pathway analysis as well as potential targets for therapeutic development.

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POB76

Analysis of cellular mediaters of oncogenic signaling originating from activated ALK in neuroblastoma cells

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We had previously demonstrated that a unique signaling pathway is activated in a subset of neuroblastoma cells under the effect of constitutive activation of ALK (anaplastic lymphoma kinase) due to gene amplification the ALK gene (Miyake et al., 2002). ALK is a receptor tyrosine kinase (RTK) originally found as an oncogenic fusion protein in anaplastic large cell lymphomas and is subsequently shown to be essential for the survival of the neuroblastoma cells with the amplified ALK gene (Osajima-Hakomori et al., 2005). Recently, several reports indicated that activating mutations of ALK alleles are critical oncogenic factors in advanced neuroblastoma, therefore ALK is highlighted as a new target of the therapy of this disease. To investigate the identity of oncogenic signals induced by activated ALK in neuroblastoma cells, we have been analyzed the modification of signaling molecules under the control of the activated ALK were examined. We have previously showed that activating ALK associated with PTB domain of ShcC, followed by the constitutive activation of downstream signals. In this study, it was revealed that ALK formed complex with P130Cas via SH2 domain of ShcC and regulated the phosphorylation of P130Cas in ALK-activating neuroblastoma cell lines. P130Cas is known to be a docking protein contributed tumorigenicity as a potent substrate of oncogenic Src tyrosine kinase. Here it was confirmed that downregulation of p130Cas suppressed the potential of anchorage independent growth and motility of neuroblastoma cells like other tumor cells. Interestingly, ALK habouring activating mutation of F1174L and K1062M, appeared to form more rigid complex with p130Cas comparing to the wild type of ALK, suggesting that this enhanced potential to associate with p130Cas might have some relationship with oncogenic activation of ALK.

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POB77

Development and characterization of ALK dependent cellular models

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Somatic and germline mutations in the ALK gene have recently been described in neuroblastoma. ALK (Anaplastic Lymphoma Kinase) is a receptor tyrosine kinase (RTK) the function of which is poorly understood. In mice, Alk transcripts have been detected in various structures of the embryonic central and peripheral nervous system (PNS). The period of Alk expression coincides with key biological events such as cellular proliferation, neuronal differentiation, programmed cell death, and neuron innervation of muscles. Our goal is to analyze various ALK mutations, localized in or outside the TK domain, in different cellular types. Plasmids expressing the following ALK mutants have been generated by site-directed mutagenesis: M596T in the second MAM domain, D1091N in the juxtamembrane domain, F1174L, R1192G, and R1275Q in the TK domain. To assess the role of these mutations, we use NIH3T3 cells as well as the 13.S.1.24 rat neuroblast immortalized cell line and human neural crest cells, which normally give rise to the PNS, obtained from pharyngulas. Following expression of WT or mutated ALK, different key cellular processes such as cell-cycle progression, survival, differentiation or cellular migration will be investigated. The colony formation ability in soft agar and the capacity to form tumors in nude mice will also be documented. The obtained tumors will be characterized with respect to their histological, genetic and biochemical properties. The ALK dependent signalosome will be analyzed in these different systems, in particular the PLCγ, PI3K/AKT, RAS/MAPK and JAK/STAT pathways. Such models will allow to identify the critical pathways involved in ALK signaling and participating in neuroblastoma oncogenesis, and should be extremely useful and powerful for testing anti-ALK therapeutics.

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POB78

Nucleotide excision repair and *in vivo* neuroblastoma chemoresistance to irinotecan

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Background: Acquired drug resistance is a major obstacle to successful treatment of neuroblastoma (NB) by chemotherapy. To study the mechanisms of resistance to irinotecan in a therapeutic setting, we have established a resistant NB xenograft model in vivo. Resistance was induced by the topoisomerase I inhibitor irinotecan through sequential and repeated administration in tumor-bearing mice. Resistance was reversible after irinotecan treatment was stopped. The classical mechanisms of topoisomerase I inhibitors resistance were not involved and lack of cross-resistance with alkylating and platinating agents suggested that cell death mechanisms downstream DNA damage remained functional (Calvet et al. Br J Cancer. 2004; 91, 1205-1212).
Methods: We investigated events leading to cell death after DNA damaging treatment. DNA damage after irinotecan exposure and irradiation were compared in resistant IGR-NB8R and sensitive parental IGR-NB8 xenografts by following the induction of γ-H2AX (a DNA double-strand break (DSB) marker). The early cell fate decision after irinotecan injection at 15, 30 and 60 minutes was investigated at RNA level, using gene expression profiling by Agilent 44K array.
Results: Irinotecan and irradiation-induced DSBs led to a more transient phosphorylation of H2AX in IGR-NB8R as compared to IGR-NB8, suggesting either an aberrant signaling of the DSBs or a faster DNA repair in resistant tumors. Moreover, gene expression profiling revealed an implication of JNK survival pathway and an activation of several DNA repair mechanisms, primarily the nucleotide excision repair (NER).
Conclusion: In vivo resistance to irinotecan is due to very efficient DNA repair that could be initiated very early through the JNK pathway. To our knowledge, this is the first report showing that NER is implicated in the in vivo resistance to topoisomerase I inhibitors. Interestingly, effectors of NER are known to participate in DSB repair and could also remove DNA-topoisomerase I cleavage complexes. Our findings provide a strong rationale for investigation of NER and JNK pathway inhibition to reverse chemoresistance in our model.

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POB79

A role of human Sgol1 on the growth of human neuroblastoma cells

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Background/Aims: Shugoshin (Sgo) protein was first identified in fission yeast as a protector of centromeric cohesion and is required for accurate chromosome segregation. Like fission yeast protein, human Sgo proteins (hSgo1 and hSgo2) are essential to accurate chromosome segregation during both mitosis and meiosis. However, little is known whether hSgo1 is involved in cancer. The aim of this study is to reveal the role of hSgol1 in neuroblastoma cells. MYCN is known to be amplified in many neuroblastoma cells.
Methods and results: We have compared the gene expression profiling using microarray among the tissue specimen from ganglion, hyperplasia and tumor respectively. According to the microarray results, we found that hSgo1 was expressed >5 folds in hyperplasia and tumor samples than in normal ganglion. Next we checked the mRNA level of hSgo1 in human neuroblastoma cell lines. We found that hSgo1 expression was higher in MYCN amplified cell lines than MYCN single copy cell lines. To assess the effects of hSgo1 expression on neuroblastoma cell lines, hSgo1 knockdown was performed by transfecting SK-N-AS MYCN single copy cell line and IMR32 MYCN amplified cell line with a short hairpin RNA expression vector. hSgo1 knockdown IMR 32 cells proliferated slowly, but hSgo1 knockdown SK-N-AS cells grew at the same level as control cells.
Conclusions: These findings suggested that MYCN regulates hSgo1 expression and that hSgo1, in cooperation with MYCN, has an important role in growth of human neuroblastoma cells.

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POB80

NF-kB signaling in neuroblastoma

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Background/Aim: The NF-kB is a complex signaling pathway controlling inflammation and immune response, and is involved in the balance between pro- and anti-apoptotic gene expressions. Recently, a major role for NF-kB in tumor proliferation and apoptosis evasion was determined. We were therefore interested whether NF-kB governs these processes in neuroblastoma. It has previously been described that chemo-resistance of neuroblastoma cell lines can be overcome by inhibition of the NF-kB pathway, but it remains to be determined whether the classical (NFKB1/RELA and -/cREL) and/or the alternative (NFKB2/RELB) routes are involved, and which target genes play a role. Affymetrix expression profiles of 88 neuroblastomas generated in our laboratory showed correlations between the mRNA expression of several important NF-kB pathway genes with patient survival and tumor metastasis. These data suggested a role for the NF-kB pathway in neuroblastoma pathogenesis and drug responsiveness.
Methods: Several NF-kB reporter neuroblastoma cell lines were generated. The expression of the five key NF-kB transcription factors (NFKB1, NFKB2, RELA, RELB, and c-REL) was manipulated with shRNA silencing. Gene knock-downs were confirmed on protein and transcriptional activity level. The effects of NF-kB ligand stimulation and TRAIL treatment were examined.
Results: We found that the NF-kB pathway can indeed be constitutively active in neuroblastoma cell lines. However, these cell lines also showed an additional increase in NF-kB activity upon stimulation with IL1B, TNFa, or TRAIL. No activation was detected with the alternative NF-kB pathway ligand BAFF. Silencing of RELA gave strong growth inhibition and abolished TNFa resistance. Silencing IKKa and NFKB2 did not change NF-kB activity, again excluding a role for the alternative NF-kB pathway in neuroblastoma.
Conclusion: We found a role for the classical NF-kB pathway in neuroblastoma cell growth. Gene expression profiling analysis on stimulated cell lines, as well as on cell lines with altered NF-kB transcription factor activity will be used to identify the NF-kB target genes involved in neuroblastoma growth and chemo-resistance.

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POB81

Activation of the transcription factor FOXO3/FKHRL1 by doxorubicin and etoposide induces reactive oxygen species production and programmed cell death in neuroblastoma cells

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Background: FOXO transcription factors are regulators of cell death, cell cycle progression, and stress resistance in neuronal cells and downstream targets of hyperactive PKB in neuroblastoma (NB). FOXO3/FKHRL1 was shown to protect against reactive oxygen species (ROS) by regulating detoxifying enzymes. We investigated the relationship between FOXO3 activation by chemotherapeutic agents, the production of ROS and its involvement in FOXO3-induced apoptosis of human NB cells.
Methods: For studying subcellular shuttling of FOXO3 by live cell fluorescence imaging an ECFP-FOXO3-allele was retrovirally expressed in NB cells. Generation of ROS was assessed by fluorescence staining using the ROS-sensitive dye reduced MitoTracker Red CM-H2XROS. NB cell lines for constitutive or conditional expression of Bim, BclXL, or shRNA directed against Bim and Sestrin3 were generated by retroviral gene transfer.
Results: Etoposide and doxorubicin treatment activates FOXO3, induces ROS and elevates the expression of the proapoptotic proteins Noxa and Bim in NB cells. Conditional activation of FOXO3 induced two sequential waves of ROS the first one being associated with elevation of Bim and Noxa. Knockdown of Bim or retroviral overexpression of the prosurvival BclXL both prevented ROS production and delayed apoptosis which implies that FOXO3-induced ROS is downstream of Bcl2 proteins. The decline after the first ROS wave correlated with increased expression of the peroxiredoxin Sestrin3. Knockdown of Sestrin3 prevented the ROS decline and accelerated cell death in NB cells.
Conclusions: The combined data suggest that programmed cell death by FOXO3 involves ROS production downstream of Bcl2 rheostat and that FOXO3 in parallel activates ROS-protection by Sestrin3. Prolonged FOXO3 activation however overcomes Sestrin3 protection, induces a secondary ROS burst and eventually leads to cell death in human NB cells.

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POB82

Histone deacetylase 10 contributes to the regulation of autophagy in neuroblastoma

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Lysine acetylation is a posttranslational modification of proteins and plays an important role in regulating gene transcription in cells. Lysine deacetylation is regulated by enzymes of the histone deacetylase (HDAC) family. Tumor relevant functions of individual HDAC family members are increasingly discovered. However, little is known about the function of HDAC10.
In order to investigate the relevance of HDAC10 in neuroblastoma cells, we knocked down the endogenous HDAC10 expression with two siRNAs targeting different regions of the respective mRNA. HDAC10 knockdown reduced both, population and clonogenic growth and increased the amount of dead cells. Furthermore, knockdown of HDAC10 expression induced cytosolic vacuolization and the accumulation of autophagosomes and autolysosomes in BE(2)-C cells as evidenced by electron microscopy, LC-3 conjugation and acridine orange staining. Bafilomycin A1, an inhibitor of autophagy, efficiently blocked this phenotype. Enforced expression of HDAC10 reduced autolysosome formation, whereas a catalytically impaired mutant did not. This indicates that the enzymatic activity of HDAC10 controls this process. Finally, autophagy-related genes were found up-regulated in a large series of neuroblastoma tumors associated with good prognosis, suggesting that autophagy may play a role in neuroblastoma tumor biology in vivo.
Our data show that knockdown of HDAC10 induces neuroblastoma cell death and that HDAC10 participates in the regulation of autophagic processes in neuroblastoma cells.

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POB83

Aberrant activation of ALK kinase by a short form ALK protein in neuroblastoma

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Background: Anaplastic lymphoma kinase (ALK) was originally identified from a rare subtype of non-Hodgkin's lymphomas carrying t(2;5) translocation, where ALK was constitutively activated as a result of a fusion with nucleophosmin (NPM). Aberrant ALK fusion proteins were also generated in inflammatory fibrosarcoma and a subset of non-small cell lung cancers, implicated in their pathogenesis. More recently, we and others reported that ALK is also activated constitutively by gene mutations and/or amplifications in sporadic as well as familial cases of neuroblastoma. Here we show another mechanism of ALK activation in neuroblastoma.
Methods: To examine the ALK status in neuroblastoma cells, we performed expression and re-sequence analyses of ALK in a total of 30 neuroblastoma-derived cell lines. Functional analyses of ALK protein were also performed using immunoprecipitation, in vitro kinase assay, colony formation assay, and RNA interference (RNAi)-mediated ALK knockdown.
Results Of the 30 samples, we found a cell line showing an aberrant ALK protein with lower molecular weight. Re-sequencing of the ALK c-DNA from the sample revealed a deletion of exons 2 and 3. The short form of ALK protein was autophosphorylated and exhibited a stronger in vitro kinase activity compared to the wild-type kinase. In addition, the short form of ALK was able to transform NIH3T3 fibroblasts as evident from colony forming capacity in soft agar and tumorigenicity in nude mice. Furthermore, we demonstrated that RNAi-mediated knock down of ALK in the cell line expressing the short from of ALK resulted in suppression of cell growth, indicating its role in the development of neuroblatoma.
Conclusions Our current findings indicate that amino-acid deletion is a novel oncogenic mechnism of ALK in neuroblastoma.

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POB84

MYCN sensitizes human neuroblastoma cells to apoptosis by HIPK2 activation through a DNA damage response

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MYCN amplification (MNA) occurs in about 20% of neuroblastomas (NBs) and is associated with early tumor progression and poor outcome, despite intensive multimodal treatment. However, MNA NBs are not intrinsically resistant to chemo- and radiotherapy, and MYCN overexpression sensitizes NB cells to apoptosis. Thus, uncovering the molecular mechanisms linking MYCN to apoptosis might contribute to designing novel more efficient therapies for MNA NBs. Here we show that, in NB cells, MYCN-dependent sensitization to apoptosis induced by clastogenic agents requires p53 and is associated with p53 proapoptotic phosphorylation at serine 46. The $p53^{S46}$ kinase HIPK2 accumulates upon MYCN expression and its depletion by RNA interference impairs $p53^{S46}$ phosphorylation and apoptosis. Remarkably, MYCN induces an oncogene-dependent DNA damage response that accounts for the inhibition of HIPK2 degradation through an ATM- and NBS1-dependent pathway. The rare occurrence of p53 mutations in NBs and the maintenance of HIPK2 expression in at least 50% of our primary MNA NBs prompted us to evaluate the role of this pathway in NB cell responses to non-genotoxic p53-reactivating compound Nutlin-3. At variance from other tumor histotypes, in MNA NB cells Nutlin-3 strongly induced HIPK2 expression, $p53^{S46}$ phosphorylation and apoptosis, and in combination with bleomycin it purged virtually the entire cell population. Altogether, our data uncover a novel molecular mechanism linking MYCN to apoptosis and add significant advancement to the molecular understanding of the therapeutic potential of p53-reactivating compounds in NB treatment, supporting their use in the most difficult-to-treat subset of NB patient.

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POB85

Expression of TWEAK/Fn14 in neuroblastoma; implications in apoptotic resistance and survival

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Background: Tumor necrosis factor-like weak inducer of apoptosis (TWEAK),a member of the tumor necrosis factor (TNF) family of cytokines, acts on responsive cells via binding to a cell surface receptor called Fn14. TWEAK binding to a Fn14 receptor or constitutive Fn14 overexpression has been shown to activate nuclear factor κB (NFκB) signaling which is important in oncogenesis and cancer therapy resistance.
Methods: Expression of TWEAK/Fn14 was analysed in neuroblastoma (NB) primary tumors and cell lines by RT-PCR, western blot, ELISA and immunohistochemistry. The effect of recombinant TWEAK on NB cells was assessed by analysis of NFκB, Survivin, Bcl2, MMP-9 and gene sliencing of TWEAK/Fn14.
Results: The treatment of NB cells with recombinant TWEAK in vitro causes increased survival and apoptositc resistance. This effect is partly due to the activation of the NFκB signaling and increased expression of anti-apoptotic proteins. TWEAK induced cell survival was significantly reduced by silencing the function of TWEAK/Fn14 by siRNA.TWEAK also induced release of MMP-9 in NB cells.
Conclusion: Expression of TWEAK/Fn14 in NB suggests that TWEAK functions as an important regulator of NB growth, invasion, and survival and that therapeutic intervention of the TWEAK/Fn14 pathway may be an important clinical strategy in helping to regulate neuroblastoma carcinogenesis

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POB86

Green Tea Catechins inhibit neuroblastoma growth in vitro and *in vivo*

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Background: Green Tea Catechins (GTC) are natural compounds with known anti-cancer activity. In recent years many reports have shown that GTC inhibit proliferation and induce apoptosis of various types of cancer. Capsaicin is a natural compound extracted from red peppers. It has been shown that capsaicin can inhibit the growth of xenotransplanted human prostate cancer cells in mice. In this study we investigated whether GTC and capsaicin could suppress the growth and survival of neuroblastoma (NB) cells in vitro and in vivo.
Methods: The effect of GTC (a GTC-containing compound called Polyphenon E) was tested on several NB cell lines in vitro. MYCN-amplified IMR-32 and LAN-1 cells and non MYCN-amplified SHSY5Y and SK-N-AS cells were exposed to GTC and capsaicin as well as combinations of both drugs for 24 hours. Cell death was assessed by trypan blue exclusion assay. In vivo, both xenograft and MYCN transgenic mouse models were used to investigate the anti-cancer activity of GTC. Gene expression profiling of GTC treated and control tumours was carried out to detect genes regulated by catechins in vivo.
Results: We found that both GTC and capsaicin are potent inducers of tumour cell death and simultaneous treatment of NB cells with combination of the drugs resulted in induction of cell death at a level comparable with that of doxorubicin in vitro. Kaplan Meyers survival analysis shows that treatment of MYCN transgenic mice with GTC significantly prolonged their life span in comparison to control group. In the xenograft model, GTC administration delayed tumour growth and reduced tumour size. Affymetrix chip analysis allowed the identification of genes differentially expressed as a result of GTC treatment in both xenograft and MYCN model.
Conclusions: The data presented in this study suggests that GTC are effective inducers of NB cell death and tumour regression in vivo and in vitro. Our results indicate that GTC could be used for chemoprevention of tumour relapse in children with neuroblastoma. Furthermore, the identification of molecular pathways modulated by GTC in vivo should result in the discovery of new targets for therapeutic approaches in neuroblastoma.

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POB87

N-myc gene expression: Impact on leukocyte infiltration in 3D neuroblastoma spheroids

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High N-myc expression is associated with advanced neuroblastoma stage and poor prognosis, but the relationship between N-myc and immunity has remained obscure. Multicellular neuroblastoma spheroids are a 3D in vitro model system that can reflect the pathophysiological in vivo situation of avascular neuroblastoma microregions and micrometastatic sites; in particular the core region of spheroids well mimics hypoxic conditions of neuroblastoma.
To investigate whether N-myc gene expression together with hypoxia affects leukocyte infiltration we used 72 hours cocoltures of peripheral blood mononuclear cells (PBMCs) and preformed 4 days old SHEP21N spheroids. Both the starting spheroids colture and the following spheroids-PBMCs cocolture was grown with (N-myc- , bare SHEP21N N-myc expression) or without (N-myc+ , high SHEP21N N-myc expression) tetracycline. The distribution of hypoxia and leukocyte infiltration was determined from 5-µm-thick paraffin-embedded spheroids sections using respectively monoclonal antibodies to pimonidazole (Hypoxiprobe kit) together with antibodies to hypoxic markers (e.g. HIF1α, HIF2α, VEGF, CaIX) and appropriate leukocyte-specific antibody (CD3, CD20 or CD68). Moreover using Affymetrix GeneChip we studied the differences in gene expression profile of N-myc+ and N-myc-SHEP21N spheroids. The gene expression data were analyzed using GeneSpring GX 7.3 software.
We found that distribution of hypoxic regions and hypoxic markers was similar both in N-myc+ and N-myc- SHEP21N spheroids while infiltration of leukocytes, especially macrophages, was detectable only into N-myc-SHEP21N spheroids. Regarding gene expression experiments we found that some chemokines (e.g. CXCL12 and CXCL14) were upregulated in SHEP21N spheroids that barely express N-myc gene relative to SHEP21N spheroids with high N-myc expression.
Our data suggest a negative correlation between overexpression of a prognostically relevant oncogene, N-myc, and leukocyte infiltrate into neuroblastoma tumors. Therefore N-myc mediated tumorigenesis may be coupled with mechanisms of immune escape that are dependent on N-myc involvement in the regulation of immunologically relevant genes.

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POB88

Use of a microgravity culture system to assess biological behavior in neuroblastoma cell lines

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Background: Clinical and biological features are used to predict outcome in NB patients. However, these assessments are inconsistent. We evaluated a microgravity culture system (rotary bioreactor), which allows NB cells to develop 3D structures (organoids), to predict biological and clinical behavior in these tumors.
Methods: We studied two characteristics of NB that impact malignant potential. First, MYCN amplification was evaluated by comparing cell lines with different MYCN copy numbers (IMR32, NLF, CHP212, SK-N-AS, and SY5Y; 100,25,2,2,2 copies/cell, respectively). Second, the impact of Trk receptor expression was assayed by comparing Trk-null cell line (SY5Y) to subclones transfected to express either TrkA or TrkB. The effect of the Trk inhibitor lestaurtinib (CEP-701) on the parental SY5Y line and its Trk-expressing subclones was also investigated. Single cell suspensions were seeded into the bioreactor (Synthecon) at 5 x 10^5 cells/ml and cultured for eight days. The organoids were evaluated for size and shape, and the single cell count in the media was plotted over time.
Results: Cells aggregated to form tumor-like organoids. Organoid size and shape correlated with malignant potential. MYCN-amplified cell lines formed larger organoids than non-amplified lines (6000, 1000 µm diameter vs. 500, 400, 350 µm), and single cells disappeared more rapidly with MYCN-amplified lines (90% at 2 hrs) vs. non-amplified lines (90% at 8 hrs), suggesting rapid aggregation. SY5Y-null and SY5Y-TrkB cells formed stellate organoids with cellular projections. In contrast, SY5Y-TrkA formed spherical organoids. Growth of SY5Y cells in lestaurtinib altered organoid size and morphology, eliminating the cellular projections when started on day 4, and reducing the size of the organoid when started on day 0.
Conclusions: This 3D culture system provides the assessment of biological and potentially clinical behavior of NB in a more refined way than traditional cell culture. Specific biological characteristics such as growth, cohesion, and morphology can be measured in a reproducible way. This would allow rapid evaluation of different biological perturbations on cellular behavior.

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POB89

A biological link between p53 and MYCN/MYC expression in neuroblastoma

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Background: We previously showed that TSA (an HDAC inhibitor) and Epoxomycin (a proteasome inhibitor) as single agents and in combination significantly suppressed growth of MYCN-amplified neuroblastoma (NB) cells. However, these compounds had contrasting effects on MYCN expression. TSA down-regulated MYCN expression, but Epoxomycin and the TSA/Epoxomycin combination led to MYCN hyper-expression (defined as markedly increased expression beyond that observed in the untreated cells). The expression of p53 was also increased in MYCN-amplified cells treated with Epoxomycin or the TSA/Epoxomycin combination. In this study, we examined (i) the pattern of gene expression induced by MYCN hyper-expression in MYCN-amplified cells, and (ii) a potential functional relationship between p53 and MYCN/MYC in NB.
Methods: Transient transfection of MYCN and TP53 into NB cells was done by electroporation. Gene expression profiling, TaqMan realtime PCR, and Western blot assays were used to detect expression patterns of genes and proteins.
Results: We confirmed that ectopic MYCN expression in MYCN-amplified IMR5 cells resulted in growth suppression. Gene expression profiling analysis revealed that the hyper-expression of MYCN in the MYCN-transfected IMR5 cells led to an increased expression of genes involved in growth suppression and apoptosis (EGR1, EPHA2, KLF2, PERP, SEL1L). The expression of PERP and EPHA2 was confirmed by TaqMan realtime PCR and Western blot assay, respectively. Co-transfection of TP53 and MYCN in IMR5 cells led to high p53 expression but a reduction in MYCN expression (below the levels of endogenous MYCN). Transfection of TP53 into IMR5, SY5Y, and SKNAS reduced endogenous MYCN and MYC expression in these cells. Consistent with these observations, treatment of IMR5 and SY5Y cells with Doxorubicin, CoCl2, or Roscovitine resulted in an increased p53 expression and a reduction of MYCN and MYC expression.
Conclusions: Although high MYCN expression sustains growth of MYCN-amplified NB, the hyper-expression of MYCN is deleterious to survival of these cells. In addition, elevated p53 expression has a suppressive effect on MYCN/MYC expression in NB cells.

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POB90

Mitochondria-related destabilization of MYC family proteins in neuroblastoma cells by OSU-03012, FCCP and Salinomycin

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Background: High-level MYCN/MYC expression is associated with the aggressive behavior of high-risk neuroblastoma (NB). Hence MYCN/MYC destabilizing agents may prove beneficial to these patients. Our previous studies have shown that several small molecules, including Ciglitazone and OSU-03012, can destabilize MYCN and MYC in NB within 3 hours. Although Ciglitazone and OSU-03012 are known to bind PPAR gamma and 3-phosphoinositide-dependent kinase-1, respectively, their effect on the stability of MYC family proteins appears to be unrelated to activity against their cognitive targets. OSU-03012 showed a rapid inhibitory action against the mitochondrial MTS (a tetrazolium salt) reducing activity. On the other hand, Ciglitazone was reported to induce reactive oxygen species though inhibition of mitochondrial electron transport systems. These observations suggest that the rapid destabilization of MYCN/MYC in response to these drugs is related to impaired mitochondrial integrity. In this study, we examined whether MYCN/MYC destabilization by these drugs in NB was linked to mitochondrial disintegrity.
Methods: Western blot assay was used to detect MYCN/MYC. Fluorescence dye based assays were used to assess mitochondrial integrity.
Conclusion: Our data collectively suggest a novel mechanism for destabilizing MYCN/MYC proteins in NB cells that involves mitochondrial disintegrity and/or inhibition of mitochondrial electron transport. We are currently exploring the underlying mechanism of these observations.

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POB91

Nutlin-3 induced miRNA expression changes in neuroblastoma cells

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Background: Less than 2% of neuroblastoma tumors harbor a p53 mutation at diagnosis. The inhibition of p53 by its antagonist MDM2 makes p53 an attractive target for molecular therapy, whereby its release from MDM2 leads to its activation and consequently to cell cycle arrest and apoptosis. A recently discovered antagonist of MDM2, nutlin-3, can specifically target MDM2 and release p53. In this study we aim at identification of potential miRNA mediators of the effects induced by targeted activation of p53.
Method/approach: Using stem-loop RT-qPCR we performed expression analysis of 670 miRNAs in a p53 knockdown model system in NB after nutlin-3 treatment. Upon transfection of NB cells with premirs and antimirs for the deregulated miRNAs, cellular effects as cell differentiation and cell viability assays were evaluated.
Results: Deregulated miRNAs after activation of p53 include mir-223, mir-449a, mir-15a, mir-15b, mir-16, mir-323, mir-424, mir-20b, and mir-133a. Functional validation of a first candidate shows that forced mir-133a overexpression induces differentiation in NGP cells. Some predicted targets of this miRNA include EGFR, NDRG1, BCL2L1, and CSF2; these genes have been reported to be involved in tumorigenesis of several types of cancer.
Conclusion: Further experiments are ongoing to functionally validate the importance of the deregulated miRNAs in the p53-mediated cellular responses. We believe that dissecting the p53 pathway and unraveling miRNAs involved in various p53-mediated cellular responses will help us to understand the pathogenesis of neuroblastoma and to delineate new targets for therapeutic intervention.

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POB92

Oncolytic effects of Sindbis virus on human neuroblastoma cells

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Background: Previously, we reported that Sindbis virus (SIN) AR339 strain has a possibility as a novel agent for human cervical and ovarian cancer therapy. In this study, we examined whether the human neuroblastoma cells are also susceptible to SIN.
Method: We evaluated the oncolytic effects of SIN on human neuroblastoma cell lines and its therapeutic efficacy in vivo against human NB cells transplanted into nude mice. And to determine the major components of SIN involved in the oncolysis, we constructed expression plasmids encoding the structural proteins, capsid, E2, and E1, which were transfected into neuroblastoma cells.
Results: SIN infection induced remarkable oncolytic effects on 14 human NB cell lines; NB69, SK-N-SH, SH-SY5Y, NB-1, CHP134, IMR32, GOTO, NMB, NLF, NGP, SMS-KAN, SMS-KCN, LAN-5, and RT-BM-1, but not on normal human keratinocytes in vitro. In nude mice, i.t. and i.v. inoculation of SIN resulted in significant regression of NB xenograft tumors. In addition, we found the oncolytic effect of UV-inactivated, replication-defective SIN against NB cell lines; IMR32, NB69, NGP, and SK-N-SH, and showed that it was induced by apoptosis. This implies that sutructural proteins of SIN play a major role in this oncolysis. Therefore, we constructed expression plasmids encoding each structural protein, capsid, E2, and E1, which were transfected into neuroblastoma cells. Transfection of the plasmid expressing E1 showed the highest cytotoxicity. The expression plasmid of E2 also showed cytotoxic effect, but the plasmid for Capsid and the vector displayed almost no cytotoxicity.
Conclusion: These results imply that the E1 protein plays an important role in the oncolysis of NB cells by UV-inactivated SIN. As the E1 protein has been considered to contribute to the viral fusion by changing the membrane permeability, it was suggested that the E1 protein of SIN causes the oncolysis of NB cells by the apoptotic signal transmitted during the fusion of membranes.

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POB93

A novel whole genome amplification approach is useful to perform aCGH in microdissected Schwannian Stromal and neuroblastic components of ganglioneuroblastomas

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Background: Tools for subtle characterization of genomic DNA (gDNA) from small clinical samples require linear whole genome amplification (WGA). We show results of Early Access Program (EAP) for Single Primer Isothermal Amplification (SPIA) of minute amounts (<10 ng) of DNA to be run on aCGH. Moreover, we demonstrate its feasibility in genotyping neuroblastic (Nb) and Schwannian Stromal (SS) cells of ganglioneuroblastomas.
Method: Our group was one of the 7 EAP partners (EAPPs). 1) Each EAPP amplified NuGEN-supplied HeLa and normal male gDNAs by a prototype Ovation WGA kit. DNAs were sent back to NuGEN for running on Agilent CGH 44K arrays. Slides were analyzed together for gain/loss calls and compared to internal controls. 2) We supplied to NuGEN normal and IMR-32 cell line gDNAs for amplification and then we run Agilent CGH 244K arrays. To test reproducibility, linearity and fidelity of the method, aCGH was also performed on unamplified gDNA as well as DNA processed by Sigma library-based GenomePlex WGA. We isolated Nb and SS cells by laser capture microdissection (LCM) and gDNA was purified from <100 cells by Qiagen QIAamp DNA Micro kit.
Results: The EAP provided a high consistent performance in beta test among the EAPPs. Array quality metrics were highly correlated between unamplified and amplified DNAs, indicating that SPIA from as little as 10 ng of starting gDNA generates robust array-CGH results. In Nb and SS cells it successfully identified 1p36-pter imbalance detected by FISH in 40% of nuclei of bulk sample.
Conclusion: a) NuGEN kit can be employed in the Agilent array-CGH workflow without any deviation from recommended protocol; b) SPIA method needs lesser amounts of starting gDNA in comparison to Sigma WGA; c) Amplified DNA produces high-quality chromosomal karyotypes that perfectly match results from unamplified gDNA; d) Such approach provides a means to decrease the amount of required DNA for aCGH, which could expand the application of the technology to analyze minute samples (i.e., cells isolated by LCM of heterogeneous tumors).
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POB94

Analysis of expression and inhibition of the Sonic Hedgehog signaling pathway in neuroblastoma

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Background/aims: The Hedgehog (Hh) signaling pathway has been implicated in the development of several types of solid tumors, including embryonic tumors such as medulloblastoma, although its role in neuroblastoma has not been fully investigated yet. The aim of this study was to analyze the role of the Hh signaling pathway, if any, in NB.
Methods: The expression of SMO, PTCH1 and GLI1 marker genes was analyzed by qRT-PCR and western blot in 12 neuroblastoma cell lines and 28 NB tumor samples. Treatment with the SMO inhibitor cyclopamine was carried out in 12 NB cell lines. Inhibition of the pathway was determined by the fold change decrease of GLI1 at RNA level. Following cyclopamine treatment we analyzed changes in cell proliferation, apoptosis and CD133, BCL2 and BAX1 gene expression.
Results: High expression levels of the pathway components in most neuroblastoma cell lines as well as in 25% of the NB tumor samples was detected, suggesting a persistent activation of the Hh pathway in a subset of NB. Genomic amplification of the GLI1 gene was detected in one tumour expressing very high levels of GLI1. Inhibition of the pathway using the SMO antagonist cyclopamine, induced a dramatic decrease in the proliferation capacity of the NB cell lines treated. An increase in the presence of apoptotic DNA fragments together with a decrease in the BCL2/BAX1 ratio was found. Moreover, cyclopamine reduced the levels of GLI1 and CD133 expression in NB cell lines.
Conclusion: Components of the Hh pathway are highly expressed in many NB tumours and cell lines. Upon Hh inhibition, the effects shown in cell proliferation and apoptosis, together with the down regulation of key genes related to tumorigenesis, suggest that the activation of the pathway could be an important feature of neuroblastoma development.

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POB95

Anaplastic Lymphoma Kinase (ALK) activates the small GTPase Rap1 via the Rap1-specific GEF C3G in neuroblastoma

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Background: The oncogene ALK RTK was discovered as the translocation product NPM-ALK in a subset of Anaplastic Large Cell Lymphomas, but more fusion proteins have been found lately. While many studies investigated the mechanisms of action employed by oncogenic ALK fusion proteins, the physiological function of ALK and its downstream targets in mammals is still unclear, but ALK is suggested to be involved in the normal development and function of the nervous system. In *D. melanogaster* ALK can be activated by the natural ligand Jelly Belly (Jeb), promoting formation of the visceral musculature of the gut and guidance cues for axons in the optic lobe. However, no Jeb exists in mammals. Neuroblastoma, which is derived from neural crest cells and can occur in the entire peripheral sympathetic nervous system, accounts for approximately 15% of all deaths in pediatric cancer. Amplification of the ALK locus as well as recently reported activating ALK point mutations have been observed in neuroblastoma cell lines and patient samples. The small GTPase Rap1 is involved in the regulation of many cellular processes, amongst others neurite outgrowth and neuronal polarization, which are mediated by sustained MAPK-pathway activation. Rap1 activity is controlled by activating GEFs and inhibitory GAPs. To date a role for Rap1 in the development of neuroblastoma has not been investigated.
Method/approach: We have developed a tetracycline inducible cell system in order to characterize downstream ALK signalling by examining whether ALK is able to activate Rap1 and contribute to differentiation/proliferation processes.
Results: Indeed, ALK activates Rap1 via C3G. The activation of the C3G-Rap1-pathway results in neurite outgrowth of PC12 cells, which is inhibited by siRNA-mediated knockdown of Rap1 or C3G. Significantly, this pathway also appears to function in the regulation of proliferation of neuroblastoma cell lines like SK-N-SH and SH-SY5Y, since abrogation of Rap1 activity reduces cellular growth.
Conclusion: These results suggest that ALK activation of Rap1 may contribute to cell proliferation and oncogenesis of neuroblastoma driven by gain-of-function mutant ALK receptors.

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POB96

Deep sequencing of the small-RNA transcriptome reveals differential expression of microRNAs in high-risk versus low-risk neuroblastoma

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Background: Small non-coding RNAs, in particular microRNAs, regulate fine-tuning of gene expression in general, and can act as oncogenes or tumor suppressor genes. Differential miRNA expression has been reported to be of functional relevance for the biology of neuroblastoma (NB) and other tumors.
Method/approach: Using next-generation sequencing (NGS), the unbiased and absolute quantification of the small-RNA transcriptome is now feasible. We here analyzed the small-RNA transcriptome in 10 NB with a maximally divergent clinical course (five favorable stage 1 versus five unfavorable MYCN-amplified stage 4) using a SOLiD NGS approach generating a total of 188,000,000 reads.
Results: MiRNA expression profiles obtained by deep sequencing correlated well with real-time PCR data. Favorable and unfavorable NB could easily be separated using cluster analysis and significant differences between the miRNA transcriptomes of favorable and unfavorable NB were retrieved. Oncogenic miRNAs of the miR17-92 cluster and miR-181 family were overexpressed in unfavorable NB. In contrast, the putative tumor suppressive miRNAs, miR-542-5p and miR-628, were present in favorable NB and virtually absent in unfavorable NB. High expression of various miR star (miR*) species was detected, and correlation of miRNA expression to the expression of the respective miRNA* varied markedly. In-depth sequence analysis also revealed extensive post-transcriptional miRNA editing. Finally, 13 putative novel miRNAs were identified using miRDeep. Three of these novel miRNAs were further analyzed, and expression could be confirmed in a cohort of 70 primary neuroblastomas.
Conclusion: NGS is a valid tool to explore the small-RNA transcriptome and to identify novel miRNAs. Our results provide absolute miRNA expression counts and novel insights into the correlation of miR/miR* expression. Furthermore, we addressed the phenomenon of miRNA editing resulting in a variety of isomiRs. The functional implication of the latter mechanism in tumor biology warrants further analysis.

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POB97

Prognostic significance of NKp30 spliceoforms in neuroblastoma

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Background: Neuroblastoma (NB) is sensitive to natural killer (NK) cell cytotoxicity, a true link between innate and cognate immunity. NKp30 is a natural cytotoxicity triggering receptor involved in NK cell-mediated cytotoxicity and in the cross-talk between NK and dendritic cells. Our unpublished data identified 3 spliceoforms (a, b and c) of the NKp30 gene able to discriminate differential profiles in adult patients with gastrointestinal sarcoma. Predominant NKp30a isoforms displayed the highest cytotoxicity while predominant NKp30b or c isoforms have respectively Th1 cytokines or IL-10 secretion with defective cytotoxic activity.
Aim: To evaluate NKp30 spliceoforms in NB patients and their influence on disease dissemination and prognosis.
Methods: NKP30 spliceoforms were analyzed by Real-time reverse transcription-PCR in peripheral mononuclear blood cells from 94 NB patients treated in Gustave Roussy Institut from 1964 to 2010. Unsupervised hierarchical clustering was applied to data obtained and correlated with patient's clinical data.
Results: Unsupervised hierarchical clustering classified patients in 3 subgroups. Forty-four out of 94 patients (47%) exhibit the predominant NKp30b isoform, 28 (30%) the predominant NKp30c isoform and 22 (23%) the NKp30a isoform. Among the 39 metastatic NB, 27 (69%) exhibit the predominant NKp30b, 6 (15%) the predominant NKp30a and 6 (15%) the predominant NKp30c isoform while among the 50 localized NB, 15 (30%) exhibit the predominant NKp30b, 14 (28%) exhibit the predominant NKp30a and 21 (42%) the predominant NKp30c isoform (p=0.001). Finally, among the 40 localized NB with a follow-up more than 2 years, 1/12 (8%) with predominant NKp30a isoform relapsed comparing with 4/28 (14%) with predominant NKp30b or c isoforms.
Conclusion: NKp30 profiles appear to correlate with dissemination of NB. Prospective studies are in progress to confirm the impact of NKp30 spliceoforms on NB prognosis. New drugs that modulate alternative splicing of NKp30 receptor may therefore represent a new therapeutic approach in NB.

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POB98

Galectin-3 binding protein/90 kDa Mac-2 binding protein stimulates interleukin-6 expression in the neuroblastoma microenvironment

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Background: There is increasing evidence that the tumor microenvironment plays a key role in cancer progression, but its role in neuroblastoma is poorly understood. We have previously reported that in neuroblastoma, the production of interleukin-6 by bone marrow mesenchymal cells (BMMC) and monocytes/macrophages contributes to the pro-tumorigenic effect of the tumor microenvironment. Here we have examined the mechanism by which IL-6 is overexpressed by stromal cells.
Method/approach: Our studies used co-cultures of human neuroblastoma cells and bone marrow derived mesenchymal cells and peripheral blood monocytes, as well as the examination of human neuroblastoma primary tumor samples.
Results: We identified galectin-3 binding protein/90 kDa Mac-2 binding protein (Gal-3BP), present in the supernate of several human neuroblastoma cell lines (in particular without *MYCN* amplification) as an inducer of IL-6 expression in human BMMCs and human monocytes. Upregulation of IL-6 by Gal-3BP occurs via the Ras/Raf-1/MEK/ERK1/2 pathway and requires galectin-3, a pleiotropic glycoprotein that binds to Gal-3BP and is expressed in BMMCs. We documented that Gal-3 BP-mediated IL-6 expression is blocked by the MEK inhibitor PD 98059, an anti-Gal-3 antibody, or downregulation of Gal-3 by siRNA. Transcriptional activation of human IL-6 by Gal-3BP/Gal-3 signaling requires a 115 bp region (-97 to -212) upstream of the TATTA box in the IL-6 promoter that contains a cAMP responsive element binding protein and a CAATT/enhancer binding protein element. The expression of Gal-3BP was also examined by immunohistochemistry in 85 primary neuroblastoma tumors with (n=25) and without (n=60) *MYCN* amplification, and preliminary results indicate a paradoxically more abundant expression in *MYCN* non-amplified tumors.
Conclusion: We have identified a novel Gal-3BP/Gal-3/IL-6 pathway that contributes to the production of interleukin-6 in the tumor microenvironment of neuroblastoma tumors. Our data suggest that this pathway may play a particularly important role in high risk neuroblastoma without *MYCN* amplification.

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POB99

TrkAIII isoform expression is associated with aggressive behavior in human neuroblastomas

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Background: TrkA is the receptor for nerve growth factor (NGF). High TrkA expression typically is associated with favorable clinical outcome in NB. Conversely, TrkAIII, a novel TrkA isoform that lacks part of the extracellular domain, is constitutively active, and unresponsive to its ligand NGF. Here we determine the prevalence and significance of TrkAIII expression in primary NBs.
Method/approach: We examined 232 representative NBs and identified 118 with high TrkA expression for TrkAIII isoform analysis. Of the 118, 46 were clinically and biologically favorable (e.g., stage 1, no MYCN amplification), and 72 were unfavorable (stage 3 or 4, over 1 year of age, with or without MYCN amplification). Validation of total TrkA and TrkAIII was determined by conventional, semi-quantitative RT-PCR and quantitative real-time RT-PCR (TaqMan). T-tests were used to compare expression between favorable and unfavorable groups, and the Pearson correlation coefficient was calculated between total TrkA and TrkAIII. We also transfected TrkAIII into the Trk-null NB line SH-SY5Y to determine the effect on proliferation.
Results: There was no difference in total TrkA expression between the favorable and unfavorable groups (P=0.38). However, the mean expression of TrkAIII was significantly higher in the unfavorable high-TrkA group compared to the favorable high-TrkA group (P=0.007). Thus, TrkAIII isoform expression may explain why some unfavorable NBs with high TrkA expression have a poor clinical outcome. TrkAIII was constitutively phosphorylated in TrkAIII-transfected SH-SY5Y clones, and TrkAIII expression significantly enhanced proliferation in vitro. However, this proliferative advantage could be abrogated by treatment with the Trk-selective inhibitor, lestaurtinib.
Conclusion: This report validates the association between expression of TrkAIII and unfavorable features and outcome, and it suggests this could be a potential tool for risk assessment in unfavorable NBs. Our data also suggest that these tumors will be sensitive to tyrosine kinase inhibition therapy.

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POB100

Evaluation of MCPIP expression patterns and their impact on the survival of neuroblastoma cell lines

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Background/Aims: The transcript coding for MCPIP (Monocyte Chemoattractant Protein Induced Protein, ZC3H12A) has been identified in the transcriptome of human monocytes stimulated with IL 1β or IL 6. MCPIP acts as an RNase, thanks to the presence of a PIN domain. It has also been shown to be a specific inhibitor of NF B, a transcription factor crucial in regulating the immune response, differentiation and tumorigenesis. The molecular mechanism(s) of this action however remains unknown. We found an interesting correlation between the expression of the MCPIP transcript and the amplification status of an important diagnostic factor in human neuroblastoma cell lines. The aim of the present study is to investigate the role of MCPIP in neuroblastoma, a common solid pediatric tumor.
Methods: Different mutant MCPIP forms were cloned into a pcDNA3 vector. Three human neuroblastoma cell lines were transfected with constructs encoding the wt MCPIP protein or a mutant form, lacking either the PIN domain, or one or two ubiquitin binding domains. Cell viability was assessed with the MTT assay and MCPIP protein expression by immunoblotting. Transcript levels were determined by real time RT-PCR.
Results: We found that the expression status of MCPIP could be important for tumor cell survival. It has been evaluated whether overexpression of different protein forms could have an impact on neuroblastoma cell survival in vitro. Also, stably transfected clones of neuroblastoma cells have been established and characterized, although MCPIP-overexpression was hard to obtain due to the instability of the protein and the diminished cell viability after its forced expression.
Conclusions: Our results implicate that the significantly lower MCPIP expression, as compared to most evaluated cell lines of different origin, might be important for neuroblastoma survival, making forced MCPIP expression a potential approach for future therapeutic approaches. The obtained results remain to be confirmed in an in vivo murine model.
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POB101

NLRR1, a direct target gene of MYCN, modulates aggressive growth of neuroblastoma by selectively enhancing EGF and IGF signals through the components of lipid rafts

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Background: Human *NLRR1* (*neuronal leucine-rich repeat protein 1*) gene was originally identified from our neuroblastoma (NB) cDNA project. We previously reported that high level of *NLRR1* mRNA expression is significantly associated with poor prognosis of NB patients. Indeed, the up-regulation of NLRR1 in both NB and non-NB cells enhances cell proliferation. However, the molecular mechanisms remain unclear.
Method: To analyze the effects of NLRR1 on downstream signals of growth factors, Western blotting, in vitro EGF binding assay, immunoprecipitation (IP) and gradient fractionation were performed. To examine the role of NLRR1 in development, NLRR1 knockout mice were generated by conventional targeting strategy.
Results: MYCN directly bound to the promoter region of *NLRR1* gene and activated its transcription. Overexpression of NLRR1 caused more phosphorylation of EGFR and IGF-IR, but not FGFR and TrkA, as well as ERK upon their ligands treatment in NB and other cell lines. Scatchard plot in EGF binding assay showed a higher Bmax value in NLRR1-overexpressing cells than control cells and the slope of the Hill plot was 0.829 in NLRR1-overexpressing cells, suggesting that NLRR1 makes the binding sites more heterogeneous and increases EGF binding. However, IP assay showed no obvious interaction between NLRR1 and EGFR or IGF-IR. Gradient fractionation revealed that NLRR1 proteins co-localized with EGFR and IGF-IR in a relatively high density fraction, the so-called $B!H(Blipid rafts$B!I(B. The treatment of mβCD, which disrupts lipid rafts, attenuated the NLRR1 function to accelerate EGF signaling. In mouse embryo, NLRR1 was expressed mainly in nervous system, myotome and branchial arch which is derived from neural crest. At weaning, NLRR1 knockout mice showed less body weight than wildtype mice, suggesting that NLRR1 expression is essential for normal development and growth.
Conclusion: NLRR1 may sensitize EGF and IGF signaling to enhance the cell growth by altering their membrane localization. The development of therapeutic agents against NLRR1 could be of benefit to the cure of high-risk NB.

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POB102

Aurora A kinase is a possible target of OSU-03012 to destabilize MYC family proteins

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Background: The 3-phosphoinositide-dependent kinase-1 (PDK1) inhibitor, OSU-03012, rapidly destabilizes MYCN and MYC proteins in neuroblastoma (NB) cells. However, OSU-03012 does not affect the phosphorylation status of AKT, suggesting that PDK1 is not the prime target of OSU-03012 in NB cells. Collective studies from our group indicate that OSU-03012 uses mechanisms that are PDK1-independent to destabilize MYC/MYCN and suppress growth of NB cells. In this study, we explore one of these mechanisms. It has been reported that Aurora A kinase phosphorylates GSK3β, leading to its inactivation, and prevents MYCN/MYC destabilization. Accordingly, Aurora A knockdown results in reduced MYC levels. Based on these observations, we hypothesize that one of the targets of OSU-03012 is Aurora A kinase.
Method: In silico molecular docking analysis was used to investigate whether OSU-03012 has any likelihood of binding to Aurora A kinase.
Results: The 3D structure of AURKA (PDB ID: 3DAJ) was obtained from the Brookhaven Protein Databank. The structures of OSU-03012 and FXG were constructed by using MOE (version 2007, CCG, Montreal, Canada). FXG is an Aurora kinase A inhibitor, a derivative of Compound 6 that was discovered through site-specific dynamic combinatorial chemistry by Cancilla et al, (Bioorg Med Chem Lett 2008;18: 3978-81). Docking simulations and interaction energy calculations were performed by MOE Dock of MOE, based on the coordinates of co-crystallization of FXG and Aurora A (PDB ID: 3DAJ). The resulting most stable docking structures between Aurora kinase A and FXG or OSU-03012 were displayed. Our analysis showed that the calculated interaction energy between Aurora kinase A and FXG was -89.273 kcal/mol, whereas that between Aurora kinase A and OSU-03012 was -109.901 kcal/mol. Thus, OSU-03012 has a high likelihood of binding to Aurora A kinase.
Conclusion: These results suggest that OSU-03012 affects multiple cellular targets including Aurora A kinase to exhibit its growth suppressive, cell death-inducing, and MYCN-destabilizing effects on neuroblastoma cells. Biochemical verification is currently pending.

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POB103

Expression and function of RET in neuroblastoma cell lines

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Background: NB is the most common extracranial tumor in children and is associated with a high mortality rate. Receptor tyrosine kinases (RTKs) have been suggested as important molecules in the development of NB. RET plays an important role in the development of normal sympathetic neurons, and it has been implicated in NB pathogenesis. RET has four ligands (GDNF, NRTN, ARTN, or PSPN) and four co-receptors (GFRα1-4), which bind to RET and begin the signaling cascade. RET is expressed in many NBs, but little is known about the expression of RET and its co-receptors in NB cell lines and tumors, or the activation of RET in response to specific ligand exposure. We examined the presence of RET and GFRα1-3 in six NB cell lines and the phosphorylation of RET in response to ligand. Methods:
Methods: IMR5) were grown. The mRNA expression of RET, and GFRα1-3 was quantified by TaqMan assay. The protein expression of RET and GFRα1-3 and phosphorylation of RET was analyzed by Western Blot. Morphologic changes induced by ligand exposure were observed and photographed over 7 days.
Results: IMR5 has the lowest RET expression, whereas SY5Y, SK-N-AS, NLF, NBLS and NB-E-Bc1 expressed very high levels of RET. The expression of GFRα1-3 was variable in the different lines. At least one or more GFR co-receptor was expressed at high levels in the lines with high RET expression. NB-EBc1 and NBLS had the most dramatic response to ligand exposure, as determined by both by Western blotting and morphologic changes. Both NB-EBc1 and NBLS underwent extensive neurite outgrowth during treatment with NRTN and ARTN. IMR5 showed no significant morphologic change in response to any ligand. Expression of the co-receptors paralleled the ligands to which the lines had the greatest response (GFRα2 for NRTN, GFRα3 for ARTN). Conclusions: Ligand activation of RET in NB cell lines required the expression of the appropriate co-receptor. RET activation caused morphologic differentiation, demonstrating that the signaling pathway was functional and may contribute to NB survival and/or differentiation.

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POB104

Irrespective of ALK mutational status, neuroblastoma tumors are sensitive to Akt inhibitor perifosine

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Background: Akt is an intracellular serine/threonine kinase that plays a key role in survival signaling pathways. Increased Akt activity mediates survival and resistance to chemotherapy in Neuroblastoma (NB) and activated Akt is more highly expressed in tumors from patients with a poor prognosis and. Anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase predominantly expressed in the developing nervous system. Mutated ALK has been identified in association with familial and sporadic NB and patients whose tumors contain amplification, mutation or express increased levels of activated ALK have a poor prognosis. Akt is frequently a downstream target of ALK. Since Perifosine inhibits Akt , the aim of this study was to assess whether perifosine was active in NB cell lines with ALK mutations.
Methods: Six NB cell lines were utilized; 4 with ALK mutations- SY5YALK F1174L, KCNRALK R1275Q, Lan5 ALKR1275Q and SKNFIALKS1136S and 2 with wild type(wt) ALK- ASALKwt and NGPALKwt. Cell survival after perifosine treatment was evaluated by MTS assay. In representative cell lines, the effects of Perifosine on tumor xenograft growth and inhibition of Alk/Akt signaling were evaluated.
Results: In vitro, perifosine treatment caused a 50-80% decrease in growth in all NB cell lines tested. Cell lines containing Alk mutations were inhibited to the same extent as ASALKwt, which expresses low levels of ALK. Moreover perifosine induces apoptosis in KCNRALK R1275Q cells, which were reported to be resistant to the ALK inhibitor TAE684. Akt activation was inhibited by perifosine in all the cell lines tested. In vivo, perifosine treatment induced regression of ASALKwt tumors and inhibited the growth of NGPALKwt tumors and KCNRALK R1275Q tumors. Perifosine inhibited Akt phosphorylation in all these tumors.
Conclusion: These data indicate that Perifosine inhibits NB tumor cell growth despite the mutational status of ALK. Perifosine may be an effective treatment for NB tumors irrespective of their ALK mutational status.

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POB105

Enhanced effect of IFNγ on the induced-apoptosis of neuroblastoma cells by cytotoxic drugs

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Background: The expressions of Caspase 8,a cysteine protease that is crucial for the apoptotic cascade, is absent in a high percentage of neuroblastoma cells. Resistance of neuroblastoma cells to cytotoxic drugs -mediated apoptosis is thought to be caused by loss of Caspase 8 expression. In this study we explored the effect of cytotoxic drugs on neuroblastoma cell line SH-SY5Y and the influence of γ-interferon(IFNγ) on the antitumor effects of cytotoxic drugs.
Methods: The expression of Caspase 8 mRNA and protein was detected with RT-PCR and Western-blot analysis . The effects of cytotoxic drugs (adriamycin, TNFα and TRAIL) CIFNγ + cytotoxic drugs, IFNγ+Caspase 8 inhibitor(zIETD-FMK)+ cytotoxic drugs on the growth and apoptosis of SH-SY5Y cells were detected with the methods of MTT and flow cytometry. The relative Caspase8 activity was measured by colorimetric assay.
Results: Caspase 8 was undetectable in SH-SY5Y cells with an increased expression of Caspase 8 after the treatment of IFNγ. SH-SY5Y cells were sensitive to adriamycin relatively but resistant to TNFα and TRAIL, while IFNγ-pretreated SH-SY5Y cells were more sensitive to the cytotoxic drugs with an increase of Caspase 8 activity but the killing effects could be diminished by zIETD-FMK.
Conclusion: IFNγ can sensitize SH-SY5Y cells to cytotoxic drugs-induced apoptosis and this may be realized by the upregulation of Caspase 8.

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POB106

JAGGED1 antogonizes NOTCH2 mediated cell migration

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Background: Notch signalling is an evolutionary conserved mechanism of intercellular communication involved in normal development and cancer. Ligands from the DELTA/JAGGED families activate NOTCH receptors, resulting in the liberation of the NOTCH intracellular domain (NOTCH-IC) signalling peptide. Previously, we identified NOTCH3 as master regulator of neuroblastoma cell migration. Therefore, we investigated whether the paralogous NOTCH2 receptor controls cell migration and analyzed the function of the ligand JAGGED1 in the regulation of NOTCH2-IC signalling.
Methods: We performed a functional analysis of the JAGGED-NOTCH signalling axis in neuroblastoma cells using lentiviral shRNA mediated gene silencing and over-expression of NOTCH2 and JAGGED1. Cell migration was tested in transwell migration assays.
Results: We show that NOTCH2 controls neuroblastoma cell migration. Unexpectedly, JAGGED1 did not activate, but rather attenuated NOTCH2-IC signalling. Consistent with this regulation, we found that cell migration is increased after JAGGED1 silencing, but is restored to control levels by co-silencing of NOTCH2. Our data reveal that the antagonistic function of JAGGED1 acts on the liberated NOTCH2-IC domain. Interestingly, JAGGED1 is strongly regulated by both NOTCH2-IC and NOTCH3-IC exerting antagonism on NOTCH-IC signalling in cis.
Conclusions: Our data implicate the importance of the JAGGED-NOTCH axis in neuroblastoma cell migration. Furthermore, we reveal an unexpected antagonistic function of JAGGED1 on NOTCH2 signalling, that occurs intra-cellular and acts on the released NOTCH2-IC. Moreover, JAGGED1 is induced by NOTCH signalling to attenuate NOTCH-IC signalling in cis, but possibly to promote NOTCH signalling in trans as well.

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POB107

A migration signature in neuroblastoma cell lines and tumours identifies YAP1 as a regulator of cell migration

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Background Neuroblastoma is a childhood tumour from the peripheral sympathetic nervous system, a tissue ultimately derived from migratory neural crest progenitors. Aggressive neuroblastoma tumours can be locally invasive, but also highly metastatic. Cell migration is a prerequisite for invasive behaviour. However, the genes that control migration of neuroblastoma cells remain largely unknown.
Methods We analyzed differential gene expression correlating with a cell migration phenotype *in vitro*. The predictive power of this 'migration signature' was assessed in migration of other cell lines. The existence of a 'migration signature' was analyzed in a series of neuroblastoma tumours. Functional analysis was performed to identify candidate genes that control cell migration.
Results Two groups of fast and slow migrating neuroblastoma cell lines were identified *in vitro*. Affymetrix gene expression profiles were generated from these cell lines and used to identify a set of 296 differentially expressed genes between the fast and slow migrating groups. Gene Ontology analysis revealed that this gene set was enriched for genes with annotated functions in cell migration, adhesion and chemotaxis. This 'migration signature' correctly predicted the cell migration phenotype in 6 other cell lines that had not been tested previously. Furthermore, the 'migration signature' was found to be strongly co-regulated in a series of 88 primary neuroblastoma tumours, suggesting that this migratory phenotype exists *in vivo*. Pathway analysis of genes expressed in fast migrating cell lines revealed expression of several members from the Hippo-pathway, a cascade of negatively regulating kinases that converges on the regulation of YAP1. Knockdown of this transcriptional co-activator reduces neuroblastoma cell migration.
Conclusions Our data reveal a set of co-regulated genes in fast migrating neuroblastoma cell lines that correctly predicts cell migration in other cell lines. Moreover, this 'migration signature' exists *in vivo* in neuroblastoma tumours. Finally, we functionally implicate YAP1 in neuroblastoma cell migration.

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POB108

Functional microRNA library screen to identify synthetically lethal interactions in neuroblastoma

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Background: Perturbation of microRNA (miRNA) function is well established as one of the possible mechanisms contributing to cancer formation and increasing evidence suggests a role in neuroblastoma. In an effort to elucidate the role in neuroblastoma, a complementary data set was generated, containing miRNA and mRNA gene expression and copy number information in a large cohort of neuroblastoma tumors and cell lines, and next-generation sequencing based mutation data is about to complement this data set. In order to understand how altered miRNA expression levels, copy numbers or sequence variants contribute to neuroblastoma pathogenesis, functional assessment of individual miRNAs is needed.
Method/approach: A functional miRNA library screen, containing miRNA mimics and antagonists for 470 miRNAs, will be performed on well characterized neuroblastoma cell line model systems for MYCN, ALK and TP53. Effects of library transfection on cell viability will be monitored using Real-Time Cellular Analysis (RTCA) technology, measuring electrical impedance of cell cultures in 96-well plates. Using in-well reverse transcription together with high-throughput RT-qPCR, monitored cells will subsequently be profiled for the expression of a set of marker genes reflecting the status of known cancer pathways. Selected miRNAs will then be further characterized, evaluating their effects on proliferation, apoptosis, differentiation and cell cycle distribution.
Results: MiRNAs displaying differential effects in the model systems will receive further attention, as they serve as potential candidates for targeted therapy. Results on the screen will be presented.
Conclusion: In analogy to high-throughput RNAi library screens for the identification of candidate therapeutic targets, we aim to identify synthetically lethal miRNA interactions with important cancer genes in neuroblastoma

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POB109

Galectin-3 protects MYCN single copy neuroblastoma cells from apoptosis: a mechanism impaired by MYCN

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Galectin-3 (Gal-3) is a β-galactoside specific lectin provided with a unique carbohydrate recognition domain. Biologically, it is involved in a number of processes, largely depending on its particular localization. Nuclear Gal-3 is related to RNA processing and DNA transcription while cytoplasmic Gal-3 is involved in controlling either apoptosis or Ras signaling. Gal-3 can also be secreted in the extracellular matrix to control cell adhesion, migration and angiogenesis. Aberrant Gal-3 expression occurs in different human malignancies with potentially opposite effects on tumor progression. In thyroid tumors, Gal-3 expression may be considered diagnostic of malignancy and appears to be controlled by aberrant p53 signaling. In particular p53 phosphorylated on S^{46} by its proapoptotic activator kinase HIPK2 transcriptionally repress Gal-3 in response to DNA damage in order to commit cells to apoptosis. Conversely, Gal-3 suppresses HIPK2-p53 induced apoptosis. MYCN gene is strictly associated to neuroblastic tumor biology and its amplification (MNA) is the most relevant adverse prognostic marker in human neuroblastomas (NBs). Paradoxically, its expression might also be associated to increased sensitivity to apoptosis triggered by different events. At least in part, this is due to the upregulation of the proapoptotic p53 kinase HIPK2 by MYCN, as we recently demonstrated. Surprisingly however HIPK2 overexpression failed to induce apoptosis and sensitize MYCN single copy (MNSC) NB cells to DNA damaging drugs, suggesting that these cells might be protected from HIPK2- p53-induced apoptosis by Gal-3. Consistent with this hypothesis, Gal-3 is strongly expressed in MNSC cells at RNA and protein level, and its depletion by specific RNA interference sensitizes these cells to apoptosis. Conversely, we detected very low levels of Gal-3 in MNA NB cells which can be made more resistant to apoptosis by transfection with a human Gal-3 construct. In an inducible cellular system, MYCN repressed Gal-3 expression, suggesting that its ability to sensitize NB cell to apoptosis might be closely dependent on the regulation of the HIPK2-p53-Gal-3 axis.

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POB110

Genome-wide analysis of favorable-stage neuroblastoma reveals discrete patterns of gene expression and alternative splicing between *MYCN* amplified and non-amplified tumors

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Background: Children with localized *MYCN* amplified hyperdiploid neuroblastoma do better that those with diploid tumors. We sought to probe the genetic signatures of these tumors to gain an understanding of their clinical behavior.
Methods: We analyzed 28 favorable stage (INSS Stage I, IIa, IIb, IVs) *MYCN*-amplified tumors from the COG Tumor Bank with the Affymetrix SNP 6.0 and HuEx 1.0 Expression array platforms. Exon array data was normalized with Affymetrix Power Tools and then analyzed in BioConductor. SNP genotyping was performed using Affymetrix Genotyping Console, and subsequent analysis was performed using dChip and custom scripts.
Results: SNP data was obtained on 24 tumors and 9 paired blood samples. Exon array data was obtained for 14 tumors. LOH was determined by comparison to a paired blood sample or a dataset of HapMap controls and copy number calculated by comparison to the average of blood and HapMap samples. Five samples reported to have *MYCN* amplification had copy numbers less than 5 and 2-4 times comparative lower expression by exon analysis. Moreover, there were too few hyperdiploid samples to allow a comparison. Unsupervised clustering of exon data revealed two groups of tumors, corresponding to *MYCN* status, with stage IVs tumors clustered tightly together among *MYCN* amplified samples. Genes whose expression contributed most to the separation along PC1 included *MYCN*, *DDX1*, *PHOX2B*, *SYN3*, and *AHNAK*. Supervised analysis of alternative splicing dependent on *MYCN* status suggested alternative splicing isoforms of *PHOX2B*, *SPARCL1*, and *AHNAK*.
Conclusion: Using complimentary genome-wide approaches, we show that among favorable-stage tumors, *MYCN* amplification is associated with a discrete profile of LOH, gene expression and alternative splicing, revealing interesting tumor suppressor gene candidates and suggesting potential mechanisms for *MYCN*-mediated tumor aggressiveness. Further study will likely reveal potential targets for better patient stratification and possible therapeutic development.

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POB111

JAG2 induction in hypoxic tumor cells alters Notch signaling and enhances endothelial cell tube formation

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Background: Several studies have revealed links between hypoxia and activation of Notch in solid tumours. While most reports have focused on icN1 stabilization by direct interaction with HIF proteins, little attention has been given to Notch ligand regulation during hypoxia. Here we aim to elucidate the contribution of hypoxia induced Notch ligands on Notch signaling in a variety of cancer forms, including neuroblastoma, breast cancer and renal cell carcinoma.
Method/approach: Tumour cell lines derived from neuroblastoma and breast cancer were grown in normoxic (21% O_2) or hypoxic (1% O_2) conditions. Notch ligand expression was analyzed using quantitative real-time PCR (QPCR) or microarray analysis. icN1 protein levels, as well as the Notch target gene HEY1 mRNA levels were used to measure Notch activity. JAG2 contribution to Notch activity was determined with use of siRNA targeting JAG2. Primary tumour microarrays were used to generate ranked JAG2-correlated gene lists, which were subsequently used for Gene Ontology (GO) analyses and Gene Set Enrichment Analyses (GSEA). Endothelial tube formation analyses were performed by co-culturing endothelial cells with hypoxic tumour cells treated with control or JAG2 targeting siRNA.
Results: Here we show that the Notch ligand JAG2 is transcriptionally activated by hypoxia in a HIF-1α dependent manner. The hypoxic JAG2 induction was confirmed in neuroblastoma, breast cancer and renal cell carcinoma. Elevation of JAG2 resulted in increased Notch activity in tumour cells. In primary tumour material, JAG2 expression correlated with vascular development and angiogenesis gene signatures. In line with this, co-culture experiments of endothelial cells with hypoxic tumour cells displayed a reduction in number of capillary-like tubes formed upon JAG2 siRNA treatment of the tumour cells.
Conclusion: Together these results suggest that a hypoxia-regulated induction of JAG2 positively contributes to Notch activity within tumour epithelial cell compartments. In addition, a hypoxic-JAG2 regulated cross-talk mediated by Notch between tumour and endothelial cells is involved in tumour angiogenesis.

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POB112

Transcriptome analysis of chromosome 1p in neuroblastoma

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Background: Chromosome 1p is frequently deleted in neuroblastoma and is thought to harbor one or more tumor suppressor genes. The goal of this study was to identify genes on ch 1p that may function as tumor suppressors in neuroblastoma.
Method: We compared the transcriptomes of two neuroblastoma cell lines, SK-N-SH (without 1p deletion) and LAN-6 (with 1p deletion) using the TranscriptionPath ChIP assay™ (Genpathway, Inc.). Both cell lines had non-amplified *MYCN* and unbalanced gain of 17q but no 11q deletion. Chromatin was isolated from cells and stabilized by cross-linking, after which immune-precipitation was carried out using specific antibodies against polymerase II to isolate protein/DNA complexes. Purified DNA was then hybridized in triplicate onto the GeneChip® Human Promoter 1.0R Array and GeneChip® Human Tiling 2.0R Array (Affymetrix). Analysis was performed using Affymetrix Tiling Analysis Software. Selected target gene expression was verified by quantitative real-time PCR (qRT-PCR).
Results: We identified 102 ch 1p genes on Promoter Array analysis and 55 genes on Tiling Array analysis that showed significantly decreased Pol II binding and subsequent transcription in the LAN-6 cell line (1p-) compared to SK-N-SH (1p+). There were 37 overlapping genes between the two arrays, of which 22 localized to 1p35-36. Among these we identified known candidate TSG genes, such as *KIF1B* and *CHD5*. The decreased expression of 17 genes in the 1p deleted cell line, LAN-6, was verified by qRT-PCR (1.2 fold change, $P<0.05$). Decreased expression of these genes was further confirmed in four additional NB cell lines with 1p deletion compared to one cell line with intact 1p.
Conclusion: Using two complementary genomic approaches, we have identified a number of candidate tumor suppressor genes on chromosome 1p. Further functional validation will reveal whether they have a role in neuroblastoma tumorigenesis.

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POB113

Expressional alterations in ultra-conserved non-coding RNA resulting from changes in MYCN levels and in response to ATRA-induced differentiation

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Background: All-trans-retinoic acid (ATRA) causes the SK-N-BE neuroblastoma (NB) cell line to undergo differentiation and leads to a significant decrease in MYCN. Transcribed ultra-conserved regions (UCRs) have been shown to be differentially expressed in cancers versus normal tissue, indicating a possible role in carcinogenesis. Here, we examine the impact of ATRA treatment and changing MYCN levels on UCR expression.
Methods: Tiling microarrays were used to profile the expression of 962 UCRs (481 in sense and antisense orientation) in ATRA treated SK-N-BE cells and in SHEP-21N cells which contain a MYCN trans-gene under the control of a tetracycline responsive repressor element. mRNA expression was analysed using mRNA expression arrays.
Results: Following ATRA induced differentiation of SK-N-BE cells, 23 UCRs had a >1.5 fold increase in expression, while 1 UCR was decreased. 19 of these UCRs were exonic and 5 were intronic, with ~70% transcribed in the same direction of the host gene. Since MYCN levels significantly decrease in these cells, we decided to determine how many of these UCRs might be undergoing expressional alterations as a consequence of MYCN depletion. We profiled UCR expression in SHEP-21N cells for both high and low (Dox-treated) MYCN states. 61 UCRs were affected by changes in MYCN levels. For MYCN depleted cells, the transcriptional activity of 32 UCRs was increased by >1.5 fold. Conversely, 29 UCRs decreased by >1.5 fold. Only two UCRs that were differentially expressed in SK-N-BE cells were also differentially expressed in the SHEP-21N cells, indicating that many mechanisms are involved with UCR regulation.
Conclusions: Our results indicate that significant numbers of UCRs have increased expression levels in response to ATRA and that an even greater number of UCRs are regulated by MYCN. The intergenic transcripts, along with the exonic/intronic UCRs that have opposite orientation to the host gene, may represent independent non-coding transcripts that are influenced by MYCN levels and ATRA treatment. The precise roles that UCRs might play in cancer or in normal development are largely unknown and an important area for future study.

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POB114

ALK signaling in neuroblastoma

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Background and aims: Activating mutations of the anaplastic lymphoma kinase (ALK) gene were recently described in familial and sporadic neuroblastoma. We aimed to analyze the effects of the F1174L and R1275Q ALK mutations in neuroblastoma cell lines to identify the downstream ALK signaling routes in neuroblastoma.
Methods: Lentivirally-delivered shRNAs were used for knockdown of the ALK gene in ALK mutant and wild type neuroblastoma cells. In addition, we have constructed stably transduced neuroblastoma cell lines with doxycyclin-inducible overexpression of wild type, the F1174L mutant or R1275Q mutant ALK. The phenotypical changes and downstream signaling of these cells were extensively analyzed. The mRNA expression profiles of the knockdown and over expression experiments were created and analyzed.
Results: Introduction of the wild type, the F1174L mutant or R1275Q mutant ALK gene were found to have different effects on the downstream signaling pathways in neuroblastoma cells. Although wild type ALK protein could be very efficiently induced in our system, no increase in phosphorylation of the wild type protein was observed. Consequently, downstream signaling pathways were not noticeably affected in these cells. Expression of the F1174L or R1275Q mutant ALK protein immediately resulted in a large increase of phosphorylated ALK protein. The mutants showed highly divergent induction of major downstream signal transduction routes. These differences were reflected in the different phenotypes, growth rate, their response to inhibitors, and in the gene expression profiles of the cell lines.
Conclusions: We demonstrate differences in downstream signaling pathways between the F1174L and R1275Q ALK mutants in neuroblastoma cell lines.

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POB115

Regulation of differentiation by estrogen receptors in neuroblastoma cells

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The aim of this study is to investigate the interplay between MYCN, miRNAs, estrogen receptor alpha (ESR1) and downstream targets in the regulation of proliferation versus differentiation in neuroblastoma (NB) cells. NB is the most common extra cranial solid tumor of childhood and the deadliest. Even though NBs have a remarkable ability to spontaneously regress and differentiate, 40 % of the high-risk patients are incurable with current treatment. One of the few prediction markers for poor outcome is amplification of MYCN, which is associated with a survival rate of less than 15%, even in patients with otherwise favorable outcome profile. In order to understand the mechanisms behind MYCN-mediated NB initiation and progression we surveyed the miRNA signature in MYCN overexpressing NB cells. The results show that several members of the miR-17-92 cluster are upregulated in MYCN overexpressing NB cell lines and in MYCN amplified NB tumor samples. Specifically, we show that these miRNAs can target and repress expression of ESR1. Restoration of ESR1 expression in NB cells resulted in growth arrest and neuronal differentiation. Bioinformatic analyses of microarray data from NB tumors revealed that high ESR1 expression correlated with increased event-free survival and favorable outcome in NB patients. Estrogen and its receptors stimulate neural differentiation in the developing nervous system by modulating the synthesis and regulation of growth factors and related receptors. These results suggest that MYCN amplification and miRNA deregulation may disrupt sensitivity to estrogen signaling in primitive sympathetic cells through deregulation of ESR1 and thereby preventing normal induction of neuroblast differentiation and subsequently initiation of NB. We are currently investigating interacting growth factors and downstream targets involved in the regulation of differentiation by ESR1 in NB cells.

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POB116

Role of p53 and p73 in neuroblastoma chemosensitivity and neuroblastoma tumor initiating cells (TIC)

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Background: Although rare at diagnosis *p53* mutations (mt) and other abnormalities of the p53/HDM2/ARF pathway are more commonly detected in relapsed neuroblastoma (NB). P53 plays a critical role in the response to chemotherapies and also recently has been shown to control self-renewal and differentiation in normal and malignant neural stem cells. In two infants with aggressive chemoresistant NB we identified germline *p53* mutations (R158H and R248W). Our objective was to determine the role of these mtp53 proteins as well as wild-type (wt) p53 and p73 in chemotherapy response in NB adherent cell lines and NB tumor initiating cells (TICs) that are isolated from the bone marrow of NB patients and have cancer stem cell properties.
Methods: Adherent and TIC NB lines were transfected with plasmids encoding wtp53, TAp73, mtp53 (R158H and R248W) or siRNA (p53 or p73). Immunoblots to detect target gene expression and Cl-PARP, luciferase assays, alamar blue and sphere formation assays were performed.
Results: We identified two patients with Li-Fraumeni Syndrome (LFS)-associated NB. Overexpression of mtp53 proteins derived from these patients diminished chemotherapy-induced apoptosis and activation of p53-target genes (p21, BAX, NOXA). P53 siRNA knockdown of endogenous mtp53 in fibroblasts derived from the patient with R158H mutation resulted in enhanced chemosensitivity. Both mtp53 proteins form complexes with wtp53 and the pro-apoptotic p73 protein TAp73, and when overexpressed inhibit p53 and TAp73-dependent p53 target gene activation. In NB TICs (with confirmed *wtp53*) transfection of either mtp53 LFS proteins resulted in more spheres that were larger in size. Similarly, p53 or p73 siRNA also resulted in larger more numerous spheres.
Conclusion: Mtp53 enhances chemoresistance in NB and interference with wtp53 or p73 contributes to enhanced self-renewal and proliferation of NB TICs. Taken together our data support a role for p53 and p73 in NB chemotherapy response and suggest that interference with wtp53 expression or function in TICs results in a survival advantage in vivo.

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POB117

Identification of proteomic changes associated with differentiated neuroblastoma using an in vitro differentiation system and an optimized proteomics *platform based* on ^{18}O peptide labeling

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Background: Neuroblastoma (NB) is the most common extracranial solid cancer in childhood. When tissue biomarkers indicate good differentiation, more aggressive therapeutic measures can be avoided and the overall survival of these NB patients is not affected. Here, in order to further characterize what biomarkers are associated with well differentiated neuroblastoma, we have employed a newly optimized proteomics platform based on ^{18}O peptide labeling to examine the structural change in an in vitro cell culture system stimulated by a γ-secretase inhibitor (GSI), N-[(3,5-Difluorophenyl) acetyl]-L-alanyl-2-phenyl] glycine -1,1-dimethylethyl ester (DAPT).
Method/approach: The ^{18}O peptide labeling technique is supposed to be a simple, inexpensive, and comprehensive strategy, but this approach has been long marred by the issue of incomplete labeling. To overcome this problem, we have devised a protocol that renders complete peptide labeling, which thus produces a homogeneous four-dalton mass increase in peptides with ^{18}O labeling. This difference can be readily detected using high-resolution mass spectrometric analyses.
Results: With this technique, we have found that there are a small group of proteins differentially expressed in GSI -treated SK-N-SH cells. The identities of these differentially expressed proteins have been unambiguously revealed using liquid chromatography-tandem mass spectrometry. Intriguingly, many of them have been previously identified in cell systems with other differentiation stimuli.
Conclusion: While this result highlights the prowess of this system in identification of novel biomarkers associated with neuroblastoma differentiation, it also indicates the convergence of cellular pathways in differentiating NB cell under distinct stimuli.

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POB118

Role of Caspase 8 and Caspase 3 in TRAIL-induced apoptosis of neuroblastoma cells

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Objective: To study the role of Caspase 8 and Caspase 3 in TRAIL-induced apoptosis of neuroblastoma cell line CHP212 cells.
Methods: The effects of TRAIL and Caspase 8/ Caspase 3 inhibitor+ TRAIL on the apoptosis of CHP212 cells was detected by flow cytometry. The relative Caspase 8 / Caspase 3 activity was measured by colorimetric assay. The morphous of the apoptosis cells was detected by using the transmission electron microscope(TEM).
Results: CHP212 cells were sensitive to TRAIL and had dose dependency. Caspase 8/ Caspase 3 inhibitor could diminish the apoptosis of CHP212 cells induced by TRAIL. The relative Caspase 8/ Caspase 3 activity of CHP212 cells increased gradually with the prolongation of TRAIL action time and reached the peak at 16h and 8h respectively. Typical features of apoptosis were seen by TEM.
Conclusion: TRAIL could induce apoptosis of CHP212 cells through Caspase - transduced signal pathway with the increase of Caspase8 and Caspase 3 activity.

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POB119

Creation of a CHD5 knockout (KO) mouse model

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Background: Neuroblastomas (NBs) are characterized by deletion of 1p36.31, and CHD5 is a tumor suppressor gene (TSG) that maps to this region. Our data strongly suggest that somatically acquired inactivation of this gene contributes to the development of high-risk NBs. CHD5 is expressed exclusively in the nervous system and may play a critical role in neural development, so an animal model with CHD5-KO may provide insight into normal neural development as well as tumorigenicity.
Method/approach: We used ES cell gene targeting to insert loxP sites in introns 11 and 13 of the human CHD5 gene. For selection, we inserted PGK-neo flanked by two FRT elements between exon 13 and the second loxP site. We used both PCR and genomic Southern blotting to identify stable integration of the targeting construct. Constitutional CHD5-KO mice were generated by mating Prm-CRE (Jackson Labs) male mice with female carrying the CHD5-KO construct. To generate the CHD5 conditional KO (CKO) mice, PGK-FLP male mice (Jackson Labs) were first mated with female mice carrying the CHD5-KO construct to remove the neo gene. Then females will be mated with TH-Cre (Jackson Labs) male mice to generate the TH-Cre/CHD5-CKO mice.
Results: We have successfully generated constitutional CHD5 +/- mice. After three weeks of observation, there is no apparent difference in the phenotype or behavior between wild type and CHD5-KO heterozygous mice. We will continue to observe them and monitor for neural abnormalities and tumorigenesis until the mice reach 24 months of age. CHD5-KO heterozygous mice will be mated to determine if total absence of this gene is embryonic lethal. We will observe the phenotype and tumorigenicity of homozygous TH-Cre/CKO mice to determine the effect on neural development and tumorigenicity.
Conclusion: The CHD5 constitutional and conditional KO mice may be used to assess the role of this gene in normal development and tumorigenesis. Assuming the mice develop NBs, this model can be used to evaluate new drugs to treat NB patients. Because 1p36 deletion including CHD5 occurs commonly in many human cancers, these animals could be used to elucidate pathogenesis and treatment as well.

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POT1

Prognostic significance of tumor and microenvironment gene expression for children with metastatic MYCN non-amplified neuroblastoma

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Background: Outcome for patients with stage 4 MYCN non-amplified (MYCN-NA) neuroblastoma who are diagnosed >18 months of age previously could not be predicted. We developed a 55-gene prediction model for these patients from microarray tumor gene expression profiling (JNCI, 2006). We now present a validated TaqMan® Low Density Array (TLDA) assay that predicts progression-free survival (PFS) using expression of genes from the microarray signature and of genes related to inflammation.
Method/approach: TLDA data for 48 genes was generated using primary tumors from patients of all ages with stage 4 MYCN-NA neuroblastoma enrolled in Children's Cancer Group (CCG) trials (training set, n=133), German Pediatiric Oncology and Hematology Group (GPOH) trials (test set #1, n=48), and Children's Oncology Group COG-A3973 (test set #2, n=32). After normalization, a multivariate regression model was developed for PFS for the training set using genes that did or did not correlate with age, and this was subjected to leave-one-out cross validation (LOOCV). The model was then applied to two test sets. Results were evaluated by the Receiver Operating Characteristics (ROC) of the model. A cut-off value based on the median score of the training set defined high risk (HR) and ultra high risk (UHR) tumors of patients diagnosed >18 months of age (training set n=94, test set #1 n=39, test set #2 n =32).
Results: Fourteen genes were selected for the final model. Expression of 4 correlated with age at diagnosis and of 10 predicted relapse independent of age. The Area Under the Curve (AUC) of the ROC for predicting PFS of children diagnosed ≥18 months of age in the training set (LOOCV results) and test sets #1 and #2 were 74% and 85%, and 64%. The 5-year PFS for those diagnosed ≥18 months of age in the molecular HR and UHR groups in these three cohorts was 42% (±9%) and 14% (±4%); 57% (±13%) and 14% (±8%); and 67% (±19%) and 22% (±8%).
Conclusion: Our prediction model using expression of 14 genes from tumor and microenvironment cells accurately identifies an ultra high risk subset of patients among those diagnosed 18 months of age with stage 4 MYCN-NA neuroblastoma.

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POT2

Genomic characterization and targeted resequencing of high-risk neuroblastoma (the neuroblastoma TARGET)

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Background: Although genomic changes are associated with clinical outcome and help define a high-risk (HR) group in neuroblastoma (NB), there are few genes known to be mutated and no genomic aberrations predictive of relapse within this group. The NB TARGET (Therapeutically Applicable Research to Generate Effective Treatments) collaborative research initiative aims to discover therapeutic targets and genomic predictors of outcome.
Methods: Copy number (CN) was assayed for using the Illumina HH550 SNP array in 631 highly annotated NB, and genes from regions of aberration were selected for resequencing. Relative CN was derived from absolute CN (OverUnder), and regions recurrently lost or gained were determined using a statistical test for significance (GISTIC).
Results: Low- and intermediate-risk NB cases showed whole chromosome (WC) gains of chromosomes 2, 7, 12, 13, and 17. In contrast, HR cases showed segmental gains in those same chromosomes (2p, 7q, 12q, 13q, and 17q). A similar pattern was found for CN losses: lower-risk cases had WC losses of 3, 4, 9, 11, 14, 16, 19, and 21 while HR cases had segmental losses of 1p, 3p, 4p, 5p, 9p, 11q, 14q, 19p, 19q, and 21q. Within the HR group, cases showing only WC changes had better outcomes (p=0.01). Candidate genomic regions were large, but 117 genes and microRNAs were prioritized for resequencing in 188 HR NB samples based on CN data. Analysis to date of over 680,000 traces revealed 841 sequence variants that passed strict filtering criteria. Non-silent sequence variants were found in 79/117 genes (range of cases with variants per gene: 1-19).
Conclusion: Although the same chromosomes have aberrant CN in both the lower-risk and HR groups, the aberration type (WC vs. segmental) clearly differed, with the HR group showing segmental aberrations and the lower-risk group showing WC aberrations for all overlapping regions. This suggests differing mechanisms for genomic rearrangement and reveals potential loci of interest. The low frequency of somatic mutation found by targeted resequencing underlines the need for unbiased whole genome sequencing approaches to discover mutated genes in NB (Morozova, ANR 2010).

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POT3

Identification of miRNAs contributing to neuroblastoma chemoresistance

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Background: The emergence of the role of microRNAs (miRNAs) in exacerbating drug resistance of tumours is recently being highlighted as a crucial research field for future clinical management of drug resistant tumours (1). The purpose of this study was to identify dysregulations in expression of individual and / or networks of miRNAs which may have direct effect on neuroblastoma (NB) drug resistance.
Method/approach: Individual subcultures of chemosensitive SH-SY5Y and UKF-NB-3 cells were rendered chemoresistant to doxorubicin (SH-SY5Y, UKF-NB-3) or etoposide (SH-SY5Y).In each validated chemoresistance model, the parental and subcultured cell lines were analysed for miRNA expression profiling, using a high-throughput quantitative polymerase chain reaction (RT-qPCR) miRNA profiling platform (2,3) for a total of 668 miRNAs.
Results: A total of seven miRNAs were found to be differentially expressed (higher than 2-fold change) within all three NB chemoresistance models. Four miRNAs were upregulated in the subcultured chemoresistant cell lines. Three miRNAs were found to be downregulated in the chemoresistant cell lines for all models.
Conclusion: Based on the initial miRNA findings, this study elucidates the dysregulation of seven miRNAs in three separate NB chemoresistant cell line models, spanning two cell lines (SH-SY5Y & UKF-NB-3) and two chemotherapeutic agents (doxorubicin & etoposide). These seven miRNAs may thus be possibly linked to chemoresistance induction in NB. Such miRNAs are good candidates to be novel drug targets for future miRNA based therapies against aggressive tumours that are not responding to conventional chemotherapy.

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POT4

Gene expression signatures of mutant *ALK* in neuroblastoma

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Background: Activating mutations in the *ALK* tyrosine kinase receptor have been identified in both familial and sporadic neuroblastomas (NB), the most common being the *ALK* F1174L and R1275Q variants. Preclinical studies have identified differential sensitivity of NB cell lines harboring these mutations to PF2341066 (Pfizer, Inc), an ATP-competitive c-MET/ALK inhibitor which is currently in clinical trials in NB patients. The goal of this study was to identify the signaling pathways that are critical for *ALK*-mediated NB cell survival and whose inhibition may underlie the efficacy of PF2341066.
Method: We analyzed the transcriptomes of NB cell lines harboring *ALK* mutations; Kelly and SH-SY5Y (F1174L) and SMS-KCNR (R1275Q), in which ALK signaling was abolished by treatment with PF2341066. PF2341066 doses were titrated to achieve inhibition of ALK phosphorylation without apoptosis induction. Cell lines were treated with either compound or DMSO in triplicate for 6 hours, total RNA isolated and submitted for microarray analysis. Gene expression profiles were generated using Affymetrix Human U133A HT array and Gene Pattern® software.
Results: Inhibition of ALK phosphorylation after treatment with PF2341066 was confirmed by western blot analysis. Gene expression profiling revealed 467 differentially expressed genes in the treated vs. untreated cell lines (161 downregulated and 306 upregulated; $p<0.05$; minimum fold change of 2 and a minimum absolute difference of 50 across all the samples). The signature included known downstream effectors of ALK such as AKT and ERK and also genes involved in regulation of apoptosis, cell cycle and signal transduction. The two cell lines with the *ALK* F1174L mutation, Kelly and SH-SY5Y, shared the highest fraction of significantly regulated genes that changed in the same direction compared to the SMS-KCNR line (R1275Q).
Conclusion: We have generated gene expression signatures of activated *ALK* in NB cell lines harboring mutant *ALK*. This represents a versatile tool to identify novel signaling networks regulated by ALK and to functionally validate suitable genes that may represent new therapeutic targets.

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POT5

Cytomegalovirus infection in neuroblastoma, high prevalence in tumors and reduced growth *in vivo* and in vitro using HCMV targeted therapies

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Background: The etiology of neuroblastomas is not completely known, but the involvement of viruses as additional trigger factors has been proposed. Neuroblastoma and other neural tumors have been related to active Human Cytomegalovirus (HCMV) infection. Recent evidence demonstrates a high prevalence of HCMV in several cancers including tumors of the brain, colon and prostate. HCMV infects 70-90% of the world's population and it possesses several oncomodulatory properties. HCMV infection affects cell cycle control and chromosome stability, increases COX-2 expression and influences tumor formation, and angiogenesis. Neuroblastomas express high levels of COX-2 and COX-2 inhibitors may reduce angiogenesis and tumor growth in vivo. Aims We aimed to determine whether HCMV is present in neuroblastomas and if targeting HCMV and COX-2 reduce tumor growth in vivo and in vitro.
Methods: Immunohistochemistry, PCR, western blot, clonogenic assay, mouse SH-SY5Y xenografts.
Results: We found HCMV proteins in 97% of neuroblastomas by immunohistochemistry (35/36) and PCR (8/11). Surprisingly, three cell lines SH-SY5Y, SK-N-BE2 and SK-N-AS were HCMV DNA positive. Co-treatment with Ganciclovir (150μM) and Celecoxib (15μM) reduced their clonogenic capacity synergistically by 50-65% ($p<0.05$) whereas each drug separately resulted in 10-25% reduction ($p<0.05$). In the mouse xenograft model, Valganciclovir significantly reduced tumor growth in a dose dependent manner. When used in combination with Celecoxib, tumor volume index was significantly reduced from 13.1±.4.4 to 6.9±2.4 ($p<0.05$), but not compared to Valganciclovir used alone.
Conclusion: We found a high prevalence of HCMV in neuroblastoma tumors and cell lines supporting an oncomodulatory role of HCMV. Xenografts were positive for both HCMV and COX-2, and combined antiviral treatment and COX-2 inhibition resulted in reduced clonogenic ability and inhibited tumor growth in vivo and in vitro. Our data indicate novel treatment options for children with neuroblastoma.

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POT6

Inhibition of lipoxygenases promotes retinoic acid induced cell death in neuroblastoma

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Background: The retinoic acid (RA) derivative 13-cisRA is used in the treatment of neuroblastoma (NB) and causes growth arrest, differentiation and apoptosis. RA has previously been found to promote arachidonic acid (AA) release. We hypothesise that inhibiting release and subsequent metabolism of AA will promote RA induced cell death.
Aims: To determine if inhibitors of AA release and its metabolism by cycloxygenase (COX) and lipoxygenase (LOX) pathways promote cell death after treatment with RA.
Methods: Cell survival was assessed in SH-SY5Y, NB-69 and NGP cells using XTT assays and apoptosis measured by flow cytometry for the % of cells with sub G_1 DNA content. The inhibitors used in combination with all-trans RA (atRA) were: AACOCF3 (PLA_2); Celecoxib (COX-2 and LOX-5); MK886 (LOX-5) and PD-176146 (LOX-15). An inducible COX-2 expression system was established to determine the influence of COX-2 on atRA and celecoxib induced cell death. Addition of the lipid messenger PG_{E2} was used to test for rescue of NB cells from atRA and celecoxib induced cell death. For *in vivo* studies, SH-SY5Y subcutaneous xenografts were established. Mice were treated daily with 10mg/kg atRA, 100 mg/kg celecoxib or a combination of the two drugs.
Results: Celecoxib synergistically promoted cell death *in vitro* and slowed tumour growth *in vivo*. Inhibiting AA release using AACOCF3 and inhibition of LOX-5 and to a lesser extent LOX-15 sensitised NB cells to atRA induced cell death. Induced expression of COX-2 in the SH-SY5Y^{tet12}COX-2 cells and addition of PG_{E2} to SH-SY5Y cells had no effect on the sensitivity of NB cells to celecoxib and RA.

		atRA IC50 (μM)	
Drug combinations	SH-SY5Y	NGP	NB-69
atRA+22μM celecoxib	4.3 ± 2.0	2.3 ± 3.2	4.4 ± 12.3
atRA+10μM AACOCF3	7.6 ±1.1	2.9 ± 1.0	4.0 ± 1.3
atRA+1μM MK886	7.0 ± 3.1	4.9 ± 2.0	1.3 ± 3.7
atRA+0.3μM PD-146176	9.9 ±0.3	9.9 ± 0.7	5.6 ± 1.8

Table 1: Summary of IC_{50} values. Cell survival for 0.5-15μM atRA alone was 95-199% in all three cell lines.

Conclusions: atRA signals for NB cell survival by PLA_2 mediated AA release and subsequent conversion by LOX-5. Inhibition of this pathway by celecoxib enhances the efficacy of RA for the treatment of MRD in high risk NB.

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POT7

Prognosis approach of one segmental chromosome aberration in neuroblastoma

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Background: Neuroblastoma (NB) presenting segmental chromosome aberrations (SCA), with or without numerical chromosome aberrations (NCA), are correlated with worse outcome. However the prognostic impact of the minimal number of SCA in NB without *MYCN* gene amplification remains elusive. We aimed to find out any differential feature in a set of NB carrying only one SCA.
Method: To detect chromosomal copy number changes, Multiplex Ligation-dependent Probe Amplification (MLPA) was carried out in 309 tumors, covering the relevant aberrant regions found in NB. In 135 cases (44%), Fluorescence In Situ Hybridization (FISH) was performed to evaluate the status and integrity of at least three chromosome regions (1p36 and/or 11q and/or 17q and *MYCN*). All cases with one SCA detected by MLPA were further validated using FISH. Frozen and paraffin-embedded tissue was used.
Results: Informative multigenomic data were obtained in 285/309 (92%) cases by MLPA, of which 154/285 (54%) showed SCA while 131/285 (46%) had NCA. Out of 154 cases with SCA, 13 cases (8%) presented one SCA by MLPA. From the 131 cases with NCA, 14 (11%) had one SCA by FISH (heterogeneous SCA cases). A total of 27/285 samples (10%) presented one SCA, the most frequent SCA detected was the gain of chromosome 17q (16/27), followed by different alterations in both arms of the chromosome 7 (5/27). Deletion of 1p was found in only one sample and *MYCN* gain in two samples. 21/27 (77%) patients were younger than 18 months at diagnosis. Percent frequencies of the stages disease were as follows: stages 1, 2 and 4S, 59% (16/27); stage 3, 30% (8/27); stage 4, 7% (2/27) and stag 4S, 4% (1/27) respectively. None of the patients suffered relapse but one carrying the 1p deletion. The OS and EFS median follow-up was 48 months both, with an OS and EFS rate of 96%.
Conclusion: Although an extended tumor cohort study will be necessary to have more categorical conclusions, our data point out that one SCA in neuroblastoma is associated with young patients harbouring localised tumors with good prognosis and excellent survival.
Grants:ISCIII(RD06/0020/0102) and FAECC(396/2009)

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POT8

Relationship between ALK expression and genetic predictive factors in neuroblastoma

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Background: Neuroblastoma, the most solid extra-cranial common tumour in childhood, remains a clinical enigma since the large heterogeneity of cases translates the biological complexity and the medical diagnostic and prognostic challenge of the pathology. Recently, Anaplastic Lymphoma Kinase (*ALK*) have been identified as a major predisposition gene as well as a potential therapeutic target for neuroblastoma. In this way, aberrant copy number or mutations in *ALK* gene and overexpression of this tyrosine-kinase receptor have been related to poor prognosis indicators of the disease. However, clinical difference appears among studies and to go more ahead of these observations, we try to define and reinforce new and existing correlations between *ALK* gene status, ALK expression, canonical genetic predictive factors and clinical outcome.
Method/approach: A total of 92 neuroblastomas was assessed for frequency of *ALK* status by FISH (*ALK* split probe, Dako) and expression by immunohistochemestry (anti-ALK C26G7 mAb, Cell signaling technology). Data were compared with clinical criteria (age, neuroblastoma stage), *MYCN* gene status, mycN protein expression, 1p36 and 11q deletions or 17q gain.
Results: We observed a strong correlation between ALK protein expression and gene status or 1p36/11q chromosome alterations, but no link between *ALK* gene/protein status and 17q chromosome alteration. However, contrary to previous reports, we found that ALK was equally expressed in all neuroblastoma stage excepting stage 2, and was significantly over-expressed in cases with *MYCN* amplification as well as in cases with mycN overexpression.
Conclusion: Our data confirm that *ALK* is one of the major predispositive marker for neuroblastoma but also suggest that more molecular studies are needed to really understand the biological function of *ALK* in neuroblastoma. This work was supported by grants from FAECC (396/2009) and ISCIII (RD06/0020/0102).

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POT9

Potential role of mesenchymal stromal cells in experimental neuroblastoma treatment

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Background/Aims: Mesenchymal stromal cells (MSC) exhibit tropism for sites of tissue damage as well as for the tumor microenvironment, where they integrate into the tumor-associated stroma supporting cancer growth. However, studies investigating the in vivo and in vitro effects mediated by MSC on tumor growth provided conflicting results, depending on the experimental model tested. Aim of this study was to investigate the role of MSC in the control of the growth of neuroblastoma (NB).
Methods: MSC were expanded in vitro from the bone marrow of healthy donors or femurs of A/J mice. In vitro NB cell proliferation was tested by 3H-thymidine incorporation after co-incubation of NB cell lines with or without MSC. In vitro invasion of MSC was investigated using Matrigel invasion chamber plate with a 8-mm pore-size. Murine (m) MSC transfection with pooled mRNA from NB cells was performed using Transmessenger Transfection Reagent. In vivo therapeutic effects by MSC were evaluated in terms of survival, tumor growth, proliferation, apoptosis and angiogenic activity in subcutaneous, pseudometastatic and orthotopic NB animal models. In vivo localization of MSC labeled with the fluorescent dye SP-DiI was evaluated by immunofluorescence.
Results: MSC had heterogeneous and marginal effects on NB cell line proliferation. However, in vitro migration and invasion assays showed that MSC were significantly attracted by NB cells as well as by soluble factors released by the latter cells. Moreover, after contact with MSC, NB cell lines invaded in vitro a microenvironment that mimicks the bone marrow. In in vivo pseudometastatic NB models, MSC localized specifically to NB metastases, but did not affect primary tumor growth. In contrast, in a subcutaneous NB model, MSC injected inside of the tumor mass induced an antineoplastic effect through inhibition of proliferation and induction of apoptosis. Finally, marginal immunomodulating and antitumor activities of mMSC transfected with NB mRNA were observed in a pseudometastatic immunocompetent NB model.
Conclusion: Our results suggest that MSC may control NB growth depending on the experimental model tested.

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POT10

Combinatory effect of 5-AZA-cytidin and Octreotide on neuroblastoma cell proliferation and apoptosis

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Introduction: Neuroblastomas have several similarities to pheochromocytomas, mostly but not exclusively, developing in adults. Knowledge from treatment of these tumors may be applied to neuroblastomas. Somatostatin receptors (SSTR) are present in neuroblastomas, and activation of these has been demonstrated to induce apoptosis in pheochromocytoma. Methylation of promotor regions in DNA may reduce expression of tumor suppressor genes involved in cell proliferation and apoptosis. We have studied effects of combinations of somatostatin analogues and the demethylation agent 5-AZA-cytidin on activation of apoptosis pathways and cell proliferation in neuroblastoma cell lines.
Materials and methods: Neuroblastoma cell lines SK-N-SH, SH-SY5Y, SK-N-AS, IMR-32, SK-N-DZ and KELLY were grown in normal conditions recommended by ATCC. Expression of SSTR was measured by quantitative PCR (Q-PCR). Methylation status of CpG island in SSTR type 2 (SSRT2) gene promoter was analyzed using methylation specific PCR (MS-PCR). Proliferation and apoptosis in cells treated by either the somatostatin analogue Octreotide or 5-AZA-cytidin alone or in combination were analyzed by ELISA based methods.
Results: Octreotide induced apoptosis in all cell lines, (SK-N-SH 63%, SH-SY5Y 16%, SK-N-AS 19%, IMR-32 69%, SK-N-DZ 73% and KELLY 59%). Q-PCR analysis revealed significant lower expression of SSRT2 in low-responsive compared to high-responsive cell lines (0,32 and 0,26 in SH-SY5Y and SK-N-AS, respectively, compared to SK-N-DZ). MS-PCR showed high methylation level of CpG islands in the SSRT2 gene promoter, while this disappeared after treatment with 5-AZA-cytidin. Proliferation rate in these cells were reduced by 28% and apoptosis was induced in 11%. Treatment with both 5-AZA-cytidin and Octreotide with minimal concentrations for 12 hours demonstrated increasingly reduced proliferation rate to > 90%. Apoptosis was induced in almost all cells.
Conclusion: Combinatory use of 5-AZA-cytidin and Octreotide at low doses can be an effective and mild choice in treatment of neuroblastoma. Further studies to reveal cytotoxic and systemic effects of this combination are required.

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POT11

ALK and PHOX2B mutations in neuroblastic tumours with highly suspected predisposition: Rare events and unexpected clinical features

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Neuroblastic tumours (NbT) may occur in a predisposition context. Two main genes are involved: PHOX2B, observed in familial NbT and frequently associated with other neurocristopathies (Ondine's and Hirschsprung's disease), and ALK, mostly in familial tumours. We have assessed the frequency of mutations of these two genes in NbT patients with highly suspected predisposition. The whole coding sequences of the 2 genes were analysed in tumour and/or constitutional DNAs. Methods: We sequenced both genes in 5 multifocal neonatal (<1 month) (MNN), 25 unifocal neonatal (NN), 10 multifocal (MF) and 12 syndromic NbT. Syndromes included 1 familial truncus arteriosus (TA), 1 familial patent ductus arteriosus (PDA), one Tetralogy of Fallot (TOF), one Hirschsrpung disease (HSCR), one Ondine's curse (CCHS), 2 obesity with late onset hypoventilation and hypothalamic deficiencies (LO-CHS/HD), 2 growth defects and 1 mental retardation. Results: The 5 MNN tumours showed no mutation. ALK variants were found in 2/25 NN (R1275Q and P228del3bp), 3/10 MF (R1275Q, NbT at 3 months, R1192G at 5 months and R1192G at 2years) and 1/11 syndromic NbT (M596T in TA). PHOX2B variants were found in 1/10 MF (676insC), 1 CCHS (618insC), and 1/2 Lo-CHS/HD (C17Y); the patient with HSCR had no mutation. Patients with the ALKR1192G and the PHOX2B676insG variants showed neuroblastoma and multiple sub-cutaneous ganglioneuromas. The PHOX2B676insG patient also showed an intestinal ganglioneuromatosis, whereas hyperplasia of myenteric plexus was observed in the MF case with the ALKT1151R mutation. Conclusion: ALK and PHOX2B deleterious mutations are rare events (6/52) in patients with a high probability of predisposition. Sub-cutaneous ganglioneuromatosis is linked to both ALK and PHOX2B alterations. The intestinal phenotype associated with such mutations disserves closer attention. The search for other predisposing genes is warranted.

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POT12

TLR9 expression and functionality in neuroblastoma delineate a novel prognostic marker and therapeutic target

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Background: the immune system to cope with pathogens. TLR9 expression has been reported in several tumors.
Aims: To evaluate TLR9 expression in Neuroblastoma (NB), both in vitro and in vivo, and to determine its functionality and biological impact.
Methods: TLR9 expression was evaluated by RTqPCR and flow cytometry. NB cells and peripheral blood mononuclear cells were treated with CpG oligonucleotides, either free (CpG) or Lipofectamine-complexed (L-CpG). Cell proliferation was assessed by 3H-Thymidine incorporation, apoptosis by phosphatidylserine exposure, mitochondrial membrane potential depolarization and detection of caspase 3 and 7 activity. Evaluation of TLR9 functionality was assessed using inhibitory oligonucleotides (iODN) and RNA interference. In a pseudometastatic mouse model of human NB, mice received intravenously either CpG-containing NB-targeted liposomes (TL-CpG) or CpG. Immunohistochemistry was applied to detect TLR9 in NB specimens.
Results: Treatment of TLR9-expressing NB cells with L-CpG inhibited cell proliferation and induced cell death, differently to cells of hematopoietic origin, in which the same treatment triggered cell proliferation. Caspase-dependent apoptotic events were induced by treatment of NB cells with L-CpG. iODN abrogated L-CpG-mediated anti-proliferative and pro-apoptotic effects, confirming TLR9 functionality. RNA interference experiments still left sufficient amount of TLR9 expression enabling functional responses to CpG. Compared to CpG, TL-CpG administration prolonged significantly survival of NB-bearing mice (P= 0.0154). TLR9 expression in human primary NB specimens was demonstrated, and found to inversely correlate (P<0.0001) with disease stage.
Conclusion: This study demonstrates the functional expression of TLR9 in NB and suggests that it might represent a novel prognostic and/or therapeutic target.

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POT13

Arguments for the intravenous application of high dose ascorbic acid for treatment of neuroblastoma

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Background: Oral application even of gram doses of ascorbic acid failed to cure cancer. One reason is that only up to ~ 200µM serum ascorbate can be reached in this way. In contrast, 5-10mM serum ascorbate can be obtained when well tolerated quantities of ascorbate are given intravenously. Chen et al showed in in vitro and in vivo experiments that millimolar ascorbate concentrations can preverentially kill malignant cells by H2O2 which is generated in dependence of transition metals outside in the micoenvironment of the cells (PNAS,2005,2007,2008).
Method/approach: Human neuroblastoma cells were analysed for ferritin content and release, glucose consumption and lactate production. Furthermore, the cytotoxic effects of ascorbate and H2O2 were analysed in detail.
Results: Based on the results obtained by these investigations a concept is presented which proposes that the preferential killing of cancer cells compared to normal cells is based mainly on two facts: a)The preferential metabolism of glucose to lactate instead to CO2 + H2O (Warburg effect), and b) the high cellular ferritin content and its release of neuroblastoma cells. Ascorbic acid was able to liberate iron from ferritin which produces H2O2 in the presence of oxygen : Fe++ + O2 --> superoxide --> H2O2 + Fe+++ (simplified scheme). The second function of ascorbic acid is to act as a redox cycler which reduces Fe+++ back to Fe++ , therby sustaining the reaction which is especially effective in an acidic (lactate) milieu. H2O2 diffuses into the cells and can kill them. The preferential sensitivity of cancer cells compared to normal cells to H2O2 is due to the reduced anti-oxidative defense system as a consequence of the Warburg effect.
Conclusion: We suppose that intravenous application of high doses of ascorbic acid may be a suitable method for control growth of neuroblastoma and other cancer cells , e.g. after a conventional therapy, without severe side effects. Reference: Deubzer et al: H2O2-mediated cytotoxicity of pharmacological ascorbate concentrations to neuroblastoma cells: Potential role of lactate and ferritin. Cell. Physiol. Biochem. (accepted)

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POT14

Molecular imaging of MYCN-amplified neuroblastoma tumorigenesis in orthotopic xenograft and transgenic TH-MYCN murine models

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Background/aims: MYCN amplification (MA) in Neuroblastoma (NB) is associated with a poor prognosis. MYCN plays an important role in the disregulation of a wide number of genes involved in cancer progression and resistance against classic chemotherapics. Thus, it is important to obtain predictive preclinical models and accurate procedures to follow up the tumor progression and response to new therapies in vivo. In this regard, we report a real-time monitoring of the tumorigenesis by non-invasive molecular imaging in two complementary MA-NB mice models: orthotopic xenograft and homozygous transgenic TH-MYCN models (Weiss WA,'97).
Methods: Orthotopic model was established by injection of 4 Luciferase-positive MA-NB cell lines in NOD/SCID mice. Bioluminescence Imaging (BLI) was used to detect any tumor burden formation starting from the day of injection, once a week. PET was performed on homozygous TH-MYCN mice with 18F-FDG. We analyzed tracer uptake in the tumors with the Standardized Uptake Value (SUV) from the 4th week of age, once every 4 days. All animals were sacrificed and each sample was used for histology, immunohistochemical and molecular analysis of MYCN and N-Myc levels.
Results: Orthotopic model created from all the 4 cell lines showed a 100% NB incidence. IMR-5 (a IMR-32 clone with higher MYCN copies) and SK-N-Be2(c) (from relapsed patient) cell lines showed the shorter latency (2 weeks) and progression periods (5 weeks). Homozygous TH-MYCN mice showed a 100% incidence, a latency of 28 days and progression period of 5 weeks. Histology confirmed the accordance between imaging results and tumor presence. Tumor samples showed MYCN amplification and overexpression.
Conclusion: The real-time monitoring by non-invasive molecular imaging allowed the early detection and evolution of MA-NB in two complementary mice models. Moreover, the definition of the tumor signal trend offers the possibility to define optimal ranges in which it will be possible to evaluate the efficacy of new therapies against MYCN-amplified neuroblastoma.

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POT15

Microarray-based pathway analysis leads to the identification of potential molecular mechanisms underlying gamma-secretase inhibitor-induced neuronal differentiation of neuroblastoma cells

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Background: Gamma-secretase inhibitor (GSI) can block Notch signaling and thus induce differentiation in neuroblastoma (NB) cells. However, other membrane proteins, including some receptors tyrosine kinase, could also be the substrates of gamma-secretase and therefore involve in the mechanisms underlying the GSI-induced differentiation of NB cells. The aims of this study are to investigate the major signaling pathways by which GSI induced NB cells differentiation.
Methods: The time-course cDNA microarray experiments (Agilent system) were conducted. Human NB cell line SK-N-SH were treated with 10 μM DAPT, a GSI, dissolved in 0.1% DMSO or vehicle alone (0.1% DMSO) for 12, 24, 48, or 72 hours. The clarified lysates derived from DMSO- or DAPT-treated cells as well as untreated cells were collected, and mRNAs were isolated and reverse-transcribed for the cDNA microarray analysis.
Results: NB cells became morphologically differentiated after DAPT treatments. Genes with a 2-fold change in mRNA transcript levels at any 4 time points between control and DAPT- or DMSO-treated cells were selected. The genes exhibiting alteration due to DMSO treatment, comparing to untreated control, were excluded for subsequent pathway analysis. Through this approach, we found 2767 differentially expressed genes in response to inhibition of gamma-secretase, and 1648 out of those genes were mapped in Ingenuity Pathway Analysis (IPA) tool. Ten canonical signaling pathways were identified to display significant changes ($P < 0.05$) in expression of their component genes by IPA. The 10 pathways, in order of statistic significance, included Wnt, GM-CSF, hepatic fibrosis, neuregulin, IL-2, synaptic long-term potentiation, circadian rhythm, Notch, insulin-like growth factor-1(IGF-1), fibroblast growth factor (FGF) signaling pathways.
Conclusion: These canonical signaling pathways could play crucial roles in modulating GSI-induced neuronal differentiation of NB cells. The present findings could pave the way for the identification of novel therapeutic targets for anti-NB drugs.

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POT16

IL-21-based immunotherapy of neuroblastoma in combination with lymphodepleting antibodies

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Background: Interleukin (IL)-21, the lastly discovered member of the IL-2 family, is a pleiotropic cytokine produced by CD4+ T cells. IL-21 has shown anti-tumor activity in several pre-clinical tumor models. Clinical phase I-II trials indicated that IL-21 has an acceptable toxicity and induces immune-activation resulting in some objective clinical responses. Thus we evaluated whether IL-21-based immunotherapy (IT) may affect NB growth in a syngeneic metastatic murine model.
Method/approach:IL-21-transfected Neuro2a cells (Neuro2a/IL-21) alone or in combination with depleting anti-CD4, anti-CD25 or anti-CD8 mAbs were used as vaccine in a therapeutic setting in order to evaluate the role of different T cell populations in mediating the therapeutic effects.
Results: Neuro2a/IL-21 IT cured about one third of syngeneic mice bearing disseminated NB, through a CD8+ T cell-dependent response. The co-administation of an anti-CD25 monoclonal antibody (mAb), targeting immune-suppressive CD4+CD25+FoxP3+ regulatory T (Treg) cells, slightly augmented its efficacy. Conversely, the co-administration of an anti-CD4 mAb produced a significant increase in the cure rate (80%). The potent synergistic effect achieved by the anti-CD4 mAb was related to a complete depletion of CD4+CD25+FoxP3+ Treg cells and possibly of other tumour-conditioned suppressive CD4+ T cell subsets. Mice receiving the IL-21-releasing vaccine+anti-CD4 mAb developed long-lasting immunity to Neuro2a cells. Moreover, the effect of anti-CD4 mAb was transient, since CD4+ T cell counts were recovered in 90 days. Preliminary data indicate that the administration of recombinant (r)IL-21 may have limited effects and cures about 16% of mice, but co-treatment with anti-CD4 mAb strongly augmented rIL-21 IT.
Conclusion: These data open new perspectives for the use of IL-21-based immunotherapy in conjunction with transient CD4+ lymphodepletion in human stage 4 NB.

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POT17

Characterisation of tumour progression, vascularisation and response to chemotherapy in transgenic mouse models of Neuroblastoma (TH-*MYCN and TH-MYCN/p53ER^{Tam}*) using magnetic resonance imaging

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Background: The TH-*MYCN* mouse model of neuroblastoma (NB) faithfully replicates high-risk human *MYCN* amplified NB by targeted overexpression of *MYCN* to the neural crest. Tumours arise most commonly from the adrenal medulla and are highly vascular. As a prelude to evaluation of novel therapeutics for NB, the TH-*MYCN* and the TH-*MYCN/p53ER^{Tam}*-haploinsufficient model have been characterised by MRI.
Method/approach: Mice were imaged on a 7T Bruker system pre- and post- treatment with cyclophosphamide (CP) (100mg/kg, two doses, 24 hours apart), or methotrexate (MTX) (25mg/kg). MRI relaxation rates T_1, T_2 and R_2*, were measured for each tumour with dynamic contrast-enhancement (DCE) using intravenous Gd-DTPA.
Results: Anatomical T_2-weighted coronal images revealed that TH-*MYCN* mice developed tumours originating from the adrenal glands and displacing abdominal organs, with enlargement of the abdominal aorta and vena cava. Histologically, untreated tumours grew as sheets of cells, divided into lobules and surrounded by thin fibrovascular septa, infiltrating all surrounding tissues. 48 hours after treatment with CP, tumours were undetectable or difficult to identify, consistent with the clinical sensitivity of NB to CP. Preliminary data suggest that TH-*MYCN/p53ER^{Tam}* mice show evidence of early tumour relapse. There was no response to MTX, consistent with the clinical insensitivity of NB to MTX. Quantitation of tumour MRI relaxation rates revealed a heterogeneous spatial distribution of T_1 (1748 ± 14ms), T_2 (55 ±1ms) and R_2* (115 ± 3s^{-1}). The relatively fast baseline R_2* is indicative of a large tumour blood volume, which was corroborated by DCE MRI as diffuse parenchymal enhancement across the tumour ($IAUC_{60}$ = 0.13 ± 0.01ms).
Conclusion: This study reinforces the TH-*MYCN* and TH-*MYCN/p53ER^{Tam}* models as useful tools that replicate human NB in its anatomical and radiological appearance, and resistance to chemotherapy. MRI of TH-*MYCN* models may accelerate development of novel therapeutics by allowing simultaneous evaluation of MRI biomarkers of treatment response and resistance.

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POT18

Development and characterization of bioluminescent orthotopic and metastatic neuroblastoma models

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Background: The treatment of metastatic neuroblastoma remains a challenge in pediatric oncology. In preclical models, evaluation of tumor development and disease progression is often limited by the inability to visualize tumor cell migration. In this study, we developed metastatic in vivo models of bioluminescent neuroblastoma which could be used for therapeutic studies.
Methods: The neuroblastoma cell lines IMR-32 and IGR-N91 were transfected with a pcDNA3 construct carrying Luc gene to express firefly luciferase. A localized and a disseminated tumor model were established in Balb-RagãC mice following orthotopic transplantation of tumor cells in the left adrenal region and intravenous injection in the tail vein, respectively. The development of the disease was followed by ultrasound and bioluminescence imaging using the Ivis50 Caliper; tumor distribution was confirmed by immunohistochemisitry.
Results: The bioluminescence imaging detected tumor cells in the orthotopic model two weeks prior to any tumor detection with ultrasound. In systemic models, bioluminescent signals were observed in liver, lungs and bone marrow in IMR32 and lung, liver, spleen and adrenal gland in IGR-N91, in both approximately nine and five weeks, respectively, before the onset of clinical symptoms or palpable tumor mass. Metastases were confirmed by histology. Finally, bioluminescence imaging permitted to monitor and quantify non-invasively and in real-time antitumor response to the treatment with the topoisomerase I inhibitor irinotecan in disseminated disease.
Conclusions: Bioluminescence is more sensitive in disease detection than traditional techniques. Moreover, it allows to monitor and to quantify, in real-time, metastatic dissemination or evolution of residual disease. Two bioluminescent neuroblastoma cell lines are available in the laboratory allowing to study tumor cell tropism, spread mechanisms and to evaluate new therapies in orthotopic and disseminated preclinical models

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POT19

The DNA-binding protein, YB-1 is a direct N-Myc target and influences repair and resistance in neuroblastoma cell lines

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Background: YB-1 is a member of the cold shock family of proteins, and regulates both gene transcription and translation. It has been shown to bind DNA repair proteins and cisplatin-modified DNA in vitro, and increase chemosensitivity of prostate cancer cells to cisplatin and paclitaxol. Increased expression and nuclear localization of YB-1 has been associated with aggressive phenotypes of several cancers.
Method/approach: YB-1 expression was examined by western blotting and immunocytochemistry. Effects of YB-1 on cell viability after cisplatin treatment were assessed in MTT assays with and without siRNA-mediated YB-1 knockdown. Capacity for double-strand break repair was assessed by counting γH2AX-positive foci. The effect of MYCN on YB-1 expression was assessed in 2 regulatable MYCN cell models (4-OHT-inducible SHEP-MYC-ER and SHEP-MYCN tet-off). MYCN binding to the YB-1 promoter was analyzed using ChIP-chip and ChIP-seq, including markers for transcriptional activation, repression and elongation.
Results: YB-1 was expressed in all 7 NB cell lines examined. Elevated expression in the nuclei and cytoplasm of a cisplatin-resistant cell line was observed in comparison to parental cells. Cisplatin treatment induced YB-1 protein expression and nuclear translocation in both cell lines. YB-1 knockdown increased sensitivity to cisplatin in both cell lines, as well as the number of γH2AX foci, indicating reduced repair capacity for double-strand breaks. YB-1 expression increased or decreased with MYCN activation or repression, respectively, in cell culture models. MYCN binding to the YB-1 promoter was observed in all 7 NB cell lines examined, together with epigenetic marks for active transcription and elongation.
Conclusion: In NB cells, YB-1 is upregulated and translocated to the nucleus after cisplatin treatment, and enhances capacity for double-strand break repair. We show that MYCN regulates YB-1 expression, implicating it as a possible target for therapeutic intervention. YB-1 may be involved in mechanisms of resistance development in NB, and expression is currently being assessed on an NB tissue microarray to correlate protein expression with tumour phenotyp

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POT20

Comparative interactomics, transcriptomics and proteomics studies of CDK inhibitors roscovitine and CR8 effects on human neuroblastoma SH-SY5Y cells converge to a central role of Myc

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Background: The 518 human protein kinases constitute a large panel of potential therapeutic targets for a variety of diseases. Our work is focused on pharmacological inhibitors of cyclin-dependent kinases (CDKs), and their applications against cancers, neurodegenerative diseases, renal diseases and inflammation.
Method/approach: Using a diversity of global '-omics' approaches, we investigated the effects of CDK inhibitors on neuroblastoma (NB) SH-SY5Y cells.
Results: CR8, a pharmacological inhibitor of CDKs recently derived from the clinical drug roscovitine, is over 100 fold more potent than its parent compound at inducing cell death in the NCI 60 tumor cell lines panel. In order to understand the intracellular mechanisms of action of these two molecules, we made use of a wide variety of '-omics' techniques which we applied to the human neuroblastoma SH-SY5Y cell line: [1] interaction assays (Ambit Biosciences) on 402 kinases, [2] quantitative cellular target profiling following competition on an affinity matrix comprising a set of broad-spectrum kinase inhibitors (Kinaxo), [3] affinity chromatography on immobilized roscovitine and CR8, [4] whole genome size transcriptomics analysis (Agilent), coupled with quantitative PCR expression studies of a selection of mRNAs, [5] global proteomics approach, associated with Western blotting analysis of a selection of proteins. Altogether the results show that these two molecules, considered as relatively specific to CDKs, have a complex mechanism of action. Their major direct targets belong to the CDKs, DYRKs, CLKs, CK1s families. They may also include PAK4, CRK7, PIP4Ks. By inhibiting CDK7 and CDK9, roscovitine and CR8 transiently reduce RNA polymerase 2 activity, which results in the down-regulation of a large set of genes. Global analysis of the transcriptomics and proteomics results point to a central role of MYC transcription factor down-regulation.
Conclusion: Taken together these results suggest the use of CDK inhibitors in the treatment of cancers dependent on MYC expression, and in particular of neuroblastoma where MYCN amplification is a clear factor of poor prognosis.

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POT21

ALK and pALK protein levels in NBL cell lines correlate with ALK mutation status and responsiveness to ALK inhibition

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Background: Responsiveness to Anaplastic Lymphoma Kinase inhibitor (ALKi) molecules is excellent in ALK mutated neuroblastoma (NBL) cell lines with high phosphorylated ALK (pALK) protein levels. Recently, it was shown that high total ALK levels correlates to bad prognosis in NBL patients, regardless of mutation status (Passoni et al. 2009). We examined the correlation between ALK and pALK protein levels and response to ALKi treatment in ALK mutated (MUT) and wild type (WT) NBL cell lines.
Method/approach: ALK (α-ALK, Thermo Fisher Scientific) and pALK (α-pALK Y1604, Epitomics) protein levels were measured in 22 NBL cell lines by western blot. Two pALK products (both around 200 kDa) were identified and quantified together. The mutation status of the cell lines was further characterized by sequencing the ALK gene (exon 20,22-25) and performing multiplex ligation-dependent probe amplification (MLPA) of the 2p arm. Sensitivity to the ALK inhibitor TAE684 (Axon Medchem) was tested in 6 NBL cell lines (3 WT, 3 MUT) by cell viability measurement with MTS/PMS after 72 hours of incubation.
Results: ALK and pALK protein levels were significantly higher in mutant than WT cell lines (p=0.003 and p=0.03, respectively). These mutant cell lines showed a significantly higher sensitivity towards ALKi (LC50 values 3.4 fold lower) than WT cell lines. For mutant cell lines, ALK and pALK protein levels strongly correlated with responsiveness to ALKi treatment (ALK 200 kDa r=0.74 , ALK 140 kDa r=0.95 and pALK r=0.80), whereas only pALK levels but not ALK levels correlated with response to ALKi treatment in WT cell lines (ALK 200 kDa r=0.42 , ALK 140 kDa r=0.35 and pALK 200 kDa r=0.68).
Conclusion: In conclusion, ALK gene mutation is strongly correlated with high ALK and pALK protein levels and high ALKi responsiveness. For ALK WT cell lines ALKi response was best predicted by the pALK protein level.

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POT22

Development of a DNA methylation array normalization method for analyzing demethylating treatment effects in paired neuroblastoma cell lines

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Background: DNA methylation is important in normal development and neuroblastomagenesis. Demethylating agents (Mi) are potential therapeutic drugs. We studied genome-wide demethylating effects of epigenetic drugs in paired neuroblastoma (NB) cell lines, before and after treatment. Conventional normalization methods correct for experimental differences between samples, however large differences can also be erased as 'noise'. We developed a normalization technique for analyzing mass methylation data after treatment in paired samples.
Method/approach: We treated 23 neural crest cell lines (NB and PNET) with 30 nM Decitabine (Mi) for 72 hours, and 25 nM Trichostatin A (HDAC inhibitor) for the last 48 hours. DNA samples were cut using MseI, linker-ligated and digested using two methylation-sensitive restriction enzymes according to the Differential Methylation Hybridization technique (Yan et al. 2002). Labeled amplicons, enriched for methylated regions were hybridized to 244 K CpG island arrays (Agilent).
Results: Conventional normalization: Loess and VSN two channel normalization showed an increase of methylation in 8% and a decrease in 10% of CpG islands after treatment. Novel normalization: Our new normalization technique is based on MseI fragments without methylation sensitive restriction sites. The data points are methylation and treatment independent and used as controls in a weighted Loess normalization. This resulted in a significant decrease of methylation in 69.1% of the CpG islands and an increase in 1% of the CpG islands after epigenetic treatment. To confirm this, we tested the effect of demethylation on known methylated genes (DcR1, DcR2, RASSF1A, DR3). The new normalization showed significant demethylation and compared best to MSP data. Global demethylating will be validated by Luminiometric methylation assay (LUMA).
Conclusion: We developed a normalization technique for high resolution methylation arrays, based on the equal distribution of uncut DNA fragments after methylation specific enzymatic digestion. We showed that this technique preserves demethylating effects in paired samples and better compares to the proven biological effec

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POT23

Expression of chemokine CCL21 and its receptor CCR7 in in neuroblastoma

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Background: Chemokines are small (8-14kDa) soluble proteins that play an important role in lymphatic metastasis by binding to their G-Protein coupled receptors. The aim of this study was to evaluate the expression of the chemokine CCL21 and its receptor CCR7 in neuroblastoma (NB) cell lines and tumours.
Methods: Protein and RNA were assessed using Enzyme-linked immunoadsorbent assay (ELISA) and reverse transcriptase polymerase chain reaction (RT-PCR) in NB cell lines, before and after treatment with 10um aTRA and from 53 frozen neuroblastic tumours, including ganglioneuroblastoma and ganglioneuromas. Immunohistochemical staining for CCL21 and CCR7 was performed in formalin-fixed, paraffin-embedded tissue samples.
Results: RT-PCR showed expression of CCR7 and CCL21 in all the NB cell lines and tumour samples. ELISA assay showed CCl21 protein in all the cell lines, which decreased after treatment with aTRA. CCL21 expression increased with stage, with GN containing the lowest (0.28pg/ug protein) and Stage 4 the highest (4.09pg/ug protein) protein levels, although there was no significant difference in levels across the stages. There was no statistical difference in CCL21 protein levels in MYCN non-amplified and MYCN-amplified NBs. Weak cytoplasmic staining for CCL21 was identified in ganglion cells and neuroblasts, while undifferentiated neuroblasts were negative. The neuropil showed weak expression. Endothelial staining was identified in <5% lymphatic channels. Strong membrane staining for CCR7 was detected in <10% poorly-differentiated neuroblasts.
Conclusion: Higher levels of CCl21 protein in advanced neuroblastomas indicate that functional studies are essential to investigate the biological role of CCL21/CCR7 axis in lymph node metastases in neuroblastoma.

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POT24

Abstract withdrawn

POT25

Hypoxia gene signature as a prognostic factor in neuroblastoma patients

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Background: Neuroblastoma is the most common extracranial solid tumor in childhood and shows notable heterogeneity with regard to both histology and clinical behavior. Hypoxia, a decrease of oxygen tension, is crucial for tumor progression inducing angiogenesis and inhibiting apoptosis and cell differentiation. Hypoxia is related to poor prognosis in cancer and it has a strong impact on neuroblastoma aggressiveness. Our aim is to define the hypoxia signature from in vitro controlled system and to test its prognostic value on the gene expression of a cohort of neuroblastoma patients.
Method/approach: l1-l2 regularization framework has been applied on gene expression profiles of 11 neuroblastoma cell lines to define the neuroblastoma hypoxia signature (NBHS). We applied k-means clustering on the expression level of the 62 probesets of NBHS to segregate 88 neuroblastoma patients and subgroups obtained by common risk factors stratification. We analyzed the classes by Kaplan-Meier curves and log-rank test for overall survival (OS) and event-free survival (EFS). Multivariate Cox analysis was performed to define the predictive power of the signature.
Results: The NBHS distinguished two groups of neuroblastoma patients classifying them as poor prognosis, those having OS rate of 25.5% and EFS rate of 27.7%, and as good prognosis, those having OS rate of 73.2% and EFS rate of 67.7%. The poor prognosis patients show an over-expression of the hypoxia probesets. Multivariate Cox analysis revealed that the NBHS is a significant independent predictor after controlling for commonly used risk factors. When applied to MYCN not amplified patients, the NBHS stratifies patients with OS rate of 24.2% and EFS rate of 27.3% for patients with poor prognosis, compared with OS rate of 81.4% and EFS rate of 74.8% for patients with good prognosis.
Conclusion: We demonstrate that the NBHS is a significant prognostic factor capable of stratify neuroblastoma patients. Furthermore, we obtained the proof of principle that the approach of hypoxia genes selection from in vitro controlled tumor cell lines is applicable to identify specific contribution of microenvironment to the tumors' biology.

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POT26

Analysis of cytotoxic drugs that selectively target cells with MYC overexpression

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Background: Expression of MYC is deregulated in a wide range of human neoplasias, and is often associated with aggressive, and poorly differentiated tumors in children such as Burkitt's lymphoma (100% c-MYC translocation) and neuroblastoma (~40% MYCN amplification). MYCN-amplification is strongly correlated with poor clinical outcome of neuroblastoma, with low survival rates despite advances in treatment strategies. Therefore, novel treatments are urgently needed and one approach is to identify compounds with selectivity for cells over-expressing MYCN.
Methods: Neuroblastoma and Burkitt lymphoma like cells with conditional over-expression of MYCN and c-MYC, respectively, were used to screen a library of 80 conventional cytotoxic drugs and small compounds for their ability to reduce tumor cell viability in a MYC dependent manner.
Results: We found that 21% of the analyzed compounds induced apoptosis and/or inhibited proliferation in a MYC-specific manner, with a large overlap between c-MYC and MYCN over-expressing cells. The majority of the positive hits were compounds belonging to two classes: tubulin targeting agents and topoisomerase inhibitors. This indicates that MYC over-expression potentiates tumor cell killing by cytotoxic drugs in a mechanism-specific manner. Treatment of the cells with topoisomerase inhibitors led to down-regulation of MYC protein levels whereas no effect was observed using the tubulin stability effectors. One of the drugs was also found to be able to disrupt MYC-Max DNA binding in an EMSA assay.
Conclusion: The MYC pathway is only targeted by a subset of conventional cytotoxic drugs currently used in the clinic. Elucidating the mechanisms underlying their specificity towards MYC may be of importance for optimizing treatment for tumors with MYC deregulation. Our data also underscores that MYC is an attractive target for novel therapies using cellular library screenings.

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POT27

Expression of the neuron-specific protein CHD5 is an independent marker of outcome in neuroblastoma

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Background: The chromodomain, helicase DNA-binding protein 5 (CHD5) is a tumor suppressor gene located on chromosome 1p36, a region recurrently deleted in high risk neuroblastoma (NB). CHD5 mRNA expression has been reported in normal neural tissues and in low risk NB, nevertheless, the distribution of CHD5 protein has not been explored. The aim of this study was to investigate CHD5 protein expression as an immunohistochemical marker of outcome in NB. With this propose, CHD5 protein expression was analyzed in normal neural tissues and neuroblastic tumors (NTs). Reactivation of CHD5 expression in response to induced differentiation processes was investigated in high risk tumors and NB cell lines.
Results: We report that CHD5 is a neuron-specific protein, absent in glial cells, with diverse expression amongst neuron types. Within NTs, CHD5 immunoreactivity was found restricted to differentiating neuroblasts and ganglion-like cells, and absent in undifferentiated neuroblasts and stromal Schwann cells. An immunohistochemical analysis of 90 primary NTs highlighted a strong association of CHD5 expression with favorable prognostic variables (age, tumor stage, histology and ploidy; P<0.001 for all), with overall survival (P<0.001) and event-free survival (P<0.001). Multivariate analysis and Predictive Value analysis showed that CHD5 prognostic value is independent of other clinically relevant parameters and could therefore represent a marker of outcome in NB. The prognostic value of CHD5 was confirmed in an independent validation cohort of 25 NB tumors. Reactivation of CHD5 expression after induction chemotherapy was observed only in high risk tumors with evident neuroblastic maturation features. These NB tumors showed good clinical response and prolonged patient survival. None of these tumors harbored 1p deletion or MYCN amplification. In vitro, retinoic acid (RA) induced neuronal differentiation demonstrated that CHD5 expression is sensitive to RA treatment in NB cell lines lacking 1p deletion and MYCN amplification.
Conclusion: The neuron-specific protein CHD5 may represent a marker of outcome in NB that can be tested by conventional immunohistochemistry.

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POT28

Sphingosine-1-phosphate signaling is a mechanism of fenretinide resistance and provides a novel therapeutic target

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Background: Fenretinide (4-HPR), is a retinoid cytotoxic to cancer cells via increased dihydroceramide levels that has clinical activity against recurrent neuroblastoma (NB). Sphingosine kinase 1 (SPHK1) generates the anti-apoptotic sphingosine-1-phosphate (S1P); S1P is thought to counter cytotoxicity mediated by ceramides.
Methods: The 4-HPR-resistant NB line KCNR-FR (IC99=19.9 μM) was selected from SMS-KCNR (IC99=4.6 μM). Cytotoxicity was by DIMSCAN, RNA expression by TaqMan quantitative RT-PCR, SPHK1 activity by thin-layer chromatography, and cell surface SP1 receptors by flow cytometry.
Results: Using a TaqMan low-density array (TLDA) for 42 sphingolipid synthesis and metabolism genes we found that SPHK1 (5.2-fold) and the S1P receptor S1PR2 was over-expressed (2.4-fold) in KCNR-FR vs SMS-KCNR. We observed a 3 to 27-fold increase ($p < 0.01$) in basal SPHK1 expression in 4-HPR *de novo* resistant (IC90 > 10μM, n = 6) vs sensitive NB lines (IC90 < 5 μM, n = 4). Forced over-expression in SMS-KCNR increased SPHK1 enzyme activity by 70-fold and induced 4-HPR resistance relative to controls ($p < 0.05$). RNAi knockdown decreased SPHK1 mRNA expression (36% of scrambled RNAi, $p<0.05$) and significantly increased apoptosis in KCNR-FR treated with 4-HPR relative to controls ($p<0.05$). S1PR2 receptor levels in KCNR-FR and SMS-KCNR were induced 2.6-fold by 4-HPR at the RNA level and on the cell surface. Safingol, a putative SPHK1 inhibitor in phase I trials, synergized 4-HPR cytotoxicity in multiple NB cell lines and partially reversed 4-HPR resistance in KCNR-FR and SPHK1-transduced SMS-KCNR (Combination Index < 1).
Conclusion: Thus, selection for resistance to 4-HPR in NB was associated with increased expression of the anti-apoptotic S1P signaling pathway. Increased generation of S1P plays a role in 4-HPR resistance, likely acting via certain S1P receptors (induced by 4-HPR) to mediate a pro-life autocrine loop. Resistance to 4-HPR can be partially overcome by targeting sphingosine kinase, providing further preclinical data in support of clinical trials combining 4-HPR with safingol.

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POT29

Omega-3 fatty acid supplementation delays the progression of neuroblastoma *in vivo*

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Background: Epidemiological and preclinical studies have revealed that omega-3 fatty acids have anti-cancer properties. We have previously shown that the omega-3 fatty acid docosahexaenoic acid (DHA) induces apoptosis of neuroblastoma cells *in vitro* by mechanisms involving intracellular peroxidation of DHA by means of 15-lipoxygenase or autoxidation. In the present study, the effects of DHA supplementation on neuroblastoma tumor growth *in vivo* were investigated using two complementary approaches.
Method/approach: For the purpose of prevention, DHA as a dietary supplement was fed to athymic rats before the rats were xenografted with human neuroblastoma cells. For therapeutic purposes, athymic rats with established neuroblastoma xenografts were given DHA daily by gavage and tumor growth was monitored. DHA levels in plasma and tumor tissue were analyzed by gas liquid chromatography.
Results: DHA delayed neuroblastoma xenograft development and inhibited the growth of established neuroblastoma xenografts in athymic rats. A revised version of the Pediatric Preclinical Testing Program (PPTP) evaluation scheme used as a measurement of treatment response showed that untreated control animals developed progressive disease, whereas treatment with DHA resulted in stable disease or partial response, depending on the DHA concentration.
Conclusion: In conclusion, prophylactic treatment with DHA delayed development of neuroblastoma in rats, suggesting that DHA could be a potential agent in the treatment of minimal residual disease and should be considered for prevention in selected cases. Treatment results on established aggressive neuroblastoma tumors suggest further studies aiming at a clinical application in children with high-risk neuroblastoma.

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POT30

'BH3 profiles' identify neuroblastomas with exquisite ABT-737/ chemotherapy sensitivity *in vivo* and Bim signaling is a critical determinant

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Background: Apoptosis evasion contributes to therapy resistance in neuroblastoma (NB). NB mitochondria release cytochrome c selectively to pro-death BH3 peptides. Such "BH3 profiles" identify Bcl2 family addiction patterns and predict sensitivity to Bcl2 antagonists in vitro (Cell Death Diff, 2010). We now show BH3 profiles are preserved in xenografts (XG) and predict XG therapy response.
Method/approach: Mitochondrial responses to diverse BH3 peptides were obtained from >15 NBs and XGs and clustered (unsupervised). Cell lines were assessed for in vitro/in vivo response to small molecule Bcl2 antagonists, and predictions were validated by siRNA and coIP.
Results: Clustering identified three groups: Mcl1-dependent (cyto c release to Noxa), Bclxl dependent (cyto c release to Bik), and BH3 resistant (minimal cyto c release). XGs clustered with their cell lines suggesting cell intrinsic signaling. Notably, BH3 resistant NBs were derived at relapse. Most NBs had activated Bim bound to pro-survival proteins, yet relapsed NBs show little activated Bim on Mcl1, Bcl2 or Bclxl by coIP. This "Loss of Bim priming"may explain therapy resistance seen clinically. Accordingly, siRNA knockdown of Bim in "primed" NB cells led to chemoresistance. XGs from each BH3 profile group were treated with cyclophosphamide (CPM), +/-ABT737 (targets Bcl2/xl/w), or +/-AT101 (targets Mcl1). ABT737 alone regressed Bclxl dependent SMS-SAN XGs ($P<0.01$), while ABT737/CPM regressed all tumors and cured >50% after a single treatment ($p<.0001$ v. CPM). CPM cured no mice. Profiles from XGs that re-grew showed stable Bclxl dependence and re-treatment led to response and ~50% cure. Mcl1-dependent IMR5 XGs and BH3 resistant BE2C XGs were insensitive to ABT737, AT101 or CPM. We also treated TH-MYCN mice (Bik BH3 profile) with palpable NBs and single agent ABT737 doubled the median survival compared to control.
Conclusion: Activated Bim is critical to cytotoxic efficacy in NB, with Bclxl or Mcl1 antagonizing in subsets. Relapsed NB has lost Bim priming. ABT737 is remarkably effective for Bclxl dependent NB identified by BH3 profiles. Profiling provides a platform to study resistance mechanisms in relapsed NB.

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POT31

Cepharanthine reverses multidrug resistance and sensitizes neuroblastoma cells to vincristine-induced cell death

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Background: Acquired multidrug resistance (MDR) is a contributing factor to the poor prognosis faced by neuroblastoma (NB) patients. Cepharanthine (CEP), an alkaloid extracted from Stephania Cepharantha Hayata, has been used in Japan to treat both acute and chronic diseases without serious side effects. We sought to determine the efficacy of CEP on NB and MDR.
Methods: The effect of CEP alone or in combination with vincristine (Vin) was evaluated in NB cell lines: SMSKCNR (SMSR), SK-N-BE2c (BE2c), and SH-SY5Y (SY5Y). Viability was determined by MTS assay after 72 hours of treatment. The effect of CEP on MDR was evaluated using fluorescent microscopy. Cells were pretreated with 10μM CEP for 1hr followed by 1hr DOX (3μM). DOX levels were determined 18 hours after drug exposure.
Results: CEP (10μM) significantly reduced viability to 11±2.2% (SMSR); 8.2±1.1% (BE2c); 0.4±4.1% (SY5Y) compared to vehicle-treated controls. To determine if CEP could potentiate Vin-induced cytotoxicity, NB cells were treated with 3μM or 300nM CEP, 50nM Vin, or a combination of CEP and Vin. At 72 hours, 3μM CEP alone reduced viability to 72±3.4% (SMSR), 83±3.7% (BE2c), and 82±7.5% (SY5Y) of vehicle-treated controls; whereas Vin had no affect on viability 92±4.3% (SMSR), 104±4.7% (BE2c), and 94±7.5% (SY5Y). Addition of 3uM CEP to Vin reduced viability to less than 10% of vehicle-treated controls (SMSR 7.2±3.8%, BE2c 8.8±1.0%, and SY5Y 2.8±7.0%). Furthermore, 300nM CEP, a concentration that had no effect on viability, plus Vin reduced viability to approximately 50% of controls (SMSR 45±2.1%, BE2c 54±2.7% and SY5Y 58±3.4%). To determine if CEP modulated Vin-induced cell death by reversing MDR, we examined the effect of CEP on DOX cellular accumulation. DOX signal was approximately 3-fold higher in BE2c cells treated with CEP (CEP+DOX: 59±19.2 vs. DOX: 20±19.1 arbitrary units; $p<0.001$) suggesting an inhibition of MDR by CEP.
Conclusion: CEP can directly induce NB cell death as well as sensitize cells to Vin at concentrations reported to be clinically achievable. CEP represents a novel addition to NB treatment regimens and may potentially improve clinical outcome.

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POT32

***ATM* deletion is a frequent event in neuroblastoma**

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Background: Evidence suggests that chromosome 11q plays a role in neuroblastoma (NB) biology. *ATM* gene resides at 11q22-23 and ATM protein mediates a kinase cascade linking DNA damage to cell-cycle progression and apoptosis. The aim of this project is to determine the prevalence of *ATM* alterations in 16 NB cell lines and 50 primary NB samples (NBT).
Methods: We determined the relative tumoral DNA copy number for the 64 *ATM* exons, using the MLPA assay (P041/P042) in NBs. *ATM* mRNA expression was measured by quantitative real-time PCR.
Results: No intragenic deletion/duplication was detected in NB. Six NB cell lines (38%) and 14 NBT (28%) had a complete *ATM* deletion (del), while 1 NBT had a complete *ATM* duplication. *ATM* mRNA levels were significantly decreased in *ATM* del cell lines ($p<0.005$). *ATM* del was more prevalent in stage 4 vs. stages 1, 2, 3 NBT (11/24; 95% CI 26-67% vs. 3/26; 95% CI 2-30%); $p=0.011$). *ATM* del appears not be associated with *MYCN* amplification: only 1 of the 11 stage 4 NBT samples with *ATM* del was found with *MYCN* amplification vs 5 of the 13 stage 4 NBT samples without *ATM* del.
Conclusions: We show here that *ATM* del is a frequent event in NB, that it is associated with decreased *ATM* mRNA expression, and that it correlates to advanced clinical stages independently from *MYCN* amplification. Since previous work has documented an indirect link between *MYCN* overexpression and ATM downregulation in NB cell lines, our observations provide additional evidence for ATM contribution in NB biology, suggesting that in non-amplified *MYCN* NB, ATM downregulation could directly result from *ATM* gene deletion.

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POT33

Computer vision in neuroblastoma: computer-aided prognosis

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Background: The diagnosis, prognosis, and treatment planning of neuroblastoma depend on classification of the tumor, which in turn depends on morphological feature differences. A key component of the Neuroblastoma prognosis classification process involves a morphology-based scheme: International Neuroblastoma Pathology Classification – INPC. Currently the Children's Oncology Group Neuroblastoma Biology Study utilizes the INPC system for patient stratification and protocol assignment. In a recent study, it has been shown that there is 20% disagreement between central and institutional reviewers using the INPC system.
Method/approach: In this study, we built upon our preliminary work in the area of image analysis for cancer to further develop computerized techniques to analyze the morphology of neuroblastoma histopathology slides. Specifically, we analyzed the the degree of Schwannian stromal development and the grade of neuroblastic differentiation to categorize the tissue sample as either favorable or unfavorable histology.We developed multi-resolution image analysis, feature selection and novel classification techniques. In addition to conventional texture features, we introduced a novel way of constructing structural features that captures the high-level perceptual patterns.
Results: The developed system was tested with an independent set of 34 whole-slide images and achieved a classification accuracy of 94.1% (32/34).
Conclusion: A combined computer-assisted neuroblastoma prognosis system can be developed. The latest cooperative effort of INRG (International Neuroblastoma Risk Group) in developing a consensus approach to risk stratification has determined to incorporate a part of the INPC criteria; such as "Schwannian stromal development" and "Grade of neuroblastic differentiation", in the factors significantly predicting clinical outcome of neuroblastoma patients. This requires a standardized/unified histopathology evaluation by participating pathologists in different countries in North America, Europe, and Japan. Computer-assisted tools, once established, could play a critical role in supporting this activity.

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POT34

Analysis of aggressive human and mouse ALK neuroblastoma mutations.

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ALK was originally identified as a partner in the NPM-ALK chromosomal translocation, found in a subset of anaplastic large cell lymphoma. The normal cellular ALK protein is a Receptor Tyrosine Kinase (RTK) and contains an extracellular ligand-binding domain, a transmembrane domain and an intracellular tyrosine kinase domain. A wide variety of ALK fusion protein have since been described in inflammatory myofibroblastic tumours, non-small cell lung cancer, diffuse B-cell lymphomas and squamous cell carcinoma of the esophagus, constituent with a function for inappropriately activated ALK in oncogenic progression. Recently, mutations in the kinase domain of Anaplastic Lymphoma Kinase (ALK) have been implicated in the progression neurobastoma. In order to clarify the molecular cause of the disease we have developed using cell culture systems and biochemical analysis to study wild type and putative gain-of-function ALK mutations. Gain-of-function ALK mutations display ligand independent activation and are auto-transphosphorylated, enabling activation of downstream targets of the ALK signaling pathway. Our studies should allow a more complete picture of the function of native and constitutively active ALK in normal situations and under uncontrolled neuroblastoma pathology. These systems should provide important information regarding ALK mediated signalling processes and will allow study of ALK function in both controlled and uncontrolled growth.

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POT35

Rapamycin upregulates osteoprotegerin and increases time to pathologic fracture in a mouse neuroblastoma bone metastasis model

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Introduction: Osteoprotegerin (OPG) is a soluble decoy receptor for RANK ligand that can inhibit osteoclastogenesis and slow the progression of osteolytic bone lesions. Rapamycin is an immunosuppresive and anti-neoplastic agent that has been shown to upregulate OPG in mouse bone marrow stroma. We tested the hypothesis that rapamycin could inhibit osteolytic neuroblastoma bone metastases through its action on OPG.
Methods: Mouse bone marrow cells were co-cultured with human neuroblastoma cells (CHLA-255 or NB-1691) and treated with rapamycin. Supernatant was collected for OPG ELISA and cells were stained to detect osteoclasts. For in vivo studies, an orthotopic model of bone metastasis was created by injecting neuroblastoma cells intra-femorally in SCID mice. Mice with established disease were treated with one cycle of standard chemotherapy, with or without rapamycin (5mg/kg IP, QD). X-rays were obtained twice a week to detect pathologic (Grade IV) fractures.
Results: OPG in co-culture medium was increased when cells were treated with 100nM rapamycin compared to control in CHLA 255(52.19 pg/mL+/-1.12, vs 11.42 pg/mL+/-0.50, p= 0.0168) and NB1691 (153.8pg/mL+/-5.05 vs. 5.02pg/mL +/-1.01 p=0.0395). The mean number of osteoclasts was significantly decreased compared to control in wells containing either CHLA255 (14.3+/-5.04 vs 30.07+/- 3.04, p= 0.004) or NB1691 (17.72+/-6.18 vs. 32.38+/-4.9, p=0.001). In vivo, tumor-bearing mice treated with rapamycin had a significantly increased serum level of OPG (82.11pg/mL+/-4.002 vs.55.83pg/mL+/-3.63 p=0.005) and longer median time to pathologic fracture compared to control with CHLA 255 (103 days+/-4.04 vs. 74.5 days+/-12.03 p=0.0139) and NB1691 (93 days +/-11.24 vs.62 days+/-8.39 p=0.0086).
Conclusion: Rapamycin treatment increased OPG expression in both bone marrow cells and tumor cells, and delayed the time to pathologic fracture in a mouse neuroblastoma xenograft model, thus demonstrating a novel anti-tumor mechanism of action for rapamycin. These results support the continued exploration of the role of rapamycin in the treatment of children with neuroblastoma, particularly if bone metastases are present.

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POT36

The addition of HIF inhibition potentiates the effects of angiogenesis inhibition in mouse neuroblastoma xenografts

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Introduction: One mechanism of resistance to antiangiogenic therapy is the upregulation of hypoxia inducible factors (HIF) in areas of tumor hypoxia. HIF-activated gene products allow cells to survive in low oxygen environments, enabling them to elude the effects of antiangiogenic therapy. We hypothesized that the addition of a HIF-1α inhibitor, topotecan, would enhance the antitumor activity of the antiangiogenic agents, bevacizumab or sunitinib, in orthotopic neuroblastoma xenografts.
Methods: Neuroblastoma xenografts were established by injecting CHLA-255 or NB1691 cells into the retroperitoneal space of SCID mice. Following the demonstration of established tumors by ultrasound, treatment was started with bevacizumab (5mg/kg 2qwk), sunitinib (40 mg/kg qd), topotecan (0.5mg/kg qd), or a combination of either sunitinib/topotecan or bevacizumab/topotecan for a total of two weeks.
Results: Montherapy with each agent (bevacizumab, sunitinib or topotecan) was largely ineffective, with final tumor volumes being similar to untreated controls. However relative volumes of CHLA-255 xenografts were significantly less in cohorts treated with sunitinib/topotecan (4.98+/- 1.34 vs. 54.76 +/-17.45 [sunitinib alone], p= 0.0291) and bevacizumab/topotecan (14.88 +/-4.33 vs. 41.69+/-10.4 [bevacizumab alone], p=0.0548). Results were similar when performed with NB1691 xenografts. Expression of the HIF-dependent factors, VEGF and GLUT3, as determined by qPCR, increased 2.4-fold and 2.0-fold, respectively, in tumors treated with bevacizumab-alone. However this increase was abrogated in tumors treated with a combination of bevacizumab/topotecan. Finally, plasma levels of VEGF in mice treated with a topotecan containing regimen were decreased relative to those treated with one of the antiangiogenic agents alone.
Conclusion: Resistance to anti-VEGF therapies can subvert their effectiveness against solid tumors. We have shown that eliminating one of these mechanisms of resistance, HIF upregulation, can potentiate the effects of antiangiogenic treatment in human neuroblastoma xenografts.

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POT37

Screening at 18 months of age using the new serum marker for reducing the mortality of neuroblastoma: Simulation using Japanese population based cohort study

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Background: Infantile screening for neuroblastoma (NBL) reduced the incidence rate (IR) of advanced disease or mortality rate (MR) due to NBL but the significant increase of IR. Then, we proposed the NBL screening at 18-months of age.
Methods: Japanese nationwide mass screening was conducted using a quantitative HPLC in 1990-2003. Cumulative IRs and MRs of NBLs diagnosed before 6 years of age were compared between children born in 1990-98 (n = 10,878,918, participation rate: 85.9%) to historical control born in 1980-1984 (n = 7,620,203) as well as between screened and unscreened children. Then, we simulated the IRs and MRs of NBLs under NBL screening at 18-months of age with the same participation rate. Moreover, the new candidate marker for unfavorable NBLs was surveyed using serum and urine samples of 45 NBL patients by LC-MS-MS using ESI TOF-MS. The candidate markers for detecting unfavorable NBLs were extracted using Mass Mapping (MM) software.
Results: The IRs of the screening cohort became significantly higher than that in the historical control (11.91 vs 30.30; P < .0001), while the IRs in unscreened subgroup of the screening cohort was similar to that in historical control. The MRs of these two cohorts were 2.82 and 5.14 (P<0.001). Simulation analysis under NBL screening at 18-months of age showed the significantly decrease of MR (under 40%) without increase of IR. After surveyed the urine substances and the serum metabolites in NBL patients using LC-MS, MM revealed the specific candidate serum marker for unfavorable NBLs (peptide, MW 1500). This marker increased only in pretreatment sera of 13 unfavorable NBLs patients. Serum levels of this marker decreased after effective chemotherapy and resection of tumor but increased at the time of tumor progression or recurrence.
Conclusion: There is a possibility that HPLC quantitative screening for NBL at 18 month of age result in significant decrease in MR by NBL of children without the increase of IR. The new marker for detecting unfavorable NBLs specifically might attribute to more effective NBL screening as well as to evaluation of therapeutic effects in unfavorable NBL.

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POT38

Effect Phosphoinositide-3-Kinase (PI3K) and mTOR dual inhibitors in Human Neuroblastomas (NBs)

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Background: The PI3K-AKT-mTOR pathway is constitutively activated by different receptor tyrosine kinases (RTKs), such as TrkB, TrkAIII, IGF-IR, EGFR and ALK in NB cells. It plays an important role in proliferation and tumorigenesis. Blocking RTK signaling by inhibiting the PI3K-AKT-mTOR pathway with specific inhibitors impairs the growth of NB cells. We studied the effect of direct targeting key signaling proteins with inhibitors of either PI3K (GDC-0941), mTOR (rapamycin-Rap) or both with dual inhibitors, such as PI-103, NVP-BEZ235 (BEZ235) in NBs. We also investigated the effect of inhibiting both PI3K-AKT and Trk receptors with the Trk inhibitor lestaurtinib.
Methods: We used a panel of NB cells, including TrkA and TrkB transfectants of SY5Y and NLF. We assessed changes in cell viability by inhibitors with MTT and RT-CES assays. We determined the expression, activation and inhibition of Trk, AKT and S6 by immunoblotting with specific antibodies.
Results: The IC50 of GDC-0941 was 2μM, and the IC50 of Rap was over 10μM. In contrast, the IC50 of PI-103 averaged 0.5μM (range 0.5 to 2μM) and BEZ230 averaged 1μM (range 0.2 to 1μM). Furthermore, BEZ235 was the most potent at inhibiting growth of wild type NB cells in vitro, compared to other inhibitors tested. The effect of the dual inhibitors did not correlate with expression of Trk receptors. Lestaurtinib and PI-103 showed no additive effect on SKNAS (endogenous TrkA) or on SY5Y-TrkB G8 (TrkB low) cells. PI-103 (1μM) abolished AKT but not Trk phosphorylation. Lestaurtinib (200 nM) completely inhibited TrkB but only partially inhibited AKT phosphorylation in G8 cells. No additive effect on phosphorylation was observed in combination of lestaurtinib and PI-103.
Conclusion: Dual inhibitors of PI3K and mTOR effectively inhibited the growth of NB cells in vitro regardless of Trk expression. BEZ235 was the most effective among the signaling inhibitors. PI-103 and lestaurtinib showed no additive effect on Trk-expressing NB cells. In summary, targeting PI3K-AKT-mTOR pathway alone is an effective way to potently inhibit NB cell growth.

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POT39

Analysis of molecular interactions between the GD2 ganglioside-specific mouse monoclonal antibody 14G2a and GD2-mimicking peptides

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Background: Children with high risk neuroblastoma (NB) are at the risk for poor outcome. This stresses the need for new treatment protocols to control minimal residual disease. Over-expression of GD2 ganglioside (GD2) distinguishes NB from benign neural tumors and most normal cells. Anti-GD2 antibodies are used in NB diagnosis and tested in passive immunotherapy approaches. GD2 can also be targeted by active immunization strategies. Our goal is to design GD2-targeting active immunotherapy of NB by replacing the weakly immunogenic GD2 with its peptide mimotopes isolated with application of a phage display technology and the mouse anti-GD2 monoclonal antibody 14G2a (mAb).
Method/approach: We performed in vitro competition tests and vaccination studies on mice to characterize the 14G2a-binding of the peptides, evaluate, and optimize anti-GD2 specific responses induced with our mimotopes.
Results: We showed that despite clear dissimilarities in amino acid (aa) sequences all five isolated peptides have overlapping binding sites on the 14G2a mAb. Moreover, these peptides mimic a unique GD2 epitope, as they do not cross-react with other ganglioside-specific mAb. We elucidated molecular mechanism of the observed mimicry for one of our best binding peptides (#94 RCNPNMEPPRCF). With consecutive aa replacements by A, we identified aa indispensable for the observed GD2 mimicry by the #94 peptide. Additionally, we compared several analogs of the #94 peptide with further replacements, truncations, or elongations, and found new longer peptides, which showed significant improvement of the 14G2a binding in the competition assays. Finally, we showed that vaccines containing our peptides conjugated to KLH induce GD2-targeting antibodies in mice, and we analyzed the level and specificity of the peptide-induced humoral responses.
Conclusion: The accumulated data allowed us to gain insight into the molecular mechanism of the observed GD2 mimicry, and can lead to improvement of anti-tumor activity of our peptides. Acknowledgments: This work was financed in years 2006 - 2009 from the research grant No. N302 034 31/3063 (awarded to Irena Horwacik).

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POT40

NLRR2 is a novel regulator of neuroblastoma cell death via ER stress

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Background: To understand the pathogenesis of neuroblastoma (NB), we previously screened for human NB cDNA libraries we generated, and identified a novel family gene *NLRRs* (neuronal leucine-rich-repeat proteins). We have so far found that, among the family members, functions of NLRR1 and NLRR3 are associated with cell proliferation and differentiation, respectively. However, the role of other member, NLRR2, remains elusive.
Method/approach: Immunocytochemistry was performed to check the localization of NLRR2. FACS, WST-8 and colony formation assays were employed to assess apoptosis. RT-PCR and Western-blot were performed to check the expressions of *mRNA* and protein, respectively. *NLRR2* promoter activity was measured by dual luciferase assay. Tunicamycin (TM) and Thapsigargin (TG) were used to induce endoplasmic reticulum (ER) stress.
Results: There was no difference in expression levels of NLRR2 *mRNA* among the subsets of NB (n=32, p>0.05). However, immunohistochemical analysis showed that NLRR2 proteins localize in the cytoplasm of primary NB cells and mainly in the ER of NB as well as other cell lines. To know the functional role of NLRR2 in the neuronal cell fate, we tested several stress responses in cells. Interestingly, *NLRR2* overexpression led to cellular apoptosis upon ER stress (TM and TG treatments) as well as oxidative stress (H_2O_2 treatment), whereas siRNA-mediated knockdown of *NLRR2* resulted in the resistance to those stresses. Moreover, the expression of endogenous *NLRR2* was induced upon ER stress, which was confirmed by the luciferase reporter assay using the core promoter region of *NLRR2* showing that ER stress enhanced transcription of *NLRR2*. Indeed, the expression of proapoptotic genes (*CHOP*, *BAX* and *BAK*) were induced by *NLRR2* overexpression.
Conclusion: Different from the functions of *NLRR1* and *NLRR3*, *NLRR2* was found to be a stress-inducible gene to cause apoptosis in cells. Notably, activation of NLRR2 is associated with cellular ER and oxidative stress responses. Thus, NLRR2 may be involved in the regulation of cellular survival and death in NB and might give clue to make a new therapeutic strategy against high-risk tumors.

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POT41

Positive feedback loop of Mycn-nlrr1-egf/egfr signals in aggressive neuroblastomas to accelerate cell growth

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Background: Neuroblastoma (NB) is one of the most common solid tumors in children. To understand the pathogenesis of NB, we screened for human NB cDNA libraries we generated, and identified a novel human gene, *NLRR1* (neuronal leucine rich repeat protein 1). We have previously reported that *NLRR1* is highly expressed in aggressive NB and significantly associated with the poor clinical outcome. However, the precise role of NLRR1 function in the progression of aggressive tumors remains unclear.
Method/approach: WST-8 assay was employed to monitor cell proliferation upon EGF and IGF treatment. RT-PCR and western-blot were performed to check mRNA and protein expression, respectively. *MYCN* promoter activity was measured by dual luciferase assay. Chromatin Immunoprecipitation (ChIP) assay was used to analyze recruitment of transcriptional factors onto the promoter region.
Results: The treatment with EGF and IGF enhanced cell growth in many NB cell lines. Enforced expression of NLRR1 enhanced EGF- and IGF-mediated proliferation of NB cells, whereas *NLRR1* knockdown inhibited it. Activation of ERK and AKT was elevated in *NLRR1*-overexpressing cells, which was decreased by ERK or AKT inhibitor, and the further inhibition was observed in cells with down-regulation of *NLRR1*. Of interest, EGF- or IGF-enhanced cell proliferation was accompanied with induction of MYCN expression, which was further accelerated after overexpression of NLRR1 in cells. The analysis of *MYCN* gene promoter region revealed that Sp1 transcription factor may be one of the main regulators for activating *MYCN* transcription. Furthermore, Sp1 was found to be phosphorylated by ERK and recruited onto the *MYCN* promoter region upon EGF treatment. Of further interest, MYCN directly targeted *NLRR1* gene for its transcriptional activation
Conclusion: Our results suggest that NLRR1 enhances cell proliferation by activating EGF and IGF signals to induce *MYCN* expression in the aggressive NB cells. Since *NLRR1* is a downstream target gene of MYCN, NLRR1 may form a positive regulatory loop with MYCN in NBs. These findings might help to develop novel chemotherapeutic tools to cure aggressive NBs.

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POT42

Application of chicken anti-human midkine IgY antibody to neuroblastoma diagnosis

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Background: We reported at previous meetings of ANR that plasma midkine (MK) level is a prognostic factor for human neuroblastoma. For the detection of MK, we used to employ a sandwich ELISA system using goat anti-human MK (hMK) antibody and chicken anti-hMK IgG antibody. As it is time-consuming to prepare chicken IgG antibody from blood and the productivity is limited, we attempted to prepare IgY from egg yolk in the present study.
Method/approach: We immunized chicken with hMK. We prepared chicken IgG antibody from chicken blood and chicken IgY antibody from chiken egg yolk. Goat anti-hMK antibody was adsorbed onto the wells of microtiter plates for 16 h at room temperature. After washing, the wells were blocked for 2h at 37°C. On the other hand, control hMK samples or supernatant from neuroblastoma cell lines were mixed with buffer containing peroxidase-labelled chicken anti-hMK-IgG antibody or chicken anti-hMK-IgY antibody. This mixture was then added into the wells of microtiter plates prepared as described above. After incubation for 1h at room temperature, the wells were washed five times. A substrate solution was added into the wells and the plates were incubated for 30 min at room temperature. The reaction was stopped and OD450 was detected using a multiplate reader. We also developed an easy-to-use equipment for measuring. This system can use both anti-hMK-IgG and anti-hMK-IgY.
Results: We could make a linear standard curve in both ELISA, i.e., anti-hMK-IgG ELISA and anti-hMK-IgY ELISA. Both ELISA could measure MK levels in several supernatant media from several neuroblastoma cell lines; IMR-32 andNB39. The determined MK levels were 13.6 ng/ml in IMR-32 media, and 14.1 ng/ml in NB39 media (cell number 3x10^5). Furthermore, both ELISA did not detect MK in culture media and a healthy plasma.
Conclusion We have developed a new ELISA employing the chicken anti-hMK IgY antibody. The performance of this ELISA was comparable to that of our previous ELISA using the chicken anti-hMK IgG antibody. From time and economic points of view, IgY is superior to IgG. Thus, the newly developed ELISA may be useful for neuroblastoma diagnosis.

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POT43

Expression and gene status of HER2 in neuroblastic tumors

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Background: HER2 is essential for normal embryonic development of autonomic and peripheral nervous system and plays a critical role in oncogenesis and progression of some types of cancer. The biological and prognostic role of HER2 in neuroblastic tumors is not well established.
Method/approach: In the current study we evaluated HER2 expression, its prognostic significance and clinicopathological correlations in series of 79 neuroblastoma. The immunohistochemical assessment of HER2 as well as FISH for HER2 copy number status were performed on tissue microarrays.
Results: In the examined group 20% of patients died of disease from 4 to 107 months (median 18) from the diagnosis and the survivors were followed up for 14 to 149 months (median 59). Sixteen cases were HER2-immunonegative. HER2 expression characterized 63 tumors (34 low and 29 high level) showing either membranous or mixed membranous-cytoplasmic pattern within neuroblastic component. Schwannian stroma disclosed low level HER2 expression. The pattern of immunolabeling depended on the maturity of neuroblastic cells. None of tumors revealed HER2 amplification. Patients' age, stage of disease, tumor location, MKI and presence of HER2 expression were statistically significantly related to survival probability as detected by the Cox proportional hazard model. In the univariate analysis Kaplan-Meier curves revealed significantly poorer outcome of HER2- negative than HER2- positive tumors (either low or high expression). The immunonegativity was associated with adverse clinicopathological parameters, including poor survival, metastatic stage of disease, un- or poorly differentiated histology and high MKI.
Conclusion: HER2 expression, not accompanied by gene amplification, is common in neuroblastic tumors. HER2- positivity seems to have a positive prognostic significance. HER2 expression with a variable pattern is a marker of the stage of neuroblastic cells differentiation and is connected to Schwannian stroma development.

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POT44

Ki-67 proliferation index is marker of poor prognosis in neuroblastoma especially in patients aged over 18 months

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Background: Proliferation index measured immunohistochemically with Ki-67 (PI Ki67) is the accepted prognostic factor in some types of cancer. Its significance in neuroblastoma (NB) is not well established. The aim of our study was to assess the prognostic impact of PI Ki67 and its patho-clinical relations in a series of NB tumors.
Methods: 103 patients followed-up from 4 to 149 (median 46) months [m] were enrolled in the study. 34 patients died of disease. Analyzed data included: patients' age, tumor localization, stage, overall survival, tumor histology, MKI, *MYCN* and ploidy status. Ki-67 immunostaining was performed on representative tissue slides and counted as percent of positive nuclei for 100-1000 neoplastic cells.
Results: Patients' age ranged 1-169 m, including 63 children older than 18 months (>18m). There were 74 neuroblastoma, 20 ganglioneuroblastoma and 9 ganglioneuroma cases. High MKI characterized 28 cases. PI Ki67 ranged from 0 to 72% (median 18%). PI was lower in >18m children (median 10%; p=0.0002).
Higher PI was correlated with higher MKI and adrenal location, and inversely with increasing tumor differentiation. In addition, it was correlated with fatal outcome of the disease (p=0.025). 5 years overall survival for patients with PI Ki67≥30% was 24% vs 59% in case of PI<30% (p=0.004), with no significance in multivariate Cox regression analysis.
In the group >18m significant correlation between higher PI and metastatic stage (p=0.0002), unfavorable histological category (p=0<0.0001), high MKI (p<0.0001), *MYCN* amplification (p=0.0004) and adrenal primary tumor localization (p=0.022) was found. Here, 5 years overall survival for PI Ki67≥10% was 36% vs 66% in patients with PI<10% (p=0.003). Cox regression analysis showed PI Ki67≥10% as the independent prognostic factor (HR=3.1; 95%CI 1.3-7.4; p=0.011).
Conclusion: Ki-67 proliferation index has a prognostic significance in NB tumors based on patients' age. Two cut-off values were identified as markers of poor prognosis: 10% for >18m children and 30% for the entire group. We propose to include the assessment of PI Ki67 to the standard histological assessment of NB tumors.

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POT45

MLPA (Multiplex Ligation-dependent Probe Amplification) and FISH comparison/validation for genetic characterization of neuroblastoma

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Background: The increasing knowledge on genetic alterations associated with neuroblastoma tumors brings also an increasing need for a method that identifies these alterations in a diagnostic multi-genomic approach. The Neuroblastoma specific salsa-MLPA (Multiplex Ligation-dependent Probe Amplification) probe is able to identify gains or losses of different genomic loci covering the ten chromosomal regions of highest interest within a given sample. The aim of this work was to compare/validate the MLPA results with a panel of already well established FISH assays.
Methods: A total of 62 tumor samples from patients diagnosed and treated in the SCMCI were analyzed by MLPA. 47 of them were evaluated in parallel with interphase FISH (MYCN, 1p, 17q, 11q) and DNA index by Flow Cytometry.
Results: Segmental alterations were associated with higher stage neuroblastoma, whereas in the low stages gain and/or loss of whole chromosomes could be detected. In 25/47 cases, the structural aberrations were detected by both methods. In 16/47 tumors, no structural aberrations were detected by any of the methods. In 3 cases aberrations were detected only by MLPA, and in another 3 cases aberrations were seen only by FISH. These discordances could be explained by sample error and low percentage of tumor cells, respectively. McNemar pairs comparison test was applied to statistically determine the significance of differences in the detection of segmental alterations by both methods. The two-tailed P value equals 0.683 which is considered to be not statistically significant, by conventional criteria.
Conclusion: MLPA enabled wider information on gain and losses than the classical cytogenetic and FISH methods. The use of this new routine technique with such customized probes sets would be a simple and easy molecular adjunctive tool in the classification of neuroblastoma tumors.

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POT46

Prolonged low-dose administration of the cyclooxygenase-2 inhibitor celecoxib enhances the antitumor activity of irinotecan against neuroblastoma xenografts

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Background: Irinotecan (CPT-11) is highly effective against neuroblastoma (NB) xenografts. However, treatment of CPT-11 alone cannot completely abolish tumor. Cyclooxygenase (COX)-2 which promotes tumor progression is overexpressed in NB. We evaluated the anti-tumor effect of CPT-11 combined with prolonged low-dose celecoxib, a selective COX-2 inhibitor, against 3 human NB xenografts.
Methods: NB xenografts used were drug-sensitive lines, TNB9, TS-N-5nu, and a multi-drug resistant line, TS-N-2nu. Five different treatment schedules were evaluated: celecoxib alone at 5 mg/kg daily for 20 days, low-dose (5.9mg/kg) CPT-11 ± celecoxib daily for 20 days , and conventional-dose (59mg/kg) CPT-11 in 3 doses at 4-day intervals ± celecoxib for 12 consecutive days. Tumor growth inhibition was evaluated for mean tumor doubling time. Cell proliferation, angiogenesis, apoptosis and protein expression in tumor tissues were analyzed.
Results: CPT-11 alone on a low-dose prolonged schedule was equally or more effective than a conventional-dose intermittent one. Celecoxib administered daily at 5 mg/kg could not prevent the growth of any NB xenografts. However, the combination of daily low-dose CPT-11 and simultaneous low-dose celecoxib resulted in highly significant suppression of tumor growth in all xenografts ($p<0.001$) compared not only with low-dose CPT-11 alone but also with the combination of intermittent conventional-dose CPT-11 and celecoxib, accompanied by decreased proliferation and increased induction of apoptosis in tumor cells. Induction of apoptosis was associated with the up-regulation of Bax and the down-regulation of Bcl-2. The enhanced anti-tumor effect of the combination of the two drugs against the NB xenografts might be partially COX-2 independent and was probably mediated through multiple factors including diminished expression of VEGF and activation of the caspase-dependent mitochondrial apoptosis pathway.
Conclusion: Prolonged low-dose CPT-11 treatment combined with low-dose celecoxib showed promising anti-tumor activity through the blockage of multiple critical targets related to NB tumor cell survival and proliferation.

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POT47

CHD5 is part of the nucleosome remodeling and histone deacetylase (NuRD) complex in neuroblastoma (NB) cell lines

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Background: Eukaryotic gene expression is developmentally regulated by chromatin remodeling, and its dysregulation has been linked to cancer. CHD5 is a tumor suppressor gene that maps to a region of consistent deletion on 1p36.31 in NBs. It is preferentially expressed in neural tissues, whereas expression is consistently low or undetectable in NB cell lines. The CHD5 gene encodes a protein with chromatin remodeling, helicase and DNA-binding motifs. CHD5 is highly homologous to CHD3 and CHD4, which are core subunits of the NuRD chromatin-remodeling complex. We performed studies to determine if CHD5 forms a similar complex.
Method/approach: NLF cells were stably transfected with CHD5 cDNA in the sense or antisense orientation. Immunofluorescence microscopy was used to demonstrate nuclear localization of CHD5 protein. NuRD components were detected by immunoprecipitation and by using GST-Fog as an affinity reagent to purify the NuRD complex. Proteins were detected by SimplyBlue staining and by Western blot. LC-MS was used to confirm the presence of CHD5 protein in the complex.
Results: We examined nuclear extracts from CHD5-transfected NLF cells to determine if CHD5 forms a NuRD complex similar to CHD4. V5/His-tagged CHD5 was immunoprecipitated from nuclear extracts with a V5 or CHD5 antibody, or pulled down with GST-Fog after CHD4 depletion. CHD5 associated with all NuRD subunits, including MTA1/2, P66, HDAC1/2, RbAp46/48 and MBD3) as determined by Western blotting and LC/MS
Conclusion: Our data suggest that CHD5 encodes an ATP-dependent chromatin-remodeling protein that forms a NuRD complex similar to CHD4. The CHD5-NuRD complex presumably plays an important role in chromatin remodeling and tumor suppression. This may occur by transcriptional repression (or activation) of genes important in regulating neuroblast growth or differentiation.

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POT48

Mechanisms of CHD5 inactivation in neuroblastomas

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Background: Neuroblastomas are known to have genetic, biological and clinical heterogeneity. High-risk neuroblastomas are characterized by several genetic changes, including 1p36.31 deletion, and we have recently identified the chromatin-remodeling gene CHD5 as a tumor suppressor gene that maps to this region. Low or absent CHD5 expression is associated with a 1p36 deletion and an unfavorable outcome, but the mechanisms of CHD5 inactivation in these tumors is currently unknown.
Patients and Methods: The sequence of CHD5 was determined in 188 high-risk neuroblastomas investigated through the TARGET initiative. If sequence variations were found, the patient's constitutional DNA was also sequenced to determine the variation was somatically acquired. In addition, we analyzed the expression of CHD5 in 610 representative neuroblastomas using TaqMan low-density array microfluidic cards (ABI). We also assessed the methylation status of the CHD5 promoter in 125 neuroblastomas with 1p36 deletion, MYCN amplification, both or neither.
Results: We found no examples of somatically acquired mutations or deletions of CHD5 in the 188 cases examined, indicating that homozygous genomic inactivation is extremely rare. High CHD5 expression was correlated with favorable biological features, such as lower stage, normal MYCN copy number and favorable risk group. High CHD5 expression was also a powerful predictor of favorable outcome. Methylation of the CHD5 promoter was found commonly in the high-risk tumors, but methylation was more strongly correlated with MYCN amplification than with 1p36 deletion. Promoter methylation correlated with low CHD5 expression.
Conclusion: We conclude that somatically acquired mutation of CHD5 is rare. CHD5 expression is strongly correlated with favorable clinical and biological features as well as outcome. Although promoter methylation is associated with low CHD5 expression, it is strongly correlated with MYCN amplification, suggesting a possible interaction between these two genes.

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POT49

Neuroblastoma cell lines, phenotype and susceptibility towards natural killer cells

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Background: Currently the prognosis for relapsed/ refractory high-risk (HR) neuroblastoma (NBL) patients is poor, warranting new treatment strategies such as immunotherapy. NBL tumour cells show low or absent expression of HLA class I making them resistant to specific, HLA restricted cytolytic T cells. Natural killer (NK) cells are bone marrow derived lymphocytes, that are cytotoxic against tumour cells and virus infected cells. The interaction of NK cells with target cells is controlled by a balance of inhibiting (KIR) and activating (e.g. DNAM-1, NKG2D) receptors on the NK cells.
Aim: Preclinical study to investigate whether NBL tumours are sensitive targets for NK cells and whether the NK cell cytolytic potential towards NBL tumors can be enhanced.
Methods: NBL cell lines have been tested for in vitro sensitivity of killing by NK cells using cytotoxicity assays. Cell lines have been phenotyped using flow cytometry (FACS) analysis and molecularly typed for HLA to document presence of possible inhibitory KIR ligands. Blocking experiments using monoclonal antibodies (MoAbs) were performed to assess the pathways involved in killing. Results: in vitro experiments using purified NK cells have indicated that NBL cell lines are sensitive to killing mediated by allogeneic cytokine (IL-15)-activated NK cells, but are barely lysed by resting NK cells. NBL cell lines variably express HLA class I and express some NKG2D ligands, all express DNAM-1 ligands. Preliminary experiments suggest that interaction of activated NK cells and NBL cells is partially blocked in vitro by MoAb directed against DNAM-1. Blocking of HLA class I did not result in enhanced killing of NBL cell lines by resting NK cells.
Conclusion: NBL cells express DNAM-1 ligands, making them a possible target for immunotherapy by infusion of activated NK cells. These findings will need to be extended using primary tumor material, in order to further support the potential contribution of infusion of allogeneic IL-15 activated NK cells for the elimination of NBL cells in vivo.

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POT50

UCHL1-Upregulation correlates with reduction of vital neuroblastoma cells by fenretinide and doxorubicin treatment

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Background: Neuroblastoma (NB) disseminating need an intensive cytotoxic chemotherapy. Studies prove the effectiveness of retinoic acid derivates (RAs) as differentiating agents on NB cells. We studied the effect of a single or combined treatment of the synthetic retinoid fenretinide (F) with the cytotoxic drug doxorubicin (D) on the vitality of the human NB cell line SH-SY5Y.
Method/approach: SH-SY5Y cells were incubated for 6 days with 50% inhibitory concentrations of F and D. Treatment groups: control (CO), F, D, F+D. Subsequently, measurements of vitality with MultiTox-Fluor Multiplex Cytotoxicity assays were performed. Differentially regulated proteins were identified by 2D-difference gel electrophoresis (2D-DIGE) and mass spectrometry (MS). Transcriptomic regulation of these proteins and the expression profiles of cell cycle regulators, apoptosis and differentiation markers were analyzed by real time PCR (RT-PCR).
Results: Single (F or D) or the combined treatment (F+D) of SH-SY5Y cells resulted in the following vitality rates: F 60±2.2%; D 69.9±2.7% and F+D 42.4±2.4% (p<0.01, n=7). 23 differentially regulated proteins (p<0.05 and average ratio <-2 or >2) were identified by MS. Among these UCHL1, KHSRP, THARAP3, ENO2, TPM2, MAPRE1, GRB2 and PATL1 were of particular interest. RT-PCR results for UCHL1, KHSRP, THARAP3, ENO2 and TPM2 confirm an up-regulation for UCHL1 by all treatment groups and a down-regulation for TPM2 by D. F+D resulted in mRNA up-regulation of Cyclin D1, p21, caspase 3 and the neuronal markers SNAP-25 and neurofilament. The markers of Schwann-cell differentiation GFAP and S100B were not influenced.
Conclusion: RAs are commonly considered as differentiating agents which inhibit the activity of cytostatic drugs. In contrast, the new synthetic retinoid F combined with D decreases the vitality of SH-SY5Y cells and induces neuronal differentiation. Both substances alone and in combination alter the regulation of different genes on protein and/or mRNA level. Up-regulation of UCHL1 was identified as a selective target of the combination therapy (F+D).

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POT51

Low dose metronomic (LDM) administration of oral topotecan and pazopanib as an effective preclinical antiangiogenic therapy in neuroblastomas

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Background: Angiogenesis plays a critical role in neuroblastoma (NB) growth and metastasis. Low dose metronomic (LDM) chemotherapy, combine with VEGF pathway inhibitors is an emerging treatment strategy
Objectives: To establish the efficacy, PK/PD of LDM topotecan (TP) with/ without pazopanib (PZ) an oral antiangiogenic tyrosine kinase inhibitor (TKI) in NB mouse model.
Methods: SK-N-BE(2) and SH-SY5Y cell lines were used to establish IC50 and NOD-SCID mice modelfor both subcutaneous primary tumour and metastatic experiments. Mice were randomized into 5 groups: control group, LDM TP (1.0mg/Kg), PZ(30mg/kg and 150mg/Kg) and the combination (1.0mg/Kg TP + 150mg/Kg PZ). Localized tumor model, animals were treated daily till 56 days; metastatic model, were treated until death. Angiogenic makrers, circulating Endothelial cells (CECs) and circulating Endothelial Progenitor cells (CEPs) were determined by flow cytometry. PK studies were conducted to determine the plasma concentration-time profiles of both the drugs.
Results: IC 50 of topotecan on cells was 125.9 ng/ml(SK-N-BE(2) and 4.0ng/ml (SH-SY5Y). Pazopanib did not induce cytotoxicity up to 10µg/ml. .In xenograft model, statistically significant efficacy was observed for single agent (TP or PZ) and combination with repsetive p values. combination (PZ 150mg/kg)(p=0.0002) > LDM TP (p=0.0008)> than PZ >than control In the SK-N-BE(2) metastatic model, all the three treatment regimens significantly prolonged animal overall survival compared to control group. PZ (p= 0.0005) >, TP(p=0.0006) > PZ+TP (p=0.006) > respectively. No toxicity was observed in any of the groups. The Cmax of PZ was 130.5µg/ml(PZ) and 125.6µg/ml, (PZ+TP). PZ plasma concentration was maintained above the optimal concentration for up to 18 h.. The combination of LDM TP and PZ reduced the levels of viable CEP (p= 0.125) and CEC (p = 0.005) after 7 days treatment.
Conclusion: Daily oral LDM TP and PZ and combination are effective and safe regimens in both localized and metastatic neuroblastoma mouse models. The reduction in CEC/CEP levels supports the anti-angiogenic activity of these drugs regimen

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POT52

Clinical significance of TRK family gene expression in neuroblastomas

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Background: NBs are characterized by clinical heterogeneity, from spontaneous regression or differentiation to relentless progression. Evidence from our laboratory and others suggests that the pattern of TRK family gene expression contributes to these disparate behaviors. TrkA is expressed in favorable NBs, whereas TrkB and its ligand BDNF are coexpressed in unfavorable NBs, representing an autocrine survival pathway. We determined the expression pattern and clinical significance of TRK family genes in a large set of primary NBs.
Patients and Methods: We analyzed the expression the following genes in 610 representative NBs using quantitative real-time RT-PCR with TaqMan low-density array cards: TrkA/NTRK1, TrkB/NTRK2, TrkC/NTRK3, NGF, BDNF, and P75/NGFR. Expression (high vs. low) for each was compared to clinical and biological variables as well as outcome. Patients were categorized into one of two groups, dichotomized by the median expression value of each gene. A Kruskal-Wallis test was used to test for association of expression of each gene with each of the dichotomized risk factors. Life table analysis and log rank tests were performed to compare the event-free survival (EFS) or overall survival (OS) of the two groups for each gene.
Results: High TrkA expression was strongly correlated with favorable age, stage, MYCN, histology and risk group (p<0.0001 for all), and weakly with favorable ploidy (p<0.004). NGF expression was correlated with favorable risk group, but no other Trk family gene correlated significantly with clinical/biological variables. TrkA expression was not significantly correlated with outcome, but low NGF was correlated with favorable EFS and OS.
Conclusion: We conclude that the high expression of TrkA was very strongly correlated with all clinical and biological risk factors except ploidy, but surprisingly did not correlate with EFS or OS in this analysis. Also, high TrkB and/or BDNF expression have correlated with unfavorable outcome in the past but did not in this study. Nevertheless, assessment of TrkA and TrkB expression will identify tumors that are likely to respond to Trk inhibitors alone or in combination with conventional agents.

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POT54

Induction of miR-183 via an epigenetic mechanism suppresses neuroblastoma malignancy

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Background and Aims: Therapy of high-risk neuroblastoma has remained difficult. Despite high-dose chemotherapy and peripheral stem cell transplantation, resistant relapses frequently occur. Histone deacetylase inhibitors (HDACi) cause differentiation of neuroblastoma cells in preclinical models. However, little is known about the underlying molecular events. Here, we investigated the role of microRNAs, known to play a key role in promoting neural development, in mediating a differentiated phenotype by HDACi.
Methods and Results: Combining the miChip and real-time quantitative PCR methodologies, we identified miR-183 as strongly induced microRNA by both the cyclic tetrapeptide *Helminthosporium carbonum* (HC)-toxin and the carboxylate valproic acid (VPA). siRNA-mediated silencing of the 11 HDACs belonging to classes I, II and IV showed that knockdown of *HDAC2* induced miR-183 expression, whereas the plasmid-mediated enforced expression of *HDAC2* repressed miR-183. Transient transfection of miR-183 into *MYCN* amplified and single copy neuroblastoma cell lines inhibited both metabolic activity and cell cycle progression and induced cell death. In *MYCN* single copy tumors, high miR-183 expression was found to be significantly associated with event-free survival of children.
Conclusions: Induction of miR-183 via inhibition of HDAC2 suppresses neuroblastoma malignancy. The data highlight both HDAC2 as relevant family member for an HDACi-mediated intervention and the administration of miR-183 as potential therapeutic agent against high-risk neuroblastoma.

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POT55

Telomere elongation and chromosomal instability in non-MYCN amplified clinically aggressive neuroblastoma

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Purpose: To assess the relationship between telomere length alterations in neuroblastoma (NB) and clinico-biological parameters.
Background: NBs have a heterogeneous pattern of somatic chromosome changes, the underlying mechanisms of which have remained elusive. Telomere shortening and dysfunction have been shown to cause genomic alterations in several other tumour types, but the role of telomeres in NB pathogenesis and progression has been little studied.
Experimental Design: Telomere-dependent chromosomal instability was first assayed in six NB cell lines and relative telomere length was then determined by quantitative fluorescence in situ hybridisation (Q-FISH) in 50 NB biopsies. The results were correlated with genomic alterations, risk groups and survival.
Results: Telomere-negative chromosome ends, dicentrics, and anaphase bridging were found in all investigated cell lines and telomere dysfunction could be directly connected to inter-cellular heterogeneity in MYCN amplicon structures through involvement in breakage-fusion-bridge cycles. Anaphase bridging was also present in tissue sections from >80% of investigated primary tumours and Q-FISH showed significant alterations in overall telomere length in the majority of primary tumours. Reduced median tumour telomere length (TTL) in vivo was significantly associated with the presence of MYCN amplification. In non-MYCN amplified tumours, a reduced TTL was associated with an excellent probability of overall survival compared to cases with increased TTL (P=0.012).
Conclusion: Most NBs exhibit disturbances of telomere length regulation and chromosomal instability through breakage-fusion-bridge events. Non-MYCN amplified tumours that fail to maintain a high overall telomere length typically have a favourable clinical outcome.

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POT56

Nanoparticle (NP) delivery of the Trk inhibitor lestaurtinib in neuroblastomas

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Background: NB is a childhood tumor that is characterized by heterogeneous clinical behavior, and the TRK neurotrophin receptors probably play a role. We have used the Trk-selective inhibitor lestaurtinib to treat NBs in xenograft models and in a phase I clinical trial. Although lestaurtinib was well tolerated and demonstrated efficacy at higher doses, some toxicities were seen. Here we explore the use of NPs for targeted delivery of lestaurtinib to improve efficacy and decrease toxicity.
Method/approach: We used the Trk-null SY5Y line transfected with TrkB for our studies. NPs were synthesized using polylactide-PEG with the far-red fluorophore BODIPY (650/665X) incorporated for optical imaging. We used the RT-CES system (AceaBio) to monitor cell proliferation. Lestaurtinib (Cephalon, Inc) was either given as free or NP-encapsulated drug. NP-lestaurtinib was delivered IV (20 mg/kg) once every 1-2 weeks, whereas free drug was given subQ (20 mg/kg) twice a day, five days a week. We also performed optical imaging studies to assess the systemic distribution of NP-lestaurtinib given IV.
Results: Initially we tested 0.05 to 10 μM of either free or NP-lestaurtinib on TrkB-expressing NB cells *in vitro.* At <0.2 μM, the two forms had an equivalent effect. However, ≥0.3 μM, NP-lestaurtinib was significantly better at inhibiting proliferation. We saw almost complete inhibition at 1 μM with NP-lestaurtinib compared to continued proliferation with the same concentration of free drug. Western analysis of Trk phosphorylation showed almost complete inhibition of phosphorylation with 0.1 μM NP-lestaurtinib, whereas it took 0.5-1.0 μM of free drug to achieve similar inhibition. Greater imaging intensity of NP-lestaurtinib correlated with better tumor response.
Conclusion: Our preliminary data show that NP-lestaurtinib was at least as effective as free drug *in vitro*, possibly due to protein binding of the free drug to protein carriers in the fetal calf serum. We also show that NP-lestaurtinib concentrates in subQ tumors, and response correlates with intensity of uptake. These findings support further investigation of NPs to deliver Trk inhibitors as well as other agents to treat NBs.

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POT57

Bio-molecular and histo-pathological characterization of Neuroblastoma in adolescent and young adults (AYA). Italian experience with 33 cases

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Background: About 3% of NB cases occurs in AYA where the disease has an indolent but fatal course. We have studied the genetic profile and the pathological characteristics of 33 AYA NBs (>10 years at diagnosis) enrolled in the Italian Neuroblastoma Registry (INBR) between 1979 and 2009.
Methods: Out of 3023 patients (pts) with NB, 151 were classified as AYA. Thirty-three tumors were eligible for molecular analyses by Multiplex Ligation Probe-dependent Amplification (MLPA) technique and 26/33 were reviewed according to International NB Pathology Classification (INPC).
Results: The median age at diagnosis was 172 months (range 120-214) 20 having disseminated NB and 13 localized disease. According to INPC, 23/26 tumors were NB and 3/26 were nodular GNB. MKI was tested in 13 tumours resulting high, intermediate and low in 3, 4 and 6 cases respectively. Segmental Chromosomal Alterations (SCA) were observed in 27 tumors (82%).MYCN oncogene was amplified in 3 cases; 2p gain (NAG, DDX1, MYCN, ALK genes) was reported in 4 cases while 2 cases showed MYCN gain. The major SCA found were: 17q gain (44%), 1p imbalance (37%), 9p loss (33%), 11q loss (30%), 7q gain (18.5%), 3p loss (11%) and 4p loss (7%). DNA ploidy was available in 11 cases (3 diploid/tetraploid, 8 aneuploid).At the moment 11 pts are in CR, 10 alive with stable/progressive disease and 12 (all stage 4) died of disease.
Conclusion: The present study represents the first multigenomic approach to identify a genomic profile of AYA NB. We confirmed the low incidence of MYCN amplification and we frequently observed 17q gain, 11q loss and 9 p loss independently of stage. In particular 9p loss was found in a higher percentage of tumours if compared to children with NB (33% vs 20%). 11 q loss was present in 7 stage 4 tumours without MYCN amplification and 5 of these pts died of disease suggesting a possible role of 11q loss in tumour progression. Finally 18.5% of AYA's tumours showed 7q gain: this region harbors genes potentially involved in drug resistance and this might explain the scarce response to chemotherapy in AYA. These are preliminary data to be further investigated in a largest multinational series of patients.

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Posters

POT58

Inhibition of PARP-1 enhances the efficacy of [131I]MIBG/Topotecan combinations *in vitro*

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Background: [131I]MIBG targeted radiotherapy induces favourable remissions in neuroblastoma patients as a single agent. However, combining [131I]MIBG with other agents may enhance its potential. Previously, we reported that inhibition of DNA repair and supra-additive toxicity to NAT-expressing cells and xenografts were achieved by [131I] MIBG and topotecan (topoisomerase I poison) in combination. Here, we assess the potential of PARP-1 inhibition to further enhance [131I]MIBG/ topotecan efficacy.
Methods: Combinations of topotecan, PJ34 (PARP-1 inhibitor) and [131I] MIBG were carried out in the laboratory in SK-N-BE(2c) (neuroblastoma), EJ138-CMV/NAT (NAT-transfected bladder cancer) and UVW-CMV/NAT (NAT-transfected glioma). Topotecan/PJ34 combinations were assessed using three schedules: topotecan given 24h before [i], after [ii] or simultaneously with [iii] PJ34. In a similar manner, PJ34/[131I]MIBG and PJ34/[131I]MIBG/topotecan combinations were also assessed.
Results: Topotecan/PJ34: In SK-N-BE(2c) cells, supra-additive toxicity was observed following schedules [ii] and [iii], but not schedule [i]. In EJ138-CMV/NAT cells, schedule [iii] was most effective, while schedules [i] and [ii] induced infra-additivity. In UVW-CMV/NAT cells, all schedules induced supra-additive effects. PJ34/[131I]MIBG: In SK-N-BE(2c) cells, supra-additivity was observed following schedule [iii] treatment, but not schedules [i] or [ii]. In EJ138-CMV/NAT cells, supra-additivity was induced by all three schedules. In UVW-CMV/NAT cells, schedules [ii] and [iii] induced supra-additive responses. PJ34/[131I]MIBG/topotecan: Supra-additivity was observed in SK-N-BE(2c) and EJ138-CMV/NAT cells following all three schedules, but only schedule [iii] induced synergy in UVW-CMV/NAT. All three agents induced G2 arrest as single agents. However, triple combinations caused less cell cycle disruption than treatment by single agents.
Conclusions: PARP-1 inhibition enhances the toxicity of [131I]MIBG/ topotecan in vitro. This suggests that anti-PARP-1/[131I]MIBG/topotecan combination therapy has the potential to improve the outcome of patients with neuroblastoma and other NAT-expressing tumours.

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POT59

CDK inhibitors Roscovitine and CR8 trigger Mcl-1 Down-regulation and apoptotic cell death in neuroblastoma cells

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Background: Protein kinases are widely investigated as therapeutic targets in a variety of diseases. We have developed pharmacological inhibitors of cyclin-dependent kinases (CDKs), with potential applications against cancers, neurodegenerative diseases (Alzheimer, Parkinson, stroke), renal diseases (glomerulonephritis, PKD), inflammation, etc... Neuroblastoma (NB) display overexpression of anti-apoptotic Bcl-2 and Mcl-1 in aggressive forms of the disease.
Method/approach: We investigated the effects of CDK inhibitors on Mcl-1 in NB cell lines using classical biochemical and cell biology methods.
Results: The clinical phase 2 drug Roscovitine (CYC202, Seliciclib), a relatively selective inhibitor of CDKs, and CR8, a recently developed and more potent analogue, induce dose-dependent apoptotic cell death of NB cells (average IC50 values: 24.2 µM and 0.4 µM for roscovitine and CR8, respectively). Both roscovitine and CR8 trigger rapid down-regulation of the short-lived survival factor Mcl-1 in the 9 investigated human NB cell lines. This effect was further analyzed in the human SH-SY5Y NB cell line. Down-regulation of Mcl-1 appears to depend on inhibition of CDKs rather than on interaction of roscovitine and CR8 with their secondary targets. CR8 is an ATP-competitive inhibitor of CDK9 and the structure of a CDK9/cyclin T/CR8 complex was solved. Mcl-1 down-regulation occurs both at the mRNA and protein levels. This effect can be accounted for by a reduction in Mcl-1 protein synthesis, under stable Mcl-1 degradation conditions. Mcl-1 down-regulation is accompanied by a transient increase in free Noxa, a pro-apoptotic factor. Mcl-1 down-regulation occurs independently of the presence or up-regulation of p53 and of the MYCN status.
Conclusion: Taken together these results suggest that the clinical drug roscovitine and its novel analogue CR8 induce apoptotic tumor cell death by down-regulating Mcl-1, a key survival factor expressed in all NB cell lines. CDK inhibition may thus constitute a new approach to treat refractory high-risk NB.

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POT60

Preclinical testing of novel kinase inhibitors in high-risk neuroblastoma

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Background and aims: Novel treatments targeting MYCN amplified neuroblastoma tumours are highly warranted since an amplification of the MYCN oncogene constitutes the single most important predictor of bad prognosis. The purpose of this study was to, from a library containing 80 kinase inhibitors, highlight the most effective substances and their targets in MYCN amplified neuroblastoma cells.
Methods: Methods used include fluorometric microculture cytotoxicity assay (FMCA), Western blot for target inhibition and the Arrayscan to determine caspase 3 clevage, fragmentation and cell density. The morphology of the cells was studied using the Incucyte, an automated imaging system to monitor live cells in culture. Kinase inhibitors were tested for cytotoxicity in six cell lines with different MYCN status (SH-SY5Y, SK-N-BE(2), IMR-32, SK-N-DZ, T21N containing inducible MYCN) when treated with substances from the tyrosine kinase inhibitor library.
Results: Initial screening identified three tyrosine kinase inhibitors with potential interest which were evaluated further since they all had an inhibitory effect on cell survival and cell proliferation. Tyrphostin 9 was more effective on the MYCN amplified cell lines SK-N-BE(2), SK-N-DZ, IMR-32 and T21N expressing MYCN with EC50 values below 5 µM and 10 µM respectively. Tyrphostin 9 targets PDGFR and to some extent EGFR proven to be important in tumour development of neuroblastoma. Treatment with Tyrphostin 9 result in an increased apoptosis in the multidrug resistant MYCN-amplified cell line SK-N-BE(2). ZM449829 and ZM39923 targets JAK/STAT pathway, not previously studied in neuroblastoma and both compounds were highly cytotoxic to all neuroblastoma cell lines tested.
Conclusion: Evaluation of a kinase inhibitor library revealed significant activity of substances that may contribute to a more specific therapy for high-risk neuroblastoma, alone or in combination with conventional chemotherapeutic drugs. Our results potentially highlight JAK/STAT signalling as an important target for future neuroblastoma therapy.

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POT61

Balance of pro- versus anti-angiogenic splice isoforms of vascular endothelial growth factor as a regulator of neuroblastoma growth

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Background: VEGF is a key mediator of angiogenesis and is up-regulated by a variety of tumours. An endogenous family of anti-angiogenic isoforms, $VEGF_{xxx}b$, has been identified in normal, non-angiogenic tissues, and in contrast with the angiogenic $VEGF_{xxx}$ isoforms is down-regulated in epithelial tumours including colorectal and prostate carcinoma. This is the first study of $VEGF_{165}b$ in neuroblastic tumours, ranging from malignant neuroblastoma (NB) to benign ganglioneuroma (GN).
Method/approach: Twenty tumour samples and 5 NB cell lines, BE(2) C, IMR-32, SHIN, SY5Y and SHEP, with different tumorigenic potential and *MYCN* amplification status were assessed for $VEGF_{xxx}$ and $VEGF_{xxx}b$ expression by RT-PCR and ELISA. To determine if $VEGF_{165}b$ inhibits NB *in vivo*, 13 nude mice were injected subcutaneously (s.c.) with 10^7 BE(2)-C NB cells. Bi-weekly s.c. injections of 100µg of $VEGF_{165}b$ or saline were initiated in each group when tumours reached a diameter of 4-5 mm.
Results: $VEGF_{xxx}$ but not $VEGF_{xxx}b$ was up-regulated in NB compared to GN at mRNA level. At the protein level NB showed a significantly lower ratio $VEGF_{xxx}b$/total VEGF (0.5±0.1) than GN (1.0±0.2). All NB cell lines expressed VEGF and $VEGF_{xxx}b$. *MYCN* amplified cell lines showed the lowest ratio $VEGF_{xxx}b$/total VEGF, and the non-tumorigenic cell line SHEP showed the highest levels of $VEGF_{xxx}b$. All cell lines secreted VEGF (1-20 fg/cell). $VEGF_{xxx}b$ secretion was 1-2 orders of magnitude lower (0.2-0.6 fg/cell). Only SHIN and SHEP secreted more than 5% of its VEGF as $VEGF_{xxx}b$ (18±8% and 10±6). $VEGF_{165}b$ significantly reduced NB tumour growth rate compared to saline.
Conclusion: This study suggests that VEGF alternative splicing in NB may contribute to its malignant characteristics and show the potential of $VEGF_{165}b$ as a therapeutic agent in NB.

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POT62

Predictive consequences of risk stratification neuroblastoma patients using FISH on TMAP

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Background: Risk classification and treatment stratification of neuroblastoma patients is mandatory because the clinical heterogeneity behaviour. Understanding of neuroblastoma genetics will improve with genome-wide techniques, which are recommend in samples with <60% neuroblastic cell content. Our aim was to evaluation the use of FISH on tissue microarrays (TMA) to detect aberrations in *MYCN* gene, 1p36, 11q and 17q chromosome regions, and for patient stratification.
Method: 369 tumors were included in TMA, 291 were primary tumors. We performed FISH assays to determine the status of *MYCN* gene and 1p36 region on TMA and compared with routine diagnosis previously known in 139 tumors to evaluate the feasibility of these assays. After this validation, 11q and 17q alterations were analyzed in 369 samples. Partial genetic instability (PGI) was defined as the ratio between segmental chromosome aberrations (SCA) detected and number of genetic markers diagnosed in each tumor. Prognostic value of currently clinical and biological variables used was evaluated to know if our cohort was statistically representative. We compared patient risk using SIOPEN classification with estimated risk using the new INRG classification system.
Results: No discordance between status of *MYCN* gene and 1p36 region by FISH on TMA and previously routine diagnosed was observed. PGI was established in 260 primary tumors, 67 of them contained <60% neuroblasts, including always *MYCN* gene status and at least 2 of the others SCA. Outcome was statistically worse for patients whose tumors presented high PGI ($p<0.0001$). Risk estimation was established in 280 patients. 32 and 13 patients with intermediate risk by SIOPEN presented low and high risk by the new INRG classification respectively.
Conclusion: In our cohort, PGI established by FISH on TMA was associated with patient outcome. PGI is a useful method to identify high risk patients whose tumors have <60% neuroblasts. Grants: Instituto Carlos III Madrid, Spain (RD06/0020/0102) and FAECC (369/2009).

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POT63

The effect of COX-2 expression on celecoxib sensitivity and tumour growth in neuroblastoma

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Background: The cyclooxygenase-2 (COX-2)-specific inhibitor celecoxib induces cell death and apoptosis in neuroblastoma and other tumour cell types. However, the contribution of COX-2 inhibition to the efficacy of celecoxib has been questioned.
Method/approach: We profiled the levels of COX-2 expression in 4 neuroblastoma cell lines: SH-SY5Y, SKNBE(2), IMR-32 and SKNAS, with their sensitivity to celecoxib *in vitro*. We have used a tetracycline-inducible COX-2 over-expression system in SH-SY5Y cells (SH-SY5Y^{tet12} COX-2) and COX-2 siRNA to investigate the effect of COX-2 expression on sensitivity of neuroblastoma cells to celecoxib. Levels of prostaglandin $(PG_{(E2)})$ release was measured using an enzyme-linked immunosorbent assay. Cell survival was determined using XTT and levels of apoptosis were determined using flow cytometry for the % cells with sub G1 DNA content. SH-SY5Y^{tet12} COX-2 subcutaneous xenografts were used to determine the effect of COX-2 expression on neuroblastoma growth *in vivo*.
Results: Although endogenous COX-2 expression varied substantially between neuroblastoma cell lines, these cell lines were remarkably similar in their sensitivity to celecoxib (IC_{50} values 26-28μM). Over-expression of COX-2 increased the levels of $PG_{(E2)}$ *in vitro*. However COX-2 over expression did not affect sensitivity to celecoxib *in vitro*. Addition of arachidonic acid increased the sensitivity to celecoxib independently of COX-2 expression. Knockdown of basal levels of COX-2 using siRNA did not alter the levels of apoptosis and cell death after treatment with celecoxib. COX-2 over-expressing SHSY5Y xenograft tumours developed more rapidly than those in the control mice ($P<0.001$). Furthermore, the majority of tumours over-expressing COX-2 metastasised into the abdominal cavity.
Conclusion: The results of this study suggest that COX-2 does not mediate the cell-death promoting effects of celecoxib *in vitro* and that the effects of celecoxib on other elements of arachidonic acid metabolism need to be better understood. COX-2 expression significantly increased the tumourigenicity of neuroblastoma *in vivo*.

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POT64

Targeting neuroblastoma and neuroblastoma tumour initiating cells with the oncolytic viruses myxoma and vesicular stomatitis virus

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Background: Neuroblastoma is the second most common pediatric extracranial malignant tumor. High-risk patients do poorly and little progress has been made in improving their outcome. A subset of tumor cells called Tumor Initiating Cells (TICs) may drive aggressive tumor behavior and treatment resistance. A neuroblastoma TIC has been identified, but their role in tumor behavior is not yet characterized and their susceptibility to existing and novel cancer treatments is unknown. Myxoma and Vesicular Stomatitis Virus (VSV) are two oncolytic viruses that have been shown to effectively destroy brain tumor cells, which share a common ancestry with neuroblastoma in that both are derived from neural crest cells. It is unknown if these oncolytic viruses can effectively destroy neuroblastoma cells or neuroblastoma TICs.
Objectives: Characterize the ability of VSV and Myxoma to target and destroy 1) neuroblastoma cells; and 2) neuroblastoma TICs.
Methods: Viability assays on infected neuroblastoma and neuroblastoma TIC lines were performed. Infection and viral protein production was assessed by intergenic fluorescent protein expression, Western blot detection of viral proteins from infected cell lysates, and measurement of infected cell lysate viral activity by plaque assay. *In vivo* viral activity was measured by intratumoral injection in an established human neuroblastoma mouse xenograft model.
Results: Both myxoma and VSV infect and kill neuroblastoma cells in vitro. Cytopathic effects and green fluorescent protein (Myxoma) or rhodamine (VSV) expression were both seen after infection. Infection of neuroblastoma cells was further confirmed by Western blot detection of myxoma and VSV viral protein expression. Most neuroblastoma TIC lines are also infected and destroyed by myxoma but appear resistant to VSV. Finally, both myxoma and VSV were found to effectively inhibit the growth of neuroblastoma in subcutaneous xenografts.
Conclusion: Myxoma and VSV effectively destroy several neuroblastoma cell lines while myxoma is able to target neuroblastoma tumour initiating cells. Oncolytic viruses may offer a novel approach to treatment of high-risk neuroblastoma.

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POT65

Survival pathways of high-risk neuroblastoma identified by functional genomics

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Background: High-risk neuroblastoma (NB) is a molecularly heterogeneous disease with amplification of the MYCN oncogene detected in about one third of high-risk cases.
Method/approach: Our aim was to identify critical survival pathways and therapeutic targets for high-risk NB utilizing functional genomics. A parallel high throughput siRNA screen was performed in high-risk neuroblastoma cell lines with and without MYCN amplification. The screen included a set of 43 genes previously implicated in neuroblastoma pathogenesis and a collection of siRNA targeting the entire human kinome (~750 genes). Network pathway analysis was utilized to integrate the gene "Hits" from the screen with available microarray data (Oncogenomics website link: http://pob.abcc.ncifcrf.gov/cgi-bin/JK).
Results: The screen highlighted differential sensitivities between MYCN amplified and non-amplified lines to inhibition of key players of WNT and NGF signaling, as well as to inhibition of mitotic kinases, such as STK6 (Aurora A), NEK2, NEK4 and WEE1. However, additional genes, not previously implicated in NB were also revealed. A complete list of "hits" will be presented and linked to both known and novel NB pathways, which could constitute potential therapeutic targets.
Conclusion: This study identified druggable genes and pathways required for proliferation of high risk NB cells in vitro. In vivo validation of these genes through stable RNAi or using available small molecule inhibitors, will provide evidence for future therapeutic target development. Furthermore, our study indicates the importance of combining functional genomics with gene expression data to select within the patient population which patients could benefit from specific biologic therapies.

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POT66

Anti-angiogenic activity of the selective VEGFR-1, -2, -3 inhibitor Axitinib (AG-013736) in human neuroblastoma xenografts

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Tumor angiogenesis is an important prognostic factor in neuroblastoma. Presence of high vascularity, sprouting of new vessels by angiogenesis growth factors, and integration of circulating endothelial cells from bone marrow origin in aggressive and disseminated disease suggest the use of anti-angiogenic agents for the treatment of high risk neuroblastoma. We evaluated the new oral pan-VEGFR tyrosine kinase inhibitor axitinib (AG-013736) against neuroblastoma cell lines and the subcutaneous and orthotopic xenograft model IGR-N91 derived from a primary neuroblastoma bone marrow metastasis. Axitinib reduced cell proliferation in a dose dependent manner with IC50 doses between 667 and 1069 nmol/L. Oral treatment with 30 mg/kg BID during 2 weeks at advanced tumor stage yielded significant tumor growth delay in median time to reach 5 times initial tumor volume of 11.4 days compared to controls (p=0.0006; Mann-Whitney test) and significant reduction in bioluminescence, respectively. mTOR inhibition using rapamycin 20 mg/kg q2d x 5 resulted in tumor growth delays, however their combination did not significantly increase single agent activities. Axitinib treatment down-regulated VEGFR-2 phosphorylation and resulted in significantly decreased microvessel density (MVD) and overall surface fraction of tumor vessels (OSFV) as measured by CD34 immunohistochemical staining (mean MVD and OSFV, respectively, at 14 days were 21.27 +/-10.03 and 0.56% in treated tumors versus 48.79 +/-17.27 and 1.29% in controls; p=0.0006). We further explored the effects of axitinib on circulating mature endothelial cells (CECs) and endothelial progenitor cells (CEPs) measured by flow cytometry. While a transient reduction was observed for CECs, CEPs were significantly reduced during and at least 14 days after end of treatment. Thus, axitinib is a potent anti-angiogenic new agent targeting tumor angiogenesis in neuroblastoma and demands further evaluation.

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POT67

Relationship between ploidy and genetic instability of neuroblastoma in children below 18 months of age

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Background: Depending on their DNA content, neuroblastoma (NB) can be divided into near diploid/tetraploid (2n/4n) tumors and near triploid/hyperdiplod except tetraploid (3n/>5n) tumors. According to INRG, ploidy is a prognostic marker for patients <18 months of age with disseminated disease. It was suggested that locoregional or metastatic 2n/4n tumors have fundamental defect in genome leading to chromosomal rearrangements. The presence of segmental chromosome aberrations (SCA) with or without *MYCN* amplification (MNA) has been related to worse outcome and is the strongest predictor of relapse. This study aimed to analyze the relationship between ploidy and genomic instability in children <18 months of age.
Method: 150 NB were analyzed by FISH, MLPA and image cytometry techniques. Data were analyzed by SPSS package v.17.0. The tumors were treated mostly according to SIOP (INES, EUNS, HR and LNESG), few following national studies and 6 cases were excluded.
Results: Numerical chromosome aberrations (NCA) were found in 56 cases (37%) and SCA in 94 cases (63%). Ploidy was 2n/4n or 3n/>5n in 51 (34%) and 99 (66%) tumors, respectively. NB patients were divided in <18 months of age (n=91), >18 months of age (n=59); and stages 1, 2 (n=35), 3 (n=36), 4 (n=43), 4s (n=12) and unknown (n=24). In patients <18 months of age, 26/91 tumors were 2n/4n and 41/91 tumors had SCA (9 with MNA). Stages 1,2 and 4s were found mainly in patients <18 months of age (p<0,001). NCA were predominant in this age and stages (p<0,001). 2n/4n tumors in younger patients with disseminated disease also showed mainly NCA, but the low number of cases didn't allow a statistic analysis (n=13/26). The study revealed that 2n/4n tumors were not associated to SCA in children <18 months; SCA were equally distributed in the tumors independently of their ploidy (p=0,517).
Conclusion: This study strengthens the independent effect of ploidy and genetic instability in patients <18 months of age and reinforces the importance and the necessity of measuring ploidy and genomic profile in successive studies.

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POT68

Bi-directional regulation of the wild type of ALK in neuroblastoma: Its high expression in stage 4s tumors and transcriptional activation by MYCN and Sp1

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Background: The dependence receptor ALK forms a complex with the receptor-type protein tyrosine phosphatase β/Δ(RPTPb/z) whose ligand is pleiotrophin (PTN). We and others have previously reported that *ALK* gene is amplified and mutated in neuroblastomas (NBs). However, the aberration was only 5-7%. The functional role of wildtype of ALK in NB remains elusive.
Method: The aberration of *ALK* was analyzed by DNA sequencing and array CGH. The mRNA expression was examined by quantitative real-time RT-PCR. The transcriptional analysis was done by luciferase reporter and ChIP assays.
Results: The analysis of 343 Japanese sporadic NBs showed that 322 (93.9%) possessed no aberration of *ALK* gene (mutation 4.7%; amplification 1.5%). We then examined the expression levels of *ALK, RPTPb/z* and *PTN* mRNAs in 79 NBs with wildtype of *ALK*. The high expression of *ALK* mRNA was significantly associated with favorable outcome of NB (p=0.04). Notably, the levels of *ALK* mRNA expression were significantly high in stage 4s tumors (n=5) as compared with other stages, that was further confirmed by using additional 9 samples in stage 4s (p<0.01). The high levels of *PTN* and *RPTPb/z* mRNA expression were also significantly associated with better survival (p=0.02 and p=0.01), and the combination of low expression of both genes showed the worst survival rate (p<0.01). The overexpression of mutant as well as wildtype of ALK enhanced cell growth, migration and invasion in NB cell lines. On the other hand, elevated wildtype ALK in PC12 cells enhanced neuritis outgrowth. These suggested that wildtype ALK may regulate cell growth and survival as well as differentiation in neuronal cells. The intracellular adaptor proteins, Shf and BMCC1 we previously identified, interacted with both TrkA and ALK, suggesting the presence of crosstalk between TrkA and ALK signaling. Surprisingly, *ALK* expression was induced by MYCN and Sp1, that was confirmed by reporter and ChIP assays.
Conclusion: Wildtype of ALK signaling may be regulated bi-directionally among the different subsets of NBs.

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POT69

CD133 regulates signal transduction pathways and prevents differentiation via RET suppression in neuroblastoma cells

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Background: CD133 (prominin-1) is a transmembrane glycoprotein expressed on the surface of normal and cancer stem cells (tumor-initiating cells) and progenitor cells. Although CD133 is widely used as a marker of various somatic and putative cancer stem cells, its contribution to the fundamental properties of neuroblastoma (NB) cells, such as tumorigenesis and differentiation, remains to be elucidated.
Results and Discussion: CD133 was highly expressed in several NB cells at protein and mRNA levels. To study the functional roles of CD133 in NB cells, we knocked down CD133 by lentivirus-produced shRNA. In the CD133 knocked down cells, neurite elongation was induced without any stimulation and cell growth was suppressed *in vitro* and *in vivo*. The molecular mechanism of CD133-related differentiation suppression in NB was dependent on neurotrophic receptor RET tyrosine kinase regulation. *RET* transcription was suppressed by CD133 in NB cells and GDNF treatment failed to induce RET in CD133-expressing cells. By analysis of signal transduction pathways, we found that p38MAPK and PI3K/Akt activation were involved in *RET* transcriptional suppression and neurite elongation in CD133-expressing NB cells. Furthermore, both the endogenous *CD133* upregulation and *RET* suppression were observed in NB spheres of IMR32 and primary NB cells. *CD133* expression patterns were negatively correlated with *RET* expression in both NB cell lines and unfavorable NB tumors. Moreover, CD133-expressing NB cells formed significantly larger sized spheres and CD133-expressing IMR32 and primary spheres contained much more viable cells than mock control. Taken together, these results suggest that CD133 had a role in p38MAPK and PI3K/Akt activation resulted in suppression of NB cell differentiation/death related to *RET* transcriptional down-regulation. Our findings indicated the functional role of CD133 in NB cell tumorigenesis and may be applied to development of differentiation induction therapy for NB patients.

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POT70

Dendritic cell-based immunotherapy using sendai virus vector - a preclinical efficacy study against neuroblastoma - : An advanced report

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Background: We have recently reported that the induction of efficient antitumor immunity to C1300 murine neuroblastoma (C1300) using DCs treated with recombinant Sendai virus (SeV/DC). The aim of the current study is to investigate the potential of SeV/DC to treat less immunogenic neuroblastoma as a preclinical efficacy study.
Methods: A/J mice were subcutaneously inoculated with C1300. Bone marrow-derived DCs treated with SeV (SeV/DC) were administrated intratumorally after irradiation of x-ray (4G/day for 3 days). Human peripheral monocyte-derived DCs were treated with SeV. 48h after the transfection of SeV, we examined the expression of the surface markers of DCs and gene transduction efficiency by flow cytometry, and the expression of inflammatory cytokines in the medium of cultured DCs by ELISA.
Results: Use of SeV/DC without preirradiation showed some efficacy on established C1300, but antitumor effect against vascularized/established tumor was weakened. The combination with irradiation and SeV/DC was effective against vascularized/established tumor (> 5 mm), and dramatically enhanced the ratio of complete elimination of established tumor (5/8=62%). Antitumor effect of SeV/DC with preirradiation was enhanced CTL activity and established tumor specific long term memory against C1300. The result of the effector cell-depletion experiment confirmed that CD4+ T cells were predominant effector cells in antitumor immunity for C1300 in the early phase and CD8+ T cells in the secondary phase. Transfection efficiency of SeV to human peripheral monocyte-derived DCs were sufficient (average>70%) and some inflammatory cytokines were induced by SeV transfection without any remarkable changes in the expression of DC surface markers.
Conclusion: These results indicate that less immunogenic neuroblastoma could be a potential target of SeV/DC-based immunotherapy. Therefore we conclude that SeV/DC system is warrant to further investigation to treat patients with intractable malignancies including far advanced neuroblastoma in clinical setting.

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POT71

***DD3*, a large non-coding RNA against the pro-apoptotic *BMCC1* gene, is a candidate target for treating neuroblastoma**

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Background: We previously identified a novel gene *BMCC1* from our neuroblastoma (NB) cDNA project (Oncogene, 2006). The *BMCC1* mRNA was preferentially expressed in human nervous tissues and prostate. BMCC1 is a pro-apoptotic protein expressed at high levels in favorable NBs and is up-regulated during the NGF withdrawal-induced apoptosis in mouse SCG neurons and PC12 cells. Here we have identified *DD3* as a large non-coding RNA targeting *BMCC1*.
Methods: The expressions were examined by semi-quantitative RT-PCR, real-time RT-PCR and immunoblot. The reciprocal co-immunoprecipitation was performed for physical protein interaction assay.
Results: *BMCC1* mRNA was down-regulated in tumorous tissues as compared with the corresponding normal tissues in many cancers, suggesting that it appears to be a tumor suppressor. BMCC1 physically interacted with both TrkA and ErbB2, and the activation of its GAP activity at the BCH domain attenuated the signals for differentiation and proliferation by regulating ERK phosphorylation. From the genomic database, we found an antisense gene, *DD3*, located at the intron 6 of the *BMCC1* gene. *DD3* was supposed to be a large non-coding RNA of 3.7 kb in size. Expression of both *BMCC1* and *DD3* mRNAs was significantly decreased in unfavorable NBs (n=100; p=0.011 and p<0.001, respectively). Decreased expression of both *BMCC1* and *DD3* mRNA was significantly associated with *MYCN* amplification (p≤0.001), low expression of *TrkA* (p≤0.001) and DNA diploidy (p=0.001). However, overexpression of *DD3* down-regulated BMCC1, whereas siRNA-mediated knockdown of *DD3* increased BMCC1 expression. In addition, accumulation of *BMCC1* mRNA was also observed by cisplatin treatment. Of further interest, the cell death induced by cisplatin was significantly enhanced by knockdown of *DD3* by using siRNA-*DD3* in both NB and prostate cancer cell lines.
Conclusion: *DD3* functions like an oncogene by targeting *BMCC1* tumor suppressor in cancer cells. In NBs, the *BMCC1* and *DD3* negative regulatory loop may contribute to the reduction of regression. In addition, targeting *DD3* may enhance the therapeutic sensitivity to anti-cancer drugs.

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POT72

The novel PDK1 inhibitor OSU03012 and the dual PI3K/mTOR inhibitor PI103 target high-risk neuroblastoma *in vitro* and *in vivo*

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Background and aims: Proteins regulating signalling through the phosphoinositide 3-kinase (PI3K)/Akt pathway are frequently altered in human cancers, including neuroblastoma. We investigated two compounds inhibiting key proteins in PI3K/Akt signalling; a phosphoinositide-dependent protein kinase-1 (PDK1) inhibitor, OSU03012, and the dual class IA PI3K/mammalian target of rapamycin (mTOR) inhibitor, PI103; on neuroblastoma *in vitro* and *in vivo*.
Methods: Primary tumours were investigated for the presence and activation status of components of the PI3K/Akt pathway by immunohistochemistry. *In vitro*, the efficacy of these inhibitors was investigated by proliferation, cell cycle and apoptosis assays whereas PI3K/Akt pathway activation/inhibition was detected by western blotting. Tet21N cells were used to examine the efficacy of the inhibitors in a low/high-MYCN setting. *In vivo*, athymic mice engrafted with human neuroblastoma cells were randomised to treatment with either inhibitor and the tumour growth was followed.
Results: Immunohistochemical analysis showed the presence and activation of the target molecules PDK1 and PI3K (the catalytic p110α and the regulatory p85 subunits) in tumour cells of primary human neuroblastomas. Both OSU03012 and PI103 inhibited neuroblastoma growth *in vitro*. In treated cells, OSU03012 induced apoptosis and an S phase cell cycle arrest, but only minor apoptosis was detected in PI103 treated cells together with a G1 arrest. Both OSU03012 and PI103 down regulated phosphorylation of Akt and its downstream targets, GSK3β and S6K1, as well as cyclin D1 and MYCN protein expression. Cell lines expressing high levels of MYCN were more sensitive to OSU03012 or PI103 compared to cells expressing low MYCN levels. Both OSU03012 and PI103 significantly inhibited the growth of established *MYCN*-amplified xenografts.
Conclusion: Our results suggest that key proteins in the PI3K/Akt signalling cascade represent clinically relevant targets for high-risk *MYCN*-amplified neuroblastoma.

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POT73

NK cells engineered to express the chimeric receptor scFv(ch14.18)-zeta specifically lyse GD2 expressing meuroectodermal tumors

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Introduction Neuroblastoma (NB) is a neuroectodermal tumor of childhood characterized by a poor prognosis. The T cell independent antigen ganglioside GD2 is highly expressed on most NB which makes it an interesting target for immunotherapeutic strategies. In order to specifically direct the cytotoxic abilities of NK cells towards NB cells, the human NK cell line NK-92 was genetically engineered to express a chimeric receptor, consisting of a GD2-specific ch14.18scFv-antibody fragment and the signal transducing zeta-chain of the CD3 complex (NK-92-scFv(ch14.18)-zeta).
Methods: In order to determine specificity of NK-92-scFv(ch14.18)-zeta (NK-92tr), FACS analysis was performed. For this, we used an anti-idiotypic-antibody (anti-Id-Ab), which mimics the GD2 epitope and is directed against the binding domain of ch14.18. In vitro cytotoxicity assays measuring chromium (Cr) 51 release with GD2+ and GD2– NB and melanoma cell lines were performed, in order to prove functionality of transduced NK-92 cells. Furthermore, cytotoxic activity of NK-92tr was blocked using the α-Id-Ab in Cr51 release assays.
Results: FACS analysis using an α-Id-Ab revealed high expression of the chimeric receptor on NK-92tr, indicating specificity for GD2. Furthermore, genetically modified NK-92 specifically lysed GD2+ tumor cells at an E:T ratio of 6.3:1, ranging from 70% (M21 cells, GD2+ melanoma) to 79% (LAN-1, GD2+ human NB) cytotoxicity. In contrast to that, cytotoxicity against GD2– NB cell line SK-N-SH was decreased (16% specific cytotoxicity). Importantly, parental NK-92 cells showed only diminished cytotoxicity towards GD2 expressing tumor cells compared to NK-92tr. Additionally, we could show that blocking the chimeric receptor on NK-92tr with the α-Id-Ab leads to a significant decrease of cytotoxicity towards GD2+ LAN-1 cells (20% specific cytotoxicity). Conclusion
Conclusion: The results achieved so far clearly demonstrate specificity and functionality of NK-92tr and provide an important base line for additional experiments to further investigate anti-tumor efficacy of NK-92tr in NB xenograft mouse models.

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POT74

Synergistic inhibition of neuroblastoma tumor development by targeting ornithine decarboxylase and topoisomerase II

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Background: Neuroblastoma (NB) is a deadly childhood cancer that arises from neural crest cells of the sympathetic nervous system. *MYCN* amplification occurs in a large number of NBs and is associated with poor prognosis. Since MYCN controls a number of genes including ornithine decarboxylase (ODC), we proposed that ODC should be considered as a novel target for MYC-driven tumors such as NB (Hawaii Med J, 2004, 63:371-4; Oncogene, 2005, 24:5606-18). We showed that ODC and polyamines are markedly elevated in NBs and targeted inhibition of ODC by alpha-difluoromethylornithine (DFMO) resulted in polyamine pool depletion and subsequent p27/Rb-mediated G1 cell cycle arrest.
Methods: We utilized *in vitro* calcien AM cell viability testing to assess the effect of DFMO drug combinations on several NB cell lines. Nude mice were injected with 10^7 SMS-KCNR cells subcutaneously and treated with 1) vehicle, 2) 40mg/kg etoposide on day 1 and day 30 intraperitoneally, 3) 2% DFMO in drinking water, or 4) the combination of etoposide and DFMO.
Results: We identified a synergistic interaction in NB cells with the combination of DFMO and etoposide, a topoisomerase II inhibitor that is commonly used in front-line therapy of NB patients. Remarkably, DFMO and etoposide, in combination, synergistically reduced the tumor burden in mice and extended tumor-free survival.
Conclusion: Given the current lack of effective therapies for relapsed/refractory NB patients, the preclinical effectiveness of this combination, and high safety profile of DFMO we have advanced DFMO and etoposide into an FDA approved Phase I NB clinical trial.

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POT75

The genetic and clinical implications of *MYCN* gain in neuroblastoma

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Purpose: The *MYCN* gene is located in chromosome 2p24, and *MYCN* amplification (*MYCN*-A) is a strong prognostic factor in neuroblastoma (NB). *MYCN*-gain which is a low level of *MYCN*-A as determined by FISH is identified as less than 4-fold additional copies of *MYCN* signals in relation to the number of chromosome 2. It is unclear whether the *MYCN*-gain is the pre-status of *MYCN*-A. Furthermore, the clinical significance of *MYCN*-gain is unclear. This study assessed the correlation of *MYCN*-A and *MYCN*-gain, and the clinical implication of *MYCN*-gain in NB.
Methods: The status of the *MYCN* gene was determined by FISH and quantitative polymerase chain reaction (Q-PCR) in 47 primary NB samples and the status of chromosome 2p in all cases was analyzed using a single nucleotide polymorphism (SNP) array.
Results: Eight of the 47 cases analyzed using FISH showed *MYCN*-A, 7 cases showed *MYCN*-gain and 32 cases showed no *MYCN* amplification (NMA). A SNP array analysis showed that 6 of 8 cases with *MYCN*-A by FISH had amplification of the *MYCN* region without distal 2p gain and other 2 cases had both amplification of *MYCN* region and distal 2p gain. All 7 cases with *MYCN*-gain by FISH had distal 2p gain without amplification of the *MYCN* region, and all 32 cases with an NMA by FISH demonstrated neither the amplification of the *MYCN* region nor the 2p gain. Three of 7 cases with *MYCN*-gain showed slight increase of *MYCN* gene dosage by Q-PCR. The 5-year overall survival rate of patients with *MYCN*-gain (n=7, 71.4%) were poor in comparison to that of patients with NMA (n=32, 90.6%) by FISH. However, no significant difference was observed (p=0.11). The SNP array analysis showed that NBs with *MYCN*-gain had more other genetic aberrance such as 1p loss, 11q loss and 17q gain than NBs with NMA by FISH.
Conclusion: These results suggest that the *MYCN*-gain represents the distal 2p gain and is not the pre-status of *MYCN*-A. NB with *MYCN*-gain by FISH have other genetic aberrances associated with the prognosis.

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POT76

A new syngeneic MYCN-overexpressing neuroblastoma mouse model and MYCN-DNA vaccine

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High-level expression of MycN protein caused by amplification of the gene characterizes the malignant phenotype of neuroblastoma. Recent studies suggest that MycN is a suitable target for immunotherapy, but up to now, a syngeneic NB mouse model over expressing MYCN is not available to examine immunotherapeutic strategies in vivo. Here, we report the development of a tetracycline inducible MYCN expressing murine NB cell line syngeneic to A/J mice and a MYCN-DNA vaccine. For this purpose, the murine NB cell line C1300, syngeneic to A/J mice, was stably transfected with a tetracycline inducible MYCN expression vector. Stable transfection was verified by real-time PCR and Western-Blot, revealing high expression levels of MycN RNA and protein, respectively. Furthermore, a MYCN-DNA vaccine, based on epitopes encoding for three peptides from the murine MycN protein sequence with high affinity to the A/J mouse MHC class I allele H2-Kk, was designed and tested in vivo for its ability to induce an antigen-specific immune response. Lymphocytes isolated from A/J mice vaccinated with the MYCN vaccine effectively lysed C1300-MYCN +tet cells in cytotoxicity assays in contrast to wild type C1300 cells and lymphocytes from control mice. Lymphocytes from minigene vaccinated mice produced significantly higher amounts of IFN-ā after stimulation with irradiated C1300 cells than lymphocytes from control mice. Interestingly, vaccine induced cytotoxic T lymphocytes also kill parental C1300 tumor cells which show low MYCN expression. We expect that this effect will be enhanced when MYCN-over expressing C1300 cells are employed in a similar cytotoxicity assay which will be reported at the meeting. In summary, we demonstrate the development of a new MYCN-DNA vaccine and a murine tetracycline inducible MYCN NB cell line syngeneic to A/J mice for in vivo evaluation of MycN directed immunotherapeutic strategies.

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POT77

Deregulation of rho/ras rnd neuronal differentiation pathways is associated with fatal outcome in high-risk disseminated neuroblastoma

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Background: Fifty percent of children with neuroblastoma (NB) have high-risk (HR) metastatic disease and show a long-term survival < 30%. In order to identify novel molecular prognostic markers useful to refine current criteria of patients´ relapse risk estimation, we performed genome- and transcriptome-wide analyses in stage 4 HR-NB.
Method: Patients older than 1 year of age at diagnosis were categorized as "short-survivors" (SS) (dead of disease within 5 years from diagnosis) and "long-survivors" (LS) (alive with an overall survival time > 5 years). Array-CGH was performed in 91 NBs, gene expression profiling in 75 NBs, and the expression study of 481 Transcribed-Ultra Conserved Regions (T-UCRs) by qPCR and of 723 miRNAs by arrays in 34 NBs.
Results: All NBs were characterized by structural aberrations on chromosomes 1, 2, 3, 7, 11, 17. The number of these alterations was significantly higher (P=0.0005) in SS compared to LS. Tumors with *MYCN* amplification showed a simpler pattern of alterations compared to *MYCN*-single copy NBs (P=0.0008), dominated by 17q gain and 1p loss. ROC and Kaplan-Meier survival analyses showed that at least 4 structural aberrations are needed to discriminate (P<0.0001) LS from SS. In tumors of SS, genes involved in cell cycle are up-regulated, whereas Rho/Ras pathway is down-expressed. Among the genes up-regulated in LS, regulators of neuronal differentiation (*DPYSL3, NTRK1, CHD5, FYN*) are enriched. An inverse correlation between expression level of T-UCRs and their complementary miRNAs targeting neuronal differentiation genes was observed in SS patients.
Conclusion: a) Structural aberrations are significantly associated with fatal outcome; b) Deregulation of Rho/Ras-mediated progression through cell cycle may explain the increase of tumor aggressiveness in SS; c) Integrative expression analysis of non-coding RNAs and host genes suggests that changes in primary transcription rates are responsible for switching off the neuronal differentiation in HR-NB pathogenesis.
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POT78

Tumor cell detection in autologous stem cell harvests in patients with high risk neuroblastoma

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Introduction: The presence of minimal residual disease (MRD) detected by real-time quantitative (RQ)-PCR in autologous stem cell harvests in children with high risk neuroblastoma (NBL) seems to be associated with an unfavourable outcome, however to date only small studies have been performed. Moreover, these studies suffered from lack of specificity of the assay due to background amplification of the PCR target in normal bone marrow (BM) and peripheral blood stem cells (PBSC). In this retrospective multicenter study, harvests of a large patient cohort are studied using a recently described optimal panel of PCR targets (1).
Methods: In total, 37 BM harvests, 75 PBSC harvests and 55 CD34+ selected harvests from 167 high risk patients were retrospectively collected at 2 Dutch and 12 German centers. In 137 patients the harvest was reinfused. Of those, 25 PBSC before CD34+ selection, 44 CD34+ selected harvests and 68 unselected harvests (28 BM and 40 PBSC) were tested. RQ–PCR was performed with six NBL-specific markers: *PHOX2B, TH, DDC, GAP43, CHRNA3*, and *DBH*. The prognostic impact of MRD in autologous harvests and the reinfusion of contaminated harvests were assessed using Kaplan-Meier plots and log-rank tests.
Results: Presence of NBL mRNA was detected in 46% (17/37) BM harvests, 12% (9/75) PBSC harvests and 14% (8/55) CD34+ harvests. This was associated with poor survival (5 years overall survival (5-y-OS), 23.4±8.2% versus 45.6±4.5%; p=0.008). In 21 % (24/112) of the patients an MRD positive harvest was reinfused, which was associated with poor outcome (5-y-OS 30.5±10.5% versus 55.4±5.7%;p=0.03). Remarkably, there was no difference in survival in patients after reinfusion of BM harvests compared to PBSC harvest (± CD34 selection) (p=0.66).
Conclusion: Our series of autologous stem cell harvests is the largest series described up till now. In this series, BM harvests were more often contaminated than PBSC or CD34+ selected harvest. Both the presence of MRD in the harvests and reinfusion of a contaminated harvest were associated with worse outcome.

Reference List

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POT79

Functional analysis of the p53 pathway in neuroblastoma cells using the small-molecule MDM2 antagonist nutlin-3

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Background: Inactivation of the p53 pathway is essential for tumor cells to survive and thrive, and improved understanding of the mechanisms behind p53 inactivation may guide the development of targeted therapeutic strategies. We set out to examine the nature of p53 pathway defects in a large panel of neuroblastoma cell lines using the selective MDM2 antagonist nutlin-3 as a tool to directly activate p53.
Methods: The entire coding region of the p53 gene from 34 human neuroblastoma cell lines was analyzed by direct sequencing. Functional integrity of the p53 pathway was probed by measuring the reduction in cell viability after treatment with nutlin-3. Activation of the p53 pathway in experiments that aimed at identification of modulators of the response to nutlin-3 was assessed by real-time quantitative RT-PCR analysis of p53 target genes and by cell viability and caspase assays.
Results: We identified 9 cell lines (26.5%) with a mutation in the p53 gene, including 6 missense mutations, 1 nonsense mutation, 1 in-frame deletion, and 1 homozygous deletion of the 3' end of the p53 gene. Sensitivity to nutlin-3 was highly predictive of absence of p53 mutation. Cell lines with wild-type p53 were subject to marked nutlin-3-induced cytotoxicity in 23 out of 25 cases, indicating that p53 downstream signaling pathways are functionally intact in the vast majority of neuroblastoma cell lines. The presence of a homozygous *CDKN2A* (*$p16^{INK4a}$/$p14^{ARF}$*) deletion in one of both nutlin-3-refractory cell lines with wild-type p53 (i.e., in SHEP cells) prompted us to investigate the role of $p14^{ARF}$ and $p16^{INK4a}$ in the response to nutlin-3. The nutlin-3-resistant phenotype of SHEP cells could not be reversed by reintroduction of $p14^{ARF}$ or $p16^{INK4a}$, but knockdown and overexpression experiments in several other neuroblastoma cell lines pointed to a stimulatory effect of $p14^{ARF}$ on the response to nutlin-3.
Conclusions: Mutational inactivation of p53 is not uncommon in neuroblastoma cell lines, whereas defects in effector pathways downstream of p53 are rare. Expression levels of $p14^{ARF}$ may modulate the response to nutlin-3, dependent on the cellular context.

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POT80

Detection of microRNAs in bone marrow from children with high-risk neuroblastoma predicts survival; a UK CCLG study.

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Background: MicroRNAs (miRNAs) are differentially expressed in tumours compared to normal tissues, resulting in an aberrant expression signature that is a hallmark of cancer. We have therefore tested the hypothesis that miRNAs might constitute a new class of biomarkers for detection of disease in bone marrow (BM) from children with neuroblastoma (NB), and potentially provide an informative signature for disease prognostication and monitoring.
Methods: miRNAs were isolated from BM samples stabilised in PAXgene™ Blood RNA tubes. The expression profile of 380 miRNAs was assessed using high-throughput stem-loop QRT-PCR (Low Density Arrays, Applied Biosystems). BMs positive (n= 30) and negative (n=10) for NB cells detected by QRT-PCR taken from children at diagnosis were analysed. BMs (n=5) from healthy children were also analysed.
Results: Eight miRNAs predicted for survival; Δ ranging from 5.08 to 9.01 ($p<0.024$) identified using ranked significance levels from Cox model analyses. High expression of miR-519a was most significantly ($p<0.003$) associated with increased risk of death (Relative Risk =7.82 [95% CI 2.03-30.11]). Of the 8 miRNAs identified, miR-450-5p and miR-708 were also detected in a 67 miRNA signature that distinguishes BM containing NB cells from that with no NB cells, and from BM of healthy children (unequal variance t-test $p< 0.01$, fold expression change > 4). Of these 67 miRNAs 11 were down-regulated and 56 up-regulated; miR-223 and miR-145 were amongst the most significantly down-regulated ($p<0.00002$) and expression of mir-137, mir-149, mir-375 and mir-10b were up-regulated > 1000-fold ($p<0.003$).
Conclusions: We have identified a panel of miRNAs that when detected in BM can predict survival. We also established a 67 miRNA signature that distinguishes BM containing NB cells from that with no NB cell contamination. This signature includes miRNAs that regulate cell cycle progression, angiogenesis and metastasis, providing novel insight into the biology of the circulating NB cell. These pilot observations require study in a larger group of children with NB.

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POT81

SKP2-mediated neuroblastoma dedifferentiation is triggered by MYCN through CDK4 induction

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Background: The cell cycle regulator, SKP2, is overexpressed in various cancers, including neuroblastoma, and plays a key role in p27 degradation, which is involved in tumour cell dedifferentiation. Little is known about the mechanisms leading to impaired SKP2 transcriptional control in tumour cells.
Aim: To study SKP2 transcriptional regulation we used neuroblastoma as a model because SKP2 transcript levels gradually increase with aggressiveness of neuroblastoma subtypes.
Methods: We used quantitative realtime RT-PCR to assess the SKP2 transcript levels in neuroblastoma cell lines and tumours. SKP2 promoter deletion/mutation constructs were used to define the regulatory sites in the SKP2 promoter. Chromatin immunoprecipitation (ChIP) with pocket proteins (pRB, p130, p107), activating and repressing E2Fs was used to characterize regulatory protein complexes at the SKP2 promoter.
Results: Highest SKP2 mRNA levels are found in neuroblastomas with amplified MYCN. Accordingly, we found 5.5-fold (range 2-9.5) higher SKP2 core promoter activity in MYCN-amplified cells. Higher SKP2 core promoter activity in MYCN-amplified cells is mediated through a defined region at the transcriptional start site (TSSR). This region includes a specific E2F-binding site that makes SKP2 activation largely independent of mitogenic signals. We demonstrate by chromatin immunoprecipitation that SKP2 activation through the TSSR in MYCN-amplified cells is associated with low abundance of pRB-E2F1 complexes bound to the SKP2 promoter. Transcriptional control of SKP2 via this regulatory mechanism can be re-established in MYCN-amplified cells by restoring pRB activity using selective small compound inhibitors of CDK4. In contrast, doxorubicin or nutlin-3 treatment - both leading to p53-p21 activation - or CDK2 inhibition had no effect on SKP2 regulation in MYCN-amplified cells.
Conclusion: Together, this implies that deregulated MYCN protein levels in MYCN-amplified neuroblastoma cells activate SKP2 through CDK4 induction, abrogating repressive pRB-E2F1 complexes bound to the SKP2 promoter.

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POT82

DHA is converted to hydroperoxides and potentiates the cytotoxic effect of chemotherapeutics in neuroblastoma

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Background: Docosahexaenoic acid (DHA) is an omega-3 polyunsaturated fatty acid that protects neural cells from stress-induced apoptosis and at the same time exerts anticancer properties on neuroblastoma (NB) cells. Here, we examined the metabolic pathway of DHA in NB cells using LC-MS/MS based lipidomics, to elucidate the mechanisms behind its toxicity. In addition, we evaluated combination treatments between DHA and standard chemotherapeutics in NB cell lines for possible potentiating cytotoxic effects, as a possible clinical application.
Method/approach: NB cells were incubated with DHA, subjected to solid phase extraction, and analyzed with LC-MS/MS for downstream products of the DHA metabolic pathway such as 17-hydroperoxy-DHA (17HpDHA) and 17-hydroxy-DHA (17HDHA), and the newly discovered DHA-generated bioactive products resolvins and protectins, which have anti-inflammatory, pro-resolving and neuroprotective properties. In addition, DHA was added in a fixed, low concentration to different concentrations of ten cytostatic drugs. Cell viability after 72h incubation with the drugs either alone or in combination was measured using a cell viability assay.
Results: NB cells converted DHA to 17HpDHA and further to 17HDHA, but not to resolvins or protectins, out of which 17HpDHA showed the highest cytotoxic potency. By adding a non-cytotoxic concentration of DHA we could demonstrate a significantly lower log IC50 for vincristine, melphalan and cisplatin in combination with DHA compared to the drug alone in both tested cell lines.
Conclusion: The toxicity of the omega-3 fatty acid DHA in neuroblastoma can partly be explained by intracellular conversion to 17HpDHA, an intermediate that can initiate radical reactions and lead to cell death. When combined with chemotherapeutics, DHA potentiated the toxic effects, especially of alkylating agents and the microtubuline stabilizing agent vincristine. The potentiating effects of DHA to several chemotherapeutics may involve increased ROS production and interactions with the glutathione system. DHA alone or in combination with cytostatics may constitute a novel option for neuroblastoma treatment

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POT83

Exploiting cell cycle aberrations in neuroblastoma by targeting checkpoint kinase chk1 using AZD7762 in neuroblastoma with p14^{ARF}/MDM2/p53 defects

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Background: AZD7762 is a checkpoint kinase inhibitor currently in clinical trials. In this study, we assessed the potential synergy between AZD7762 and chemotherapeutic agents in the treatment of neuroblastoma (NB) cell lines which harbor cell cycle aberrations because of p14^{ARF}/MDM2/p53 pathway defects.
Method/approach: AZD7762 was tested *in vitro* and *in vivo* with chemotherapeutic agents against a representative panel of p14^{ARF}/MDM2/p53 pathway-defective NB cell lines encompassing p53 mutations, *MDM2* amplification, or p14^{ARF} deletion. p53/p21 functional assay, cell cytotoxicity, S/G_2 checkpoint abrogation, and tumor growth in NB xenografts were examined.
Results: All four p53 mutant lines failed to show endogenous p21 induction after DNA damage (defined as p53 pathway nonfunctional), two of the three *MDM2* amplified lines and one of the three p14^{ARF} deleted lines were p53 nonfunctional as well. While p53 pathway-nonfunctional lines failed to show G_1 checkpoint arrest, the remaining S/G_2 checkpoint arrests were abrogated by AZD7762 *in vitro*. In cytotoxicity assays, p53 nonfunctional lines were more resistant to the cytotoxic effects of DNA-damaging agents when compared to p53 functional lines, and synergy between AZD7762 and DNA-damaging agents was strong in all p53 nonfunctional lines. Moreover, AZD7762 treatment abrogated DNA damage-induced S/G_2 checkpoint arrest in NB xenografts and potentiated antitumor activity of DNA-damaging agents in these mouse models.
Conclusion: AZD7762 potentiated both the *in vitro* and *in vivo* antitumor effects of DNA-damaging agents against NB. These results suggest that the addition of AZD7762 may reverse or prevent drug resistance, especially if cell cycle checkpoint defect is present.

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POT84

Preoperative analysis of 11q loss of heterozygosity using circulating tumor-released DNA in serum: A novel diagnostic tool for therapy stratification of neuroblastoma

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Background: Because the loss of heterozygosity in the long arm of chromosome 11 (11q LOH) is independently associated with the prognosis of neuroblastoma (NB), routine assessment of 11q LOH status is required for therapy stratification of NB. Here we examined the use of serum DNA, which predominantly originates from tumor-released DNA, for a preoperative, non-invasive assessment of 11q LOH.
Method/approach: We screened serum, primary tumor and nontumor DNA, and primary culture samples from 24 NB patients. The allelic intensity score was calculated with a panel of polymorphic markers located on 11q23 and 11p to determine the unbalanced 11q LOH, using capillary electrophoresis and PCR with fluorescence-labeled primers. The existence of 11q LOH was confirmed by two-color FISH analysis using primary culture samples.
Results: The allelic intensity score of polymorphic markers in 11q23 in serum DNA was significantly different between11q LOH-positive group and -negative group. The 11q LOH-positive and -negative group did not overlap when a cut-off value of 0.5 or 2.0 was chosen for the allelic intensity score of the STS marker on 11q23. With these cut-off values, the sensitivity and specificity of the serum-based 11q LOH analysis as a diagnostic test to distinguish 11q LOH-positive and -negative patients were both 100% for our limited number of patients.
Conclusion: Our serum-based 11q LOH analysis could predict 11q LOH in tumors. The analysis is a surgery-free, rapid, sensitive, and specific genetic assessment tool that should help choose risk-adopted therapy preoperatively.

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POT85

Neuroblastoma tumor initiating cells express CD22 making them susceptible to HA22 anti-CD22 immunotoxin induced cell death

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Background: Neuroblastoma Tumor Initiating Cells (NB-TIC) have been isolated from short-term primary cultures of bone marrow (BM) from NB patients with poor prognoses. NB-TICs contain cytogenetic alterations and NB tumor-associated antigens and are capable of self-renewal and multi-lineage differentiation. Precursor frequency analysis indicates 1/4.5 cells initiates a tumor when implanted supra-adrenal in SCID/beige mice. Detection of tumor cells in BM is often the first indication of tumor relapse but is not known whether NB-TICs are responsible for it. Cell surface analysis reveals that NB-TICs but not NB cell lines express a mixed lineage phenotype (B-cell/monocyte), expressing CD19, -22, -45 cell surface antigens. To ascertain whether NB would be sensitive to anti-CD22 targeted therapy, we utilized 3 NB-TIC cultures (NB12, NB67, NB61) and 2 NB cell lines (KCNR, AS). Almost 95% of NB12, NB67, and NB61 cells express membrane CD22 while less than 1% AS (0.88%) and KCNR (0.25%) cells express membrane CD22.
Methods: NB-TICs and NB cell lines were incubated with different concentrations (0.1-1000ng/ml) of HA22, a recombinant anti-CD22 immunotoxin comprised of a truncated derivative of pseudomonas exotoxin A (PE) engineered to an anti-CD22 Fv fragment. ERB38, a PE immunotoxin targeting ErbB2 served as a negative control and HB21, a PE immunotoxin targeting the transferrin receptor served as a positive control.
Results: After 7 days HA22 induced cell death in NB-TICs to the same extent or better than the positive control HB21: NB67 (HA22IC50= 0.3ng/ml vs. HB21 IC50=3ng/ml), NB12 (HA22IC50= 10ng/ml vs. HB21IC50=15ng/ml), NB61 (HA22IC50= 30ng/ml vs. HB21IC50=30ng/ml). HA22 did not induce cell death in the NB cell line KCNR although the positive control HB21 did (HA22 IC50>1000ng/ml vs. HB21IC50=0.1ng/ml). The negative control ERB38 did not induce death in NB-TICs or NB cell lines.
Conclusion: These data indicate that NB-TICs are sensitive to the anti-CD22 immunotoxin HA22. HA22 is in clinical trials for adult and pediatric patients with CD22+ hematologic malignancies. Our study raises the possibility that strategies targeting CD22 may prove to be active against NB.

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POT86

Genotype-guided neuroblastoma therapy, CP751,871 or Rapamycin

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Background/Aims: Remarkable heterogeneity in neuroblastoma (NB) genotype regulates different prognosis and drug response. We aimed to develop new strategies targeting mTOR and IGF-1R pathways based on the diversity of NB genotypes. We also investigated the efficacy of CP751,871 and rapamycin on NB tumor initiating cells (TICs) since these cells are responsible for maintenance of the malignant phenotype in NB.
Methods: Both NB cell lines and TICs were used for drug evaluation. CP751,871 and rapamycin, as well as their combination with vinblastine were evaluated both in vitro and in vivo. IGF-1R/mTOR expression was measured by Western blot and RT-PCR. Tumor angiogenesis was evaluated by Von Willebrand Factor immunostaining, and angiogenic factors were screened with Angiogenesis cDNA Microarray.
Results: NB tumors overexpressing IGF-1R was responsive to CP751,871 due to the suppression of tumor angiogenesis instead of cell proliferation. In cells overexpressing IGF-1R, with addition of CP751,871, cytotoxicity of vinblastine was significantly enhanced with decreased IC50 values by 2 - 3.4 times. In xenograft models, both CP751,871 and vinblastine inhibited tumor growth as single agents; while the combined treatment significantly improved antitumor activity ($p<0.05$). Differential antitumor effect of rapamycin was observed among NB cell lines. NB cells with low mTOR expression are sensitive to mTOR inhibitors, while limited response was observed in mTOR overexpressing cells. Synergistic antitumor effects were obtained by combining rapamycin and vinblastine ($p<0.05$). Remarkably, we found that NB TICs, NB12 and NB88R2, are responsive to mTOR inhibitors.
Conclusions: IGF-1R and mTOR expressions could be used as predictive biomarkers for drug response to CP751,871 or rapamycin (Tab. 1). mTOR inhibitors served as a promising antitumor agent effectively targeting TICs. Adding CP751,871/rapamycin to traditional chemotherapy could result in significant advances in NB treatment.

IGR-1R expression	mTOR expression	NB cells	Reactive drugs
High	Low	NUB-7, SH-SY5Y	CP751,871 & Rapamycin
High	High	SK-N-BE(2), LAN-5	CP751,871
Low	Low	NB12, NB88R2	Rapamycin
Low	High	N/A	

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Posters – Clinical
POC1–POC51

POC1

Neuroblastoma in children-experience in Croatia

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In the past decade, considerable modifications in the management of neuroblastoma in children have resulted in continuously rising rate of recovery.
Purpose: To describe the experience in the management of children with neuroblastoma in Croatia, staged and treated at a single institution.
Patients and Methods: Since January 1998 till December 2009, 12 children with neuroblastoma (7 male and 5 female) were treated at Department of Hematology and Oncology, Universitiy Department of Pediatrics, Universitiy Hospital Center, Zagreb, Croatia. The patients were treated with protocol NB 97 and NB 2004.
Patients were allocated to treatment groups by disease stage. Patients distribution by stage was as follows: stage I 1 (8,33%), stage II 1 (8,33%), stage III 3 (25%), stage IV 6 (50%) and stage IV S 1 (8,33 %). MYCN status was investigated in 8 patients (positive in 6) . Median of follow up is 28 months.
All patients had surgery (biopsy or partial or complete resection), underwent 2, 4, 6 or 10 cycles of chemotherapy, in high risk also autologous stem cell/bone marrow transplantation, radiotherapy, and in all maintenance therapy (consolidation with cyclophosphamide or 13-cis-retinoic acid).
Results: Remission was achieved in all patients; in 6 (50%) (HR) with relapse of disease, highly aggressive cytostatic therapy and radiotherapy (MIBG) was introduced. 4 patients (33,33%) died . 8 patients (66,66%) are still alive in the first or second remission. There were severe side effects but there was no case of secondary malignancies in any of patients.
Conclusion: Better treatment results were achieved in patients with stage I, II III and IV S. Combined modality therapy using surgery, chemotherapy, ASCT, radiotherapy and consolidation is optimal treatment for the majority of children with high risk neuroblastoma. New treatment approaches should be promoted for high risk patients.

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POC2

Phase I study of single agent perifosine for recurrent pediatric solid tumors

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Background: Perifosine, a synthetic alkylphospholipid, inhibits Akt while also affecting JNK and MAPK signaling pathways. Perifosine is cytotoxic to neuroblastoma and glioma cell lines at uM concentrations. Phase I trials of perifosine in adults have demonstrated responses in patients (pts) with renal cell carcinoma, brain tumors, sarcomas, hepatocellular carcinoma, and hematologic malignancies (e.g. multiple myeloma and Waldenstrom's macroglobulinemia).
Methods: Pediatric pts less than 18 years of age with recurrent solid tumors were enrolled in a phase I, open-label, dose-escalating study to assess pharmacokinetics (PK) and maximally tolerated dose. Cohorts of 3 pts were treated at three dose levels: (1) 25 mg/m2/day, (2) 50mg/m2/day and (3) 75 mg/m2/day using 50mg tablets, all after a loading dose of 100-200mg/m2/day on D#1.
Results: 9 pts (4 male, 5 female) with high-grade glioma (n=5), medulloblastoma (n=2) and stage 4 neuroblastoma (n=2) were enrolled, at a median age of 13 years (range 5-18). Most were heavily pretreated, with a median of 3 prior treatment regimens (range 1-10). No dose limiting toxicities, or ≥ grade 3 toxicities have been encountered to date (CTCAE v3.0). Grade 2 toxicities that were possibly related to perifosine included asthenia (22%), transaminase elevations (22%), neutropenia (33%), leukopenia (11%), hyperglycemia (22%), hypomagnesemia (22%), hypophosphatemia (11%), and colitis which resolved despite drug continuation (11%). Preliminary PK analyses revealed steady state serum levels of 14.1±4μM at dose level #1, 32.8±8.1 μM at dose level #2, and 31.6±7.8μM at dose level #3. Two patients with stage 4 neuroblastoma were high risk (ages of 4 y and 5 y at diagnosis, bone and marrow metastases, both with recurrent widespread disease, persistent despite salvage with multiagent chemotherapy and anti-GD2 immunotherapy). Both are now clinically well at 9+ and 11+ months, with improved and stable 123I-metaiodobenzylguanidine scans, respectively.
Conclusions: Perifosine is well tolerated in children with advanced solid tumors. Perifosine may have antitumor activity as a single agent in neuroblastoma

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POC3

Does the amount of bone marrow disease determinate the outcome of patients with stage 4 neuroblastoma?

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Background: The methods for detecting bone marrow disease in neuroblastoma patients have been considerably improved during the recent two decades. This study investigates the clinical relevance of the amount of bone marrow infiltration on the outcome of the patients.
Methods: Bone marrow samples of 192 stage 4 neuroblastoma patients (trial NB97, age 0.27 - 21.5 years) were investigated by conventional cytology and by GD2 immunocytology in one central lab according to international standards. The cytological/ immunocytological amount of bone marrow disease at diagnosis and during treatment was correlated with outcome. For categorization, the results with the highest percentage of infiltration were used for the calculations.
Results: Cytology and immunocytology results were congruent in 89.8 %. Discrepancies (+/ - or -/ +) were mainly observed in patients with marrow infiltration < 1 %.
At diagnosis 15.1 % of patients had 0 % bone marrow infiltration, 15.7 % < 1 %, 23.3 % between 1 and 10 %, 12.3 % between 10 and 30 % and 33.7 % between 30 and 100 % tumor cells in bone marrow. A higher infiltration grade was associated with gradually worse 5 year event free survival (EFS) and overall survival (OS) ($p < 0.001$).
Following 2-6 cycles of chemotherapy the incidence of patients without residual bone marrow disease increased to 82.4 %. 13.3 % of patients still had < 1 % bone marrow infiltration, and 4.2 % of children between 1 - < 10 %. None had ≥ 10 % residual neuroblastoma cells. The cellular bone marrow response to chemotherapy after 2, 4, 6 cycles had no impact on event free and overall survival. Similarly, neither mIBG response at metastastic sites nor tumor marker response were prognostically informative. Limitation of analyses to patients > 12 and > 18 months of ages at diagnosis yielded the same results.
Conclusion: This study suggests that the outcome of patients with stage 4 neuroblastoma is influenced by the amount of bone marrow disease at diagnosis, but not by the bone marrow response to chemotherapy. It challenges the clinical use of MRD investigations.

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POC4

Outcome of metastatic neuroblastoma treated with multi-modality approach including murine antiganglioside-2 monoclonal antibody (3F8)

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Background: The prognosis of metastatic neuroblastoma remains to be poor despite surgery, intensive chemotherapy and radiation therapy. Immunotherapy with monoclonal antibody appears to provide an additional advantage to the current multi-modality treatment strategy. We previously reported our preliminary results with this approach and would like to present our update information with longer follow-up.
Methods: This is a single arm prospective study from Jan 1996 to Dec 2007 and the results were compared to our historical control from 1990 when a common data registry was started. Uniform treatment protocol was adopted by 5 public hospitals since 1996. Our treatment was based on a modified N6 & N7 protocol adopted from MSKCC which included intensive chemotherapy + autologous BMT +/- immunotherapy with murine monoclonal anti-ganglioside-2 antibody (3F8, anti-GD2 provided by MSKCC after 1999) for patients with stage 4 neuroblastoma. The analysis of outcome was by Kaplan-Meier analysis.
Results: 61 children (all >12 months) with metastatic neuroblastoma were diagnosed and treated in our centers. The 5yrs OS were 12.5+/-6.1% and 49.2+/-10.1% after treated with chemotherapy +/- auto-BMT (n=34) and chemotherapy + auto-BMT + 3F8 (n=27) respectively (p=0.016). The 5 yrs EFS were 10+/-12% versus 43.5+/-14% of the 2 groups respectively (p=0.014). Their median survival were 1.8yrs vs. 3.5yrs respectively (p=0.015). The most frequent encountered side effect of 3F8 was transient but severe pain and allergic reaction. The minimal follow-up period was 2.3yrs.
Conclusion: Patients with stage 4 neuroblastoma treated with 3F8 antibody containing regimen appeared to have a higher and longer survival as compared to the historical control in using similar regimen without 3F8. Side effects including pain and allergic reactions were manageable in our cohort.

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POC5

Plasma fractionated total metanephrines for biochemical diagnosis of neuroblastoma

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Background: Urine catecholamines and their metabolites are used for diagnosis and follow-up of patients with neuroblastoma. O-methylated metabolites of catecholamines (plasma free or total metanephrines) are the most sensitive tests for diagnosis of pheochromocytoma. We postulate that neuroblastoma transforms catecholamines into metanephrines which may be more sensitive and specific than urine catecholamines for diagnosis and follow-up of the disease. The aim of the study was to establish reference values for plasma fractionated metanephrines in children and provide preliminary results on their diagnostic values.
Methods: We included into the study 191 healthy children aged 0-17 years needing a venous puncture. Two ml of heparinized blood were drawn to measure plasma total metanephrines. Additionally, we included ten patients with neuroblastoma to evaluate the diagnostic utility of the test.
Results: Upper reference limit of total plasma normetanephrine (NMN), metanephrine (MN) and methoxytyramine (MT) were: 9.67 nmol/l (95% CI: 8.32-10.1) for NMN, 4.95 nmol/l (95% IC: 4.19-6.05) for MN, 5.8 nmol/l (95% IC: 4.85-7.37) for MT. There was no significant difference between boys and girls. A linear trend gradually decreasing from birth to 17 years was observed for NMN and MT which was statistically significant for both. Plasma concentration of MN showed a non-linear relationship to age. In patients with neuroblastoma, plasma total MT and NMN measurements were highly sensitive and specific at diagnosis. The highest sensitivity and specificity (100% and 99.48%, respectively) were achieved for plasma total NMN with a cutoff at 19.64 nmol/l (Odds Ratio 1.18, CI 1.06-1.31) and for plasma total MT (sensitivity 100% and specificity 98.95%) with a cutoff at 9.43 nmol/l (Odds Ratio 1.8, CI 1.32-2.46).
Conclusion: These preliminary results provide pediatric reference limits for total plasma metanephrines and suggest a promising role in diagnosis and follow-up of neuroblastoma patients. Further data are necessary to confirm its validity.

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POC6

Apoptotic and adjuvant effects of triterpene-containing Viscum album L. extracts in neuroblastoma

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Viscum album L. (mistletoe) is one of the most widely used complementary cancer therapies. Differential effects of defined lectin and triterpene containing mistletoe extracts on neuroblastoma (NB) are largely unknown.
In the present study, we determined for the first time the effect of clearly defined mistletoe extracts, containing either lectins (aqueous extract) or triterpenes (STE) such as oleanolic - and betulinic acid and combinations thereof (viscum TT) against NB in vitro and in vivo.
For this purpose, we used the well established syngeneic NXS2 mouse model and tested efficacy and mechanisms of the treatment with these preparations in vitro and in vivo. NXS2 neuroblastoma cells were incubated with increasing concentrations of mistletoe preparations and tested for their cytotoxicity in vitro. Apoptosis was determined using mitochondrial potential, DNA fragmentation and Annexin/PI assays.
In vivo efficacy was determined in the spontaneous NXS2 metastases model. For this purpose, 1x106 murine NXS2 cells were injected s.c. into groups of syngeneic A/J mice (n=6) and STE extracts were administered three times per week for 14 days by intraperitoneal injection. After removal of primary tumors and continuing treatment for two weeks, the level of spontaneous liver and lymph node metastasis was analyzed by measuring the weights of affected organs. We could demonstrate that Viscum album L. extracts inhibited cell proliferation and show cytotoxic properties in vitro. The highest level of apoptosis with a decrease of the mitochondrial potential was observed with STE preparation at a concentration of 60µg/ml (IC50), whereas we detect only a moderate effect in lectin-treated cells (IC50).
Based on these data, we investigated the effect of STE extract on the level of spontaneous NB metastases in vivo. For this purpose 60mg/kg oleanolic acid were administered. In line with in vitro results, mice treated with triterpenes showed a significant decrease of spontaneous metastases in contrast to control groups. In conclusion, we believe that triterpene containing Viscum album L. extracts may provide a promising approach for the adjuvant treatment of neuroblastoma.

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POC7

Phase I study of vincristine, irinotecan, and ^{131}I-MIBG for patients with relapsed or refractory neuroblastoma: A new approaches to neuroblastoma therapy consortium study

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Background: ^{131}I-metaiodobenzylguanidine (MIBG), vincristine, and irinotecan each have activity in neuroblastoma. Irinotecan is a known radiosensitizer. This phase I study aimed to determine the maximum tolerated dose of MIBG in combination with fixed doses of vincristine and irinotecan.
Method/approach: Patients 1-30 years old with relapsed or refractory neuroblastoma and MIBG-avid tumors were eligible. All patients had hematopoietic stem cells (PBSCs) available and met standard phase I requirements. Prior vincristine and irinotecan, but not prior MIBG, were allowed. Patients received cefixime (8 mg/kg/day) on days -5 to +21 to abrogate diarrhea. Irinotecan (20 mg/m2/dose IV) was given on days 0-4 and 7-11, with vincristine (1.5 mg/m2) on days 0 and 7. MIBG was given on day 1 following a 3+3 phase I dose escalation design with dose levels of 8 mCi/kg, 12 mCi/kg, 15 mCi/kg, and 18 mCi/kg. PBSCs were administered at dose level 8 mCi/kg for myelosuppression and for all patients at > 12 mCi/kg.
Results: 18 evaluable patients (median age 6.7 years; range 3.4-19.7 years; 14 boys and 4 girls) enrolled and received 25 cycles. Myelosuppression and diarrhea were the most common toxicities observed. 72% of patients experienced diarrhea, with grade 3 diarrhea in 4 cycles. No dose limiting toxicities (DLTs) were observed at dose levels 8 and 12 mCi/kg. At 15 mCi/kg, 1 patient had DLT of grade 3 diarrhea. At 18 mCi/kg, 1 patient had DLT with hallucinations and hyponatremia. One patient had protocol-defined DLT with prolonged mild ALT elevation that was not deemed clinically significant. No patients treated with 18 mCi/kg MIBG had > grade 3 diarrhea. 5 patients received 2-3 courses to cumulative MIBG doses of 24-36 mCi/kg without DLT. The objective response rate based on institutional report was 28% (95% confidence interval 12-53%), with 2 complete responses and 3 partial responses (central review pending). An additional 6 patients will be treated at the defined maximum tolerated dose of 18 mCi/kg.
Conclusion: MIBG is tolerable and active at doses of 18 mCi/kg with vincristine and irinotecan. This combination may provide a treatment option for patients with advanced disease.

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POC8

Five-day courses of irinotecan in chinese patients with refractory neuroblastoma

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Purpose: To evaluate the efficacy, safety and effect on quality of life of irinotecan in refractory neuroblastoma.
Patients and Methods: 7 patients aged between 3 years and 22 years with refractory neuroblastoma, received irinotecan at 40-50 mg/m2/d administrated as a 60 min infusion for 5 consecutive days, every 3 weeks. Tumor response was evaluated every by conventional radiological scans. Hematological and non-hematological toxicities were evaluated according to National Cancer Institute Common Toxicity Criteria. Karnofsky or Lansky performance status and face rating pain scale were used to evaluate quality of life preliminarily after first course of treatment.
Results: 21 cycles were administrated, with a median of 3 cycles per patient (range, 1-5 cycles). Stable disease were observed in 3 of 7 patients (42.9%). Median time to progression was 2 months. Most common grade 3-4 toxicities were myosuppressive hematological toxicities, neutropenia, anemia, thrombocytopenia were observed in 2 of 7 patients (28.6%), 2 of 7 patients (28.6%), 1 of 7 patients (14.3%) respectively. Grade1-2 nausea, vomiting, abdominal pain or cramping, diarrhea were the common non-hematological drug-related toxicities observed. Quality of life was improved in almost all patients after one course of treatment.
Conclusion: Irinotecan as a single agent administrated 40-50 mg/m2/d intravenously 5 consecutive days, every 3 weeks was well tolerated and was a very safe regimen in Chinese patients. Although this regimen induced no objective response in refractory neuroblastoma, the clinical benefit rate was 42.9% .This regimen could alleviate pain and improve quality of life of Chinese neuroblastoma patients.

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POC9

Gefitinib (GFB) and Irinotecan (IRN) for children with high-risk (HR) neuroblastoma

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GFB, an oral EGF and ABC transport receptor inhibitor has been shown to be a potent inhibitor of NB cell proliferation in vitro (Brodeur et al Ca Res, 2005) and in combination with IRN have greater than additive activity against NB xenografts (Stewart et al, Ca Res, 2004). For these reasons we evaluated the combination of GFB and IRN in newly diagnosed children with HR NB.
Aims: The primary objective was to estimate the response rate to two courses of intravenous IRN [(dailyx5)x2] at a dose of 15mg/m2/day combined with 112.5mg/m2/day of oral GFB for 10 days in untreated children with HR NB.
Patients and Methods: 23 children were enrolled and 20 were evaluable for the primary endpoint. Pharmacokinetic studies for IRN and metabolites were done in consenting patients.
Results: The median age at study enrollment was 3.15 years (range, 18 days - 12.67 years). Most were > 24 months (n=20; 87%), male (n=18; 78%), white (n=16; 70%), had INSS 4 disease (n=19; 83%), had adrenal primaries (n=18; 78%) and 9/23 had amplified MYCN tumors. Three patients did not receive the IRN/GFB therapy: one had the primary tumor resected prior to enrollment and thus had no primary disease evaluable, one had spinal cord compression at study enrollment and one was too young (age 18 days old at study enrollment). Toxicity associated with IRN/GFB was evaluable in all 20 patients who received this therapy. Common non-dose limiting toxicities ascribed to the combination included nausea (5/20), diarrhea (8/20), vomiting (7/20) and hypokalemia (3/20). Five had partial responses and 9 others had between 11-60% decrease in primary tumor volume and/or improved MIBG, improved tumor status in the bone marrow, decreased pain and improvement in performance activities. Median (range) IRN and SN-38 AUC values were 282 ng/ml*hr (162 to 889 ng/ml*hr) and 28 ng/ml*hr (3.6 to 297 ng/ml*hr), respectively. Expression of EGFR, MRP2-4, BCRP, and Pgp, in tumor samples did not correlate with antitumor activity.
Conclusions: Although the combination of IRN/GFB was very tolerable in these children and there were clinical responses, sufficient additive activity was not observed to warrant further investigation .

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POC10

Does tumor histology after induction therapy predict outcome in patients with high-risk neuroblastoma?

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Background: Histopathology at diagnosis predicts outcome in patients with neuroblastoma (NB). The aim of our study was to determine whether histopathological response to induction chemotherapy is useful in predicting outcome in patients with high-risk NB.
Method: Newly diagnosed high-risk NB patients treated at our institution between 1994 and 2002 for whom tumor material was available were studied. Patients were treated with 5 cycles of multiagent induction chemotherapy, primary tumor resection after the 4th/5th cycle, followed by consolidation therapy and stem cell rescue. Tumor histology sections at diagnosis and resection were retrospectively reviewed (APA, REG) and scored according to specific morphological features. Features prognostic of overall survival (OS) and disease free survival (DFS) were identified using a log rank test.
Results: Specimens from 43 patients were analyzed. Sixteen patients had available specimens at both diagnosis (D) and resection (R) which were characterized as follows: stroma-poor tumors: 94% (D), 75% (R); intermediate/high MKI: 56% (D), 6% (R); minimal neuropil: 69% (D), 19% at (R); differentiating tumors: 6% (D), 81% (R); >10% tumor necrosis: 37% (D), 56% (R). Histological features in 32 resection specimens were: stroma poor: 23/32 (72%); high/intermediate MKI: 2/32 (6%); differentiating tumors 21/32 (66%); minimal neuropil 10/32 (31%); >10% necrosis: 23/32 (72%) and 14/32 (44%) with ganglioneuromatous elements. At resection, intermediate/high MKI and ≥90% viable tumor (compared to <10%) were significantly predictive of poor OS and DFS (both $p<0.05$). At resection, there was a trend towards a lower survival for patients with <10% tumor necrosis (OS $p=0.09$, DFS $p=0.08$).
Conclusion: High proliferative tumor activity following induction therapy portends a poor outcome in patients with high-risk NB. If confirmed in a larger cohort, these patients may benefit from further stratification to more intensive therapy.

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POC11

Aromatic hydrocarbon receptor down-regulates MYCN expression and promotes neuronal differentiation of neuroblastoma

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Background/Aims: MYCN amplification is an adverse prognostic factor of neuroblastoma (NB). However, how MYCN expression is regulated in NB cells remains unclear. This study aims at defining the machinery to regulate MYCN expression in NB cells.
Methods: Ten MYCN amplified and 10 MYCN non-amplified NB tumors were subjected to oligonuleotide microarray analysis. Signaling pathways related to MYCN expression were analyzed by IPA. The relationship between genes with the highest score in the pathway analysis and MYCN expression were further evaluated and verified in 85 NB tumor samples by quantitative PCR and immunohistochemistry as well as in NB cell lines by functional studies.
Results: IPA analysis revealed that aromatic hydrocarbon receptor (AHR) had the highest score to be reversely related to MYCN expression. AHR expression in 85 NB tumors correlated well with histological grade of differentiation but reversely correlated with advanced disease stages and MYCN amplification. Positive AHR immunostaining predicted a favorable prognosis in NB patients independent of other prognostic factors. Ectopic expression of AHR in SK-N-DZ cells promoted neuronal differentiation by directly inhibition of MYCN promoter activity with the cooperation of E2F1 transcription factor.
Conclusion: AHR may negatively regulate MYCN expression and promotes NB cell differentiation in vivo and in vitro. Further study of the role of AHR expression in NB may not only shed light to the tumorigenesis but also the novel targeted therapy of NB.

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POC12

Results of treatment strategy of stage 4 infantile neuroblastoma based on born metastasis and Mycn amplification

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Background: Subgroups of infants in stage 4 neuroblastoma showed heterogeneity. The purpose is to investigate the validity of treatment of the infants in stage 4 neuroblastoma based on born metastasis and MYCN amplification (MNA).
Patients and Methods: Fifty seven stage 4 infants aged <1 year were treated in the Japanese prospective Study from 1994 to 2004. Patients with MNA, classified into a high risk group, were taken most intensive treatment. Patients without MNA were assigned chemotherapy by the presence of the bone metastasis.
Results: The number of stage 4 neuroblastoma patients with MNA is 12. Over all survival rate was poor in cases with MNA (5-year-OS 40.0%) than in cases without MNA (5-year-OS 95.0%, $p<0.001$). In cases without MNA, the number of patients with bone metastases is 23. In cases without MNA, over all survival rate was better in cases without bone metastases (5-year-OS 100%) than in cases with bone metastases (5-year-OS 85.1%, $p<0.05$). On the other hand, the number of patients with and without bone marrow metastases is 19 and 22. The over all survival rates between the patients with and without bone marrow metastases were almost equal (5-year-OS 93.8%, and 95.7% respectively, $p=0.94$).
Conclusion: The infants without MNA and bone metastases have excellent prognosis. Therapy reduction for these patients is possible. For patients with MNA or bone metastases, new therapeutic approaches are needed.

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POC13

Is there an Ultra-High-Risk neuroblastoma group? Report from the Children's Oncology Group (COG) Clinical and Biological Risk Factors (CBRF) Task Force

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Background: Current prognostic factors do not predict treatment failure for high-risk (HR) NB patients. The goal of the COG CBRF Task Force was to identify factors at diagnosis or during therapy (response-based) that define an Ultra-High-Risk (UHR) group for whom upfront novel therapies may be justified.
Method/approach: We defined UHR as including >10% of HR patients with an EFS<15%. We performed univariate analyses to identify an UHR group, using COG A3973 HR study population (N=487) to control for therapy received. Candidate factors analyzed were (1) at diagnosis: age and stage, MYCN status, specific genomic alterations (whole-genome SNP), RT-PCR based expression profile, and MIBG score; and (2) response-based: bone marrow (BM) clearance by histology, PBSC MRD (post-cycle 2; detected by 5 NB-specific Q-PCR transcripts), and quantitative MIBG scores.
Results: MIBG score >5 at end-induction predicted an UHR group (13% of A3973 patients with a 3-yr EFS=8.3+4.6%; Yanik,ANR 2010). A 14-gene signature applied to non-amplified NBs predicted an UHR group of >40% of patients enrolled on CCG, GPOH and COG trials with PFS of 14±4%, 14±8% and 22±8%, respectively (Asgharzadeh,ANR 2010). Detection of MRD in PBSC was also predictive of poor outcome, but did not define an EFS<20% (Seeger, ANR 2010). As single variables neither age, stage, initial MIBG score, MYCN status or BM clearance defined an UHR group. Genome alterations (11q or 3p loss and 17q or 9q gain) defined through the TARGET initiative (Attiyeh,ANR 2010) did not define an UHR group; nor did MYCN amplified NBs with whole chromosome gains/losses. Additional analyses will determine whether segmental CNAs, ALK status (Mosse,ANR 2010) or MRD in PB or BM can expand the UHR group. Rational combinations of such factors will also be assessed.
Conclusion: In addition to progressive disease, MIBG score at end-induction and a multi-gene tumor signature at diagnosis predict an UHR group. We plan to identify UHR patients prospectively within COG HR trials using MIBG response and perhaps other biomarkers and test novel therapies in this group with predicted dismal outcome.

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POC14

False negative studies of neuroblastoma metastatic to the central nervous system (CNS)

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Purpose: CNS NB was once considered rare and lethal. Although the outlook for these patients (pts) has improved with a salvage regimen incorporating intrathecal targeted radioimmunotherapy (RIT) (J Neuro Oncol 2009), early detection may reduce morbidity. We present an analysis of the largest series of pts with CNS NB.
Patients and Methods: As a referral center for RIT, we assessed the clinical, biologic and radiographic features in 61 pts evaluated for CNS NB at MSKCC since 2000. 34 pts had MYCN amplified disease; 9 had a lumbar puncture at initial diagnosis, known risk factors.
Results: 59 of 61 pts had confirmed CNS NB, 47 by pathology and 12 radiographically (multiple enhancing parenchymal lesions and/or nodular leptomeningeal spread.) 2 pts had neurosurgery for isolated enhancing lesions on routine MR found to be gliosis and necrosis, but not NB. 2 pts had CNS disease at initial NB presentation, both with headaches and high intracranial pressure requiring shunts. The other 57 pts had CNS NB at 5-61 months (median 21.7) from diagnosis, median 18.5 months in the MYCN amplified cohort. 23 pts (41%) were asymptomatic, with CNS NB detected on routine MIBG (4), CT head (8), MR brain (8) or MR spine (3). CNS NB was undetectable in 31 of 37 (84%) CSF analyses. Presenting symptoms in 36 pts included headache +/- vomiting in 15 (25%), seizures in 6 (10%), gait disturbance in 6 (10%), altered mental status in 5 (8.5%), visual disturbance in 2 (3%), facial palsy in 1 (<2%) and depression in 1 (<2%). 40 pts (68%) had isolated CNS relapse including 26 (44%) with a single parenchymal focus. Leptomeningeal spread occurred in 19 (32%), the remainder having multifocal disease.
Conclusions: As the natural history of CNS NB has changed, so too has the clinical evaluation and management. Isolated CNS relapse remains a complication for pts with high-risk NB. Given the false negative rates of routine studies, periodic CNS MR imaging in high-risk patients is indicated. Routine screening detects >40% of asymptomatic lesions. Biopsy to confirm disease is mandatory for isolated non-MIBG avid lesions.

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POC15

Evolution of treatment strategies and risk stratification in management of neuroblastoma over two decades at tertiary cancer centre in India

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Background: With increasing amenities of risk stratification and treatment optimisation of NB over three distinct era,we present our observations and lessons learnt in the evolution of treatment strategies over two decades at Tata Memorial Hospital(TMH),Mumbai,India.
Methods: Retrospective analysis of patients with NB presenting at TMH between 1987-2008 was performed and has been reported.
Results: Three distinct eras (1987-2000,2000-2004 & 2004-2008) have been identified based on treatment and risk stratification availability.
In 128 children of neuroblastoma in first era(1987-2000); only age(<1/= yr v/s >1yr) and stage were considered for risk stratification.Access to optimal surgery for gross total ressection (GTR) ,ABMT & 13 Cis retinoic Acid (13 Cis RA) was limited.I131 MIBG imaging was introduced in diagnostic armamenterium in second half.DFS for 1 yr is 19%.In second era (2000-2004) 51 children were evaluable.Besides age(18months) & stage,histopathology was incorporated in appropriate cases for risk stratification.Access to optimal surgery (GTR),ABMT & 13 Cis RA was still limited. However induction chemotherapy was more dose intense and MIBG therapy was introduced .DFS for 18 months is 20%.In third era (2004-2008) 62 children were evaluable. Optimal surgery (GTR) & 13 Cis RA were routinely employed in treatment strategy ; however ABMT was done occasionally due to cost constrains.MIBG therapy was more organised.N-myc first by PCR technique followed by FISH has been introduced in the risk stratification.OS for this group is 65% with 18 months median follow up.
Conclusion: As risk stratification improves treatment gets optimised. Improved survival can be obtained in low and intermediate risk group accounting for approximately 60% of children with NB.Management of High risk NB remains a challenge in cost constrained environment.

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POC16

High-dose cyclophosphamide (Cy)-irinotecan (CPT-11)-vincristine (VCR) (HD-CCV) for primary refractory neuroblastoma

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Background: HD-CCV is a novel regimen for NB resistant to standard chemotherapy which now includes topotecan in either induction or salvage therapy.
Patients and methods: We retrospectively studied 37 unselected patients (pts) treated with HD-CCV for high-risk stage 4 NB that responded incompletely to induction, but had never progressed. Their prior therapy included topotecan and 32 pts (86%) had already received 2nd-line chemotherapy. Treatment was outpatient and comprised high-dose Cy (140 mg/kg)-CPT-11 (250 mg/m2)-VCR (0.067 mg/kg). Pts received a 2nd course if the 1st showed anti-NB activity but assessable disease remained. Response was scored by international criteria, including extensive bone marrow evaluations and MIBG scan. The overall goal was to achieve a minimal disease state and then use the anti-GD2 3F8 antibody and 13-cis-retinoic acid (13-cis-RA). Progression-free survival (PFS) was calculated from day 1 of HD-CCV.
Results: Myelosuppression was grade 4. The only common non-hematologic toxicity was self-limited or readily manageable diarrhea. Objective responses (4 CR, 3 PR, 4 MR) occurred in 11/27 (41%) pts treated <9 months from diagnosis, but in only one (MR) (10%) of 10 pts on therapy for >10 months. HD-CCV was the initial 2nd-line therapy after topotecan-containing induction in 5 pts, achieving 1 CR, 1 MR, 3 stable disease (NR). All 5 pts previously treated with CPT-11/temozolomide had responses (2 PR, 3 MR) to HD-CCV. In contrast, all 6 pts with incomplete responses to HD-CCV who were then treated with CPT-11/temozolomide had NR. 20 pts remain progression-free at 2+-to-40+ (median, 11+) months, including 9 in 1st CR/VGPR at 6+-to-32+ (median, 14+) months after HD-CCV and subsequent therapy. The latter depended on response to HD-CCV and included 3F8, 13-cis-RA, targeted radiotherapy using 131I-3F8, and/or chemotherapy. PFS was 59% (SE+10%) at 24 months and 25% (SE+19%) at 36 months.
Conclusion: HD-CCV offers a treatment option against topotecan-resistant NB. The results suggest that CPT-11 may synergize with high-dose alkylators.

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POC17

A proposal for antibody based immunotherapy combined with haploidentical stem cell transplantation for high risk neuroblastoma

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Pediatric patients with relapsed solid tumors have a poor prognosis and additional therapeutic strategies are warranted. We present preliminary results of our institution with haploidentical stem cell transplantation (SCT) in 25 pediatric patients (soft tissue sarcomas, n=5, Ewings sarcomas, n=5 and high risk neuroblastomas, n=10) who relapsed after previous autologous stem cell transplantation as well as a proposal for posttransplant immunotherapy in neuroblastoma patients. 10/25 patients reached complete or very good partial remission after various chemotherapies, 15/25 patients had partial remission or were not in remission prior to transplantation. The conditioning regimen comprised Flud, TT, Mel and OKT3. The CliniMACS® device was used for complete T and B cell depletion. Median time to reach >500 neutrophiles/µl was 11 days. Acute GvHD grade 0-1 occurred in 60%, 36% had GvHD grade II and 4% had grade III. Chronic GvHD occurred in 28%. No transplant related mortality (TRM) was observed. Over all survival after 2 years was 30%. Thus, transplantation itself seems to be not sufficient for most patients but may be a platform for further immunotherapies. In vitro stimulation with cytokines of donor derived patient NK cells posttranplant and/or use of chimeric antiGD2 antibodies resulted in excellent lysis of neuroblastoma cells. Thus, we propose a phase I/II study to evaluate the feasibility and safety of anti GD2 administration in combination with low dose interleukin 2 after haploidentical SCT. Study design: 6 cycles of mAb CH14.18/CHO (20mg/m2 infusion for 5 days; in cycles 4-6, 1x10E6 units /m2 IL2 will be given additionally on days 6, 8 and 10). Up to now, 4 patients received a total of 8 courses in a pilot phase. Side effects were fever and pain in 4/4 patients. No GvHD occurred. Conclusions: haploidentical SCT represents a therapeutic option for patients with relapsed high risk neuroblastoma and other solid tumors and may be a basis for further immunotherapeutic approaches. The proposed study will adresss the question, whether the use of the monoclonal antibody CH14.18/CHO in combination with interleukin 2 will be feasible after this transplant procedure.

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POC18

Illness experience and factors that constitute resilience in families with a neuroblastoma child

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Background: Neuroblastoma (NBL) is a cancer of the sympathetic nervous system. It manifests as a malignant tumor that originates in the adrenal glands, neck, chest, abdomen, or pelvis. Neuroblastoma often affects very young children, and survival rate in the highest-risk group is about 30%. Aim: The purpose of this qualitative study was to explore the illness experience and treatment process of families with a high-risk group NBL child.
Methods: A purposive sample of eight caregivers was selected for in-depth interviews and participant observation. Data were analyzed and integrated into seven themes using the Giorgi (1997) phenomenological analysis method.
Results: 1. The results of this study show that the experiences of families of children undergoing NBL treatment can be classified by 2 categories: sequence of treatment course and utility of resources. The former includes shock and impact experienced when the illness is first discovered, the uncertainty of the characterization of this disease, the experiences related to the treatment course and the learning process, and the responses after treatment courses. The later includes support derived from family cohesion, family resilience, and assistance from the health care team and social resources. 2. Ten resilience factors were derived from the results to support family function: courage, life style adjustment, learning, positive outlook, spirituality, comparison with other families, family communication, support, expectation adjustment and collaboration in family members. The results show that family resilience exists in every family, that is, every family has potential ability to ask for assistance and adjust itself to function it as well as it can.
Conclusion: As the concepts of integrated treatment and the health-care team are in the developing stages in Taiwan, the results of this study can aid health professionals to implement appropriate interventions as well as provide a work outline for social workers. In addition, these results may be referenced as a guide to family centered care and the development of family resilience to aid families cope with medical treatment processes.

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POC19

The impact of a multidisciplinary team approach in the case management of neuroblastoma

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Background: Protocol-based therapy of neuroblastoma had been adopted in our hospital, a 2000-bed tertiary medical center, for decades. In 2002, we incorporated a new protocol, TPOG-N2002, modified from the United States CCG-3891, with high-risk patients undergoing chemotherapy, radiotherapy, and autologous stem cell transplantation or continuing intensive chemotherapy per parental decision.
Method: To improve the quality of care in children with neuroblastoma, a multidisciplinary team consisting of specialists in pediatric oncology, pediatric surgery, radiation oncology, pediatric nursing, radiology, and nuclear medicine was organized in our institution in 2007. The demographic data, treatment adherence, surgical resectability, complications, and treatment outcome of patients before and after this approach were analyzed.
Results: Since 2002, total 54 patients (38 boys and 16 girls) with neuroblastoma have been treated at our institution, with 46 (85%) of them received the N2002 protocol as frontline therapy. Among them, 35 (65%) were diagnosed during 2002-2007 (group 1), and the other 19 (35%) were diagnosed during 2008-2009 (group 2). The mean age (3.52 *vs.* 3.01), sex, staging, and MYCN amplification rate at diagnosis between the two groups were not significantly different. In group 2, the adherence of the 19 children to recommended treatment courses was almost 100% after diagnosis at our institution; the gross total resection rate was not different from that in group 1 (44% *vs.* 52%, p=0.694); and there was a trend toward lower rate of nephrectomy (0% *vs.* 17%, p=0.181). The 2-year overall survival and progression-free survival approached 76.9% and 70.3% in group 2, in comparison to those of 68.6% and 45.4% in group 1 (p=0.5580 and 0.9161, respectively).
Conclusion: A case management program using a multidisciplinary team approach might have improved quality of care and provided a management model for children with neuroblastoma. The findings of outcome survey need further follow-up to confirm benefits.

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POC20

Second stem cell transplantation for relapsed high-risk neuroblastoma in Japan

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Background: We have previously presented that total body irradiation (TBI) based preparative regimen for autologous stem cell transplantation (SCT) of advanced neuroblastoma has significant superiority in event-free survival (EFS). There are few reports concerning about preparative regimen and transplantation source for second SCT after relapse of advanced neuroblastoma.
Patients/Method: 116 patients who achieved second SCT between 1995 and 2005 were analyzed retrospectively based on the transplantation registry of Japanese Society of Pediatric Hematology. We excluded 66 patients with planned tandem transplantation, 3 transplanted for graft failure and 5 without detailed information. Finally 42 patients with relapsed neuroblastoma were analyzed.
Results: EFS after second transplantation at 3 and 5 years were 16.7±6.0% and 5.64.7% respectively. Three (one with allogeneic SCT and two with autologous SCT) out of 42 patients survived without disease sfter second SCT for 1492, 555 and 449 days. 3-year-EFS of 11 patients with TBI containing regimen was 18.2±11.6%, and EFS of 25 without TBI was 19.7±7.6% (p=0.896). Allogeneic transplantaion for the second SCT was performed for 15 patients with 3-year-OS of 0.0±0.0%, which is significantly lower survival rate compared with 24.4±8.0% for autologous SCT (p=0.003). Fourteen out of 15 allogeneic transplantation died of disease relapse (n=12), respiratory failure (n=1;non TBI) and multi-organ failure (n=1; TBI). Twenty-four out of 27 autologous SCT died of relapse (n=21), respiratory failure (n=1; non TBI) and unknown cause (n=2; non TBI). We compared 42 secondary transplanted patients with one hundred one patients who did not perform second SCT after relapse (CHEMO). Five-year over-all survival (OS) was 8.8±4.7% for SCT and 16.0±4.5% for CHEMO, and 8 year-OS was 0.0±0.0% and 12.0±4.1% respectively (p=0.606).
Conclusion: TTBI based preparative regimen does not have significant superiority in EFS for second SCT of relapsed neuroblastoma. For second SCT, allogeneic SCT does not seem to have better survival than autologous SCT, so it might be possible that Graft-versus-Tumor effect would not work properly after second SCT for relapsed neuroblastoma

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POC21

Role of minimal access surgery in children affected by neuroblastoma

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Background: Minimal Access Surgery (MAS) is one of the cornerstones in the fast-track organization model and is nowadays applied to almost all pediatric surgical fields. This study presents possible MAS approaches in a series of pediatric patients affected by neuroblastoma.
Method/approach: This 2-year (2008-2009) prospective on-going study includes all patients suspected to be affected by neuroblastoma who were diagnosed and/or treated through a MAS approach. Attention has been specifically focused on surgical risk factors (SRFs) and complications.
Results: A total of 44 MAS procedures were performed in children affected by cancer during the study period, 16 of whom presenting with neuroblastoma. Biopsies were performed in 7 out of 16 patients, in 4 patients a tru-cut biopsy being the procedure adopted, whereas an incisional biopsy was performed in the remaining 3 patients. Nine out of 16 procedures consisted in a complete resection of the mass associated with lymph-nodes sampling whenever indicated. Intraoperatively, 2 episodes of bleeding occurred and were successfully managed without conversion. One case required a conversion to traditional open surgical technique to achieve complete excision of the mass. On discharge, after a median hospital stay of 3 days (range 1-7 days), all patients were judged eligible to proceed to further treatments.
Conclusion: As far as oncological criteria are respected in terms of SRFs and risk of tumor spreading, MAS can represent the mean to obtain an accurate diagnosis, staging, complete resection, palliation or management of oncological complications in children affected by cancer.

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POC22

Role of nursing in the implementation of chimeric anti-GD2 antibody with immunotherapy (ANBL0032) into clinical practice

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Background/Aims: Interim analysis of the Children's Oncology Group (COG) (ANBL0032) phase III randomized trial of the chimeric anti-GD2 antibody ch14.18 with GM-CSF and IL2 for high-risk neuroblastoma demonstrated superiority of the experimental immunotherapy arm. After ANBL0032 was reopened as a single arm clinical trial, enrolment at our institution was significantly increased. As we had not used this study in more than two years, a comprehensive implementation plan was developed to facilitate the reintroduction of this complex and nursing care intensive therapy. The planning process required the collaboration of Nurse Practitioners (NP), Nurse Educators, Nurse Managers and the inpatient nursing staff as well as the development of standardized order sets, educational materials and patient management guidelines.
Methods: The need for extensive education was identified as a high priority. Consequently, a detailed knowledge transfer plan was developed and implemented using required COG education modules as well as additional tools developed specifically for this protocol. The required observations and the delivery of care on this protocol necessitated precise timing of admissions and planning of resources necessary to deliver care as well provide accurate documentation of therapy. Clinic visits and detailed coordination of required testing and clinical assessments was organized by the outpatient Neuroblastoma NP.
Results: Since ANBL0032 was reopened we have had three children complete all cycles of the protocol. We have generated standardized orders and educational materials. Fifty-nine nurses have completed the education component of the knowledge transfer plan. The NP continues to serve as a resource regarding this protocol.
Conclusion: The implementation of this plan has resulted in meeting the ongoing learning needs of the nursing staff. Patient care has been enhanced by the coordination of inpatient stays and outpatient evaluations.Our education program has been adapted and modified for other caregivers in our institution. An analysis is underway to assess the success of our educational knowledge transfer plan and determine the frequency for training updates.

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POC23

Phase I trial of Lestaurtinib for children with refractory neuroblastoma: A new approaches to neuroblastoma therapy (NANT) study

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Background: TrkB acts as an oncogenic kinase in a subset of human NBs. Lestaurtinib, a multi-kinase inhibitor with potent activity against Trk kinases, has demonstrated anti-tumor activity in preclinical models of human NB.
Method/approach: We performed a Phase I trial of Lestaurtinib in subjects with recurrent or refractory high-risk neuroblastoma starting at 80% of the adult recommended Phase 2 dose. Subjects received lestaurtinib twice daily for 5 days out of 7 in 28-day cycles. Lestaurtinib dose was escalated using a 3+3 design. Pharmacokinetics and plasma phospho-TrkB inhibitory activity were evaluated in the first cycle. Response data were obtained after the first and then every other cycle.
Results: Forty-seven subjects were enrolled, and 10 dose levels were explored starting at 25 mg/m^2/dose BID. All subjects were evaluable for response, and 42 subjects were evaluable for dose escalation. Asymptomatic and reversible grade 3-4 transaminase elevation was dose limiting in 4 subjects. Reversible pancreatitis (grade 2) was observed in 3 subjects after prolonged treatment at higher dose levels. Other toxicities were mild and reversible. Pharmacokinetic analyses revealed rapid drug absorption, however inter-patient variability was large. Plasma inhibition of phospho-TrkB activity was observed 1 hour post-dosing at 85 mg/m^2 with uniform inhibition at 120 mg/m^2. There were two objective responses and 9 subjects had prolonged stable control of disease at dose levels ≥5, (median: 12 cycles) before disease progression. We established a biologically effective and recommended phase II dose of 120 mg/m^2/dose BID.
Conclusion: Lestaurtinib was well tolerated in subjects with refractory NB, and a dose level sufficient to inhibit TrkB activity was established. Safety and signs of activity at the higher dose levels warrants further evaluation of this drug or other Trk-directed therapies in NB. The tolerability of this drug without hematologic toxicity makes it suitable for combination therapy with conventional cytotoxic agents.

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POC24

Arsenic trioxide as radiosensitizer for 131I-MIBG therapy: Results of a pilot phase II study

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Background: Arsenic trioxide (ATO) has *in vitro* and *in vivo* radiosensitizing effects. Given its non-overlapping toxicity profile with the known anti-NB radiotherapeutic agent 131I-MIBG, we hypothesized that ATO would enhance the efficacy of the latter and tested the combination in a pilot study.
Method/approach: Patients with heavily pretreated recurrent or refractory stage 4 NB were treated on an IRB-approved pilot phase II study (NCT00107289) Planned treatment was 131I-MIBG 12 or 18mCi/kg intravenously (IV) on day 1 plus ATO 0.15 or 0.25mg/m2 IV days 6-10 and 11-15. Toxicities were measured using NCI CTCAE version 2.0 and responses were assessed using International NB response criteria (INRC).
Results: Nineteen patients were enrolled: 14 received 131I-MIBG and ATO at maximal dosages, 2 received 12mCi/kg 131I-MIBG plus 0.15mg/kg/dose AT; 1 received 18mCi/kg 131I-MIBG plus 0.15mg/kg/dose ATO; 1 (at 131I-MIBG dose of 12mCi/kg) did not receive ATO due to transient central line-induced cardiac arrhythmia, while another (at 131I-MIBG dose of 18mCi/kg) received only 6/10 doses of AT due to significant diarrhea. All patients experienced grade 4 myelosuppression, though none required autologous stem cell rescue. Other >grade 2 adverse events were transient and included: hyperamylasemia from transient sialoadenitis (12/13 evaluable patients), hypokalemia (3), hyperbilirubinemia and hepatic transaminitis (1), and hyponatremia (1). By INRC, 14 patients had no response while 5 had progressive disease (PD) including 5/6 patients who entered the study with prior PD. However, objective improvements in one or more NB markers were observed in 12 patients. 18/19 patients were continued on further chemotherapy and/or immunotherapy. Three-year progression free survival (PFS) was 37±11% with a median PFS of 9.5 months.
Conclusion: The combination of 131I-MIBG plus ATO was well tolerated with adverse event profile similar to that of 131I-MIBG therapy alone. Objective responses were observed in most patients. However, the addition of ATO to 131I-MIBG did not significantly improve response rates when compared to historical data with single agent 131I-MIBG therapy.

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POC25

Comparison of I-123 and I-131 mIBG scans in predicting survival in patients with stage 4 neuroblastoma

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Background: Historically, I-123 mIBG scans are preferred to I-131 for neuroblastoma (NB) imaging as they deliver less patient radiation yet have greater sensitivity in disease detection. Both I-123 and I-131 mIBG scans were used for disease assessments of NB patients (pts) treated on Children's Oncology Group (COG) front-line high-risk study A3973. We tested for differences in survival prediction by Curie score for I-123 vs. I-131. The hypothesis was that I-123 and I-131 mIBG scans were sufficiently similar for clinical purposes in terms of sensitivity, specificity, and ability to predict survival.
Method/approach: Pts enrolled on COG A3973 with INSS stage 4 disease who completed I-123 or I-131 mIBG scans at any of the following time points - diagnosis, post-induction, post-transplant, or post-biotherapy - were analyzed. The performance of the Curie score for each mIBG scan type in predicting an event was evaluated using receiver operating characteristic (ROC) curve and area under the curve (AUC) analyses. At each time point, AUCs and survival curves for I-123 vs. I-131 were compared, using the t-test and log-rank test, respectively.
Results: Of the 413 pts on A3973 with at least one mIBG scan, 350 were stage 4. Median age was 3.1 years (range 6.8 months–29 years) at diagnosis, and 5-year event-free survival (EFS) and overall survival were 33.4 ± 3.6% and 45.6 ± 4.0% (n= 350). No statistically significant differences in EFS were found with respect to age, *MYCN* amplification, ploidy, and histology. There were 243/350 (69%; 5-year EFS: 34.1 ± 4.6%) pts with exclusively I-123 scans and 79 (23%; 5-year EFS: 39.9 ± 7.1%) with exclusively I-131 scans throughout treatment (P= 0.4945). At post-induction, ROC (P= 0.7740) and EFS (P= 0.3501) comparisons for I-123 vs. I-131 were not statistically significantly different. Similarly, comparisons at the 3 other time points were non-significant.
Conclusion: Curie score predicted the occurrence of an event equally well for each type of scan. For future survival analyses of mIBG Curie scores, I-123 and I-131 results may be combined and analyzed overall, without adjustment for scan type.

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POC26

Transverse myelopathy in neuroblastoma patients. Retrospective comparison of initial chemotherapy (CT) and neurosurgery (NS)

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Background: Prospective trials on neuroblastoma associated myelopathy are lacking. Therefore, we have retrospectively analyzed patients registered by the German neuroblastoma trials.
Method/approach: Neuroblastoma patients diagnosed between 1990 and 2007 were included. Clinical data were available in the trial data base. Additional details on neurology (initial symptoms, treatment, and outcome) were collected using a questionnaire.
Results: Among 2617 neuroblastoma patients, 280 had intraspinal tumor, 123 had symptoms of myelopathy, and 97 patients with complete follow-up were finally included in the analysis. These 97 patients were younger, had more localized tumors, and a lower rate of MYCN amplification than patients without myelopathy. They first presented with motor impairment (95%), sensible impairment (59%), neuropathic pain (55%), bladder dysfunction (44%), and/or constipation (34%). The median time between first symptoms of myelopathy and diagnosis was 12 days. A total of 51 patients underwent initial NS, 46 patients immediately started CT, none had initial radiotherapy. A total of 59 patients received additional corticoids. Symptoms improved after NS in 35/51 patients and after the first CT cycle in 30/46 patients (p=.882). Additional corticoid treatment was associated with better symptom relief (p=.026). After complete risk adapted neuroblastoma treatment, symptoms were improved in 41/51 patients who had initial NS and in 34/46 patients with initial CT (p=.749). After a median time of 7.8 years, 70/97 patients have residual impairments: Growth delay (CT 13% vs NC 16%), scoliosis (28% vs 35%), motor impairment (41% vs. 42%), sensible neuropathy (13% vs. 22%), neuropathic pain (4% vs 6%), impaired bladder function (26% vs 28%), and constipation (28% vs. 12%). The mode of initial treatment or application of additional corticoids had no impact on the frequency of late effects although a selection bias for treatment cannot be excluded.
Conclusion: The retrospective data show no advantage of either initial CT or NS. Concomitant corticoids are of short term benefit but have no impact on long term outcome. Many patients have residual symptoms of myelopathy.

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POC27

Decision of treatment reduction in selected children aged less than 18 months with a neuroblastoma without MYCN amplification and a numerical genomic profile

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Background: In neuroblastome (NB), children aged less than 18 months have a good outcome in the absence of MYCN amplification. It has recently been shown that a genomic profile characterized by numerical chromosome alterations (NCA), without any segmental alterations detected by array-CGH, is associated with an excellent overall survival. For some of these patients treatment reduction might be possible. The aim of this study was to analyse if the knowledge of a favourable genomic profile, characterised by NCA only, influenced the treatment decision for individual children < 18 months.
Methods: This is a retrospective study of children aged less than 18 months with a localised unresectable or metastatic NB, with a genomic tumor profile characterised by NCA only as determined by array-CGH, and treated at Institut Curie between 08/2004 and 07/2008.
Results: Among 17 children, 4 had INSS stage 2, 10 stage 3, 2 stage 4 and 1 stage 4s disease. Twelve of these children had clinical symptoms at diagnosis, including 4 children with spinal cord compression and 2 with respiratory distress. After treatment for life-threatening symptons when required, in 14/17 children, chemotherapy was reduced with regard to previous treatment protocols or trials. With a median follow-up of 23 months, overall survival was 100%, with 9 children in complete remission, including 4 who did not have surgery, and 5 others in very good partial remission.
Conclusion: The knowledge of a favourable genomic profile was taken into account for treatment decisions in 14/17 children, enabling a reduction of chemotherapy while maintaining the excellent overall survival. Prospective clinical trials are urgently required to confirm these results.

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POC28

Retinoids (RA) relieve EZH2-mediated epigenetic suppression of neuroblastoma differentiation

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Tumors from high-risk NB patients have an undifferentiated histopathology while those from low-risk patients have histologic and molecular genetic evidence of differentiation. Moreover, RA induces differentiation in tumor cells from high-risk patients. This indicates that NB tumors may have a block in their developmental programs. We hypothesized that polycomb group (PcG) repressive complex proteins (PRC1 & PRC2) suppress NB differentiation. PcG proteins have are aberrantly expressed in breast and prostate tumors. Enhancer of zeste homolog 2 (EZH2) is a methyltransferase and the catalytically active component of PRC2 (EED, YY1). EZH2's primary target is methylation of Histone3 on lysine 27 (H3K27me3) which leads to transcriptional silencing. Microarray data show elevated levels of expression of EZH2 (P=6.4x10-9), EED (P=4x10-9) and YYI (P=5x10-4) and low expression of PRC2 target genes like RARβ (2.0 x10-5) in tumors from high risk NB. Since studies indicate that RARβ primarily mediates RA induced arrest of tumor cell growth and induction of differentiation, we used the EZH2 target gene, RARβ, as a marker of EZH2 activity. Using Chromatin immunoprecipitation (ChIP), the chromatin over the RARβ 5'proximal promoter and intron1 is enriched in EZH2 protein and H3K27me3 in NGP, KCNR and SY5Y cells which fail to express RARβ mRNA. Within 6hrs of RA treatment, the global H3K27me3 levels decreased 50%. The levels of phosphorylated EZH2 (EZH2-P) increased at 6hrs (2.8-fold) and EZH2-P has decreased binding to H3. ChIP assays revealed a 2-6 fold decrease in EZH2 binding and H3K27me3 at RARβ2 promoter and intron1. Coincidently, there was enrichment in H3K27 acetylation, an epigenetic marker of gene activation, over the RARβ2 promoter region in SY5Y (25-200 fold) and NGP (4-45 fold) and a 100-fold increase in RARβ2 mRNA expression. Silencing of EZH2 with EZH2siRNA led to increases in RARβ2 expression. These data are consistent with a model in which EZH2 mediated epigenetic silencing of key developmental genes blocks neuroblast differentiation and contributes to tumorigenesis. Strategies to relieve EZH2 mediated suppression may be clinically active.

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POC29

Development of an automated quantitative method for scoring Metaiodobenzylguanidine (mIBG) scans in patients with neuroblastoma

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Background: Radiolabeled metaiodobenzylguanidine (mIBG), a norepinephrine analog, is concentrated in cells of the sympathetic nervous system and is utilized in both diagnostic imaging and treatment of neuroblastoma. mIBG scans are typically interpreted qualitatively or semi-quantitatively by radiologists, often with poor inter-reader reliability. We have developed an automated quantitative method for scoring mIBG scans to overcome this inter-reader variability.
Objective: Treatment decisions for children with neuroblastoma are often made based on results of mIBG scanning, and current methods are semi-quantitative at best. We sought to develop a computerized, automated segmentation and scoring algorithm for mIBG scan analysis, thus enhancing therapeutic decision-making.
Design/Methods: With IRB approval, data from 70 mIBG scans from 17 patients with neuroblastoma treated at the University of Chicago Medical Center were collected for evaluation, and of these, raw data needed for further analysis was available for 25 scans from 11 patients. Images were scored by two experienced radiologists, according to the currently accepted standard of assigning each of 9 body segments a segmentation score of 0-3, depending on uptake. An automated, computerized segmentation algorithm was developed to divide the scan image into 9 segments and assign an extension score by relative mIBG signal intensity when compared to physiologic mIBG uptake in the liver.
Results: Of a possible 250 events (25 scans with 9 segments and 1 total score each), our algorithm agreed with one or both of the radiologists 84.4% (211/250) of the time. The agreement between both radiologists was 44.4% (111/250) and between all three 39.6% (99/250). For total score, there was one false positive and no false negative events.
Conclusion: An automated, quantitative method for recognizing mIBG uptake in patients with neuroblastoma is feasible and has superior reliability when compared to that of two subjective experienced radiologists. A broader, prospective assessment of this method is warranted and should be incorporated into a clinical trial.

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POC30

Is retroperitoneal lymphadenectomy for high risk abdominal neuroblastoma relevant

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Objectives: Complete tumor removal in cancer surgery includes organ specific regional nodal dissection. However the extent of resection of primary tumor in treatment of high-risk neuroblastoma itself is controversial, therefore the efficacy of retroperitoneal lymphadenectomy has not been assessed. We reviewed our experience of retroperitoneal lymphadenectomy in high risk abdominal neuroblastoma in terms of its relevance, safety, local control and overall survival. Method: Thirty eight patients with high risk abdominal neuroblastoma with primary site restricted to the adrenal gland or the adjoining sympathetic ganglia operated between October 2004 and November 2009 are included in this prospective analysis. There are 26 males and 12 females, with a median age of 4 years (1 to 14 years). Retroperitoneal lymphadenectomy was performed in 48 patients and lymph node sampling in 3 patients.
Results: The median number of lymph node dissected was 8 and the median number of positive lymph node was 5. The median duration of surgery was 4 hours and 30 minutes; the median intraoperative blood loss was 205 ml. There were no major intraoperative complications leading to visceral insufficiency or perioperative deaths. The median duration of hospital stay was 7.5 days. Postoperative complications included intestinal obstruction (2 patients), chyle leak (9 patients) and infection (1 patient). Nineteen patients are alive without any disease; four patients are alive with disease. 14 patients died due to disease progression and one patient died due to chemotherapy related toxicity. There was no local recurrence in patients who died of disease except in one patient who had extensive metastases along with local recurrence.
Conclusion: There is high incidence of lymph node metastases in high risk neuroblastoma. Complete excision of abdominal neuroblastoma and retroperitoneal lymphadenectomy can be performed safely with acceptable morbidity and is associated with excellent local control and better survival.

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POC31

Concurrent ipsilateral nephrectomy versus kidney-sparing surgery in high-risk, intra-abdominal neuroblastoma

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Background: Surgical resection of the primary tumour is important in the management of high-risk neuroblastoma (NB). However, it is not clear what the effect is of a concurrent ipsilateral nephrectomy at the time of resection of the primary intra-abdominal NB. The purpose of this study was to determine if concurrent unilateral nephrectomy in high-risk NB has an impact on recurrence rate, renal function, morbidity and overall survival.
Methods/approach: A retrospective cohort review was performed on all patients with high-risk NB at one institution between 1998 and 2008. Research Ethics Board approval was obtained. Analysis was done using t-test for continuous variables and Chi-square/Fischer's exact test for categorical ones.
Results: A total of 62 patients with high risk NB with intra-abdominal tumors were eligible: 56 had kidney-sparing surgery and 6 had nephrectomy. Patient characteristics (gender and age) and tumor characteristics (side and size) were not significantly different between the two groups. The following table summarizes the results:

	Nephrectomy (N=6, ± SD)	Kidney-sparing (N=56, ± SD)	P Value
EBL (ml)	650 ± 480	110 ± 174	0.001
Operating time (hrs)	9.2 ± 4.0	5.9 ± 2.4	0.005
GFR (ml/min) post-operative/ pre-BMT	104 ± 30	148 ± 36	0.006
GFR (ml/min) post-BMT	90 ± 17	128 ± 39	0.02
Complications: intra-operative	0/6 (0%)	13/56 (23%)	0.1
Complications: renal-related at time of BMT	0/6 (0%)	13/52 (25%)*	0.5
Local recurrence	0/6 (0%)	13/56 (23%)	0.1
Metastatic recurrence	4/6 (67%)	32/56 (57%)	0.7
Overall survival	3/6 (50%)	26/56 (46%)	0.7

*GFR: Glomerular filteration rate; EBL: estimated blood loss; BMT: Bone marrow transplant;*4 patients were not eligible for BMT*

Conclusions: In this cohort the intra-operative blood loss and operating time were significantly higher in the nephrectomy group. While renal function was better in the kidney-sparing group, there were no differences with regard to renal-related complications at time of BMT, distant metastasis, or overall survival. There was a trend towards fewer intra-operative complications and less local recurrences in the nephrectomy group; however, based on small sample size, these did not reach statistical significance.

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POC32

Pilot study of high-dose chemotherapy using a novel preparative regimen with Busulfan, Melphalan, and Topotecan (TBM) followed by autologous hematopoietic stem cell transplant in high-risk neuroblastoma and other advanced stage and recurrent tumors

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Background/aims: High-dose chemotherapy followed by autologous hematopoietic stem cell transplant (AHSCT) has been used as consolidation therapy for pediatric solid tumors, including neuroblastoma (NB). Relapse from resistant disease, traditional acute (veno-occlusive disease (VOD), mucositis, and ~ 6% toxic death) and late (hearing loss) toxicities following AHSCT suggest that novel regimens are needed to improve outcome and minimize morbidity.
Methods: 19 patients (4 Ewing's, 7 NB, 3 Wilm's, 1 PNET, 3 Rhabdomyosarcoma, 1 undifferentiated sarcoma) were treated with Topotecan (continuous infusion days -8 thru -4) combined with Bulsulfan (q 6 hours x 16 doses during Topotecan) and Melphalan (days -3 and -2).
Results: 0/19 patients experienced toxic death compared to ~ 6% mortality reported from classic carboplatin, etoposide, melphalan (CEM) regimens. Specifically for NB patients, only 1/7 experienced any VOD with a maximum total bilirubin of 1.6 mg/dl and elevation of liver enzymes by < 2.5x normal. Grade 3 functional mucositis (symptomatic/unable to eat/ drink adequately) occurred in 6/7 NB patients with only 3/7 NB patients having grade 3 mucositis by clinical exam (confluent ulceration/bleeding). 3/7 NB required home total parenteral nutrition. Hearing loss requiring hearing aids has not been seen (0/7 NB patients) compared to~20-50% reported for NB patients consolidated with AHSCT using CEM (p = 0.016 by Binomial distribution test). 0/19 patients have clinical signs of pulmonary fibrosis. Toxicities in the 12 non-NB patients are similar with manageable VOD, mucositis, need for home TPN and lack of hearing aid use. Engraftment of absolute neutrophil count > 500/ µL (cell dose 9 x 106 +/- 8 x 106 CD34+/kg) ranged 10 +/- 2 days for the 7 NB patients. Progression Free Survival (PFS) is 56% for all 19 patients and 86% for the 7 NB cases with 6/7 NB patients demonstrating no evidence of disease for 23 +/- 5 months.
Conclusion: VOD, milder mucositis and absence of hearing loss requiring hearing aids) than classic CEM regimens. Study continues accrual in a phase 2 setting.

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POC33

Immunocytological GD2 negativity in treated and untreated neuroblastoma patients with bone marrow metastases

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Background
The expression of the GD2 ganglioside on the surface of neuroblastoma cells has important implications for bone marrow assessment and for treatment of minimal residual disease. Earlier detection of three cases prompted us in 2006 to study GD2 negativity prospectively.
Methods: Paired bone marrow samples of neuroblastoma patients with cytological bone marrow involvement were investigated by routine light microscopy and by GD2 immunocytology according to published standards. Only cytologically unquestionable tumor cell clumps with complete loss of GD2 staining were considered GD2 negative. Cytospin immunostaining results were controlled by additional immunostaining directly on bone marrow smears in most cases.
Results: During 42 months, 493 bone marrow samples of 366 patients were investigated. In 28 cases lacking immunocytological GD2 expression of cytological unequivocal neuroblastoma tumor cell clumps was detected. Of the 28 patients, 14 were at initial diagnosis, 6 under therapy and 8 at recurrence. In 10 cases all tumor cells and in 18 a varying number of cells were GD2 negative. The frequency of patients with GD2 negative cells at diagnosis was 7.2 % in stage 4 (12/ 166) and 3.4 % in stage 4S (2/ 59). No association to other diagnostic criteria like mIBG uptake, urinary catecholamine excretion, NSE levels, LDH, MYCN amplification, 1p aberration, and INPC histology was detected. GD2 negative stage 4 patients (at diagnosis) had a worse outcome (EFS logrank p= 0.019, OS p < 0.001).
Conclusion: GD2 negativity is a more frequent phenomenon in neuroblastoma than currently known and has important implications for diagnosis, monitoring disease and for treatment.

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POC34

Hematopoietic stem cell transplantation for high risk neuroblastoma in children

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Neuroblastoma is a malignancy of neural crest cells which usually give rise to the sympathetic nervous system. Patient with high risk NB have a very poor results after standard treatment. High-dose chemotherapy followed by HSC may improve their prognosis.
Patients and methods: From October 1995 until December 2009 30 patients (17 f\13 m) with high risk NB were referred to Russian State children hospital. The median age at diagnosis was 3.39 y.o (range 6m-15y.). The primary sites were adrenal glands, followed by retroperitoneum in 24pts. and thoracic cavity in 6pts. All patients had stage IV of the disease, 19pts had bone lesions. Their were 12 pts with N-myc positive BM, 7pts were negative and 11pts unknown. Metastatic site was positive in initial MIBG scan in 19pts; in 2pts was negative and in 9pts was unknown. The chemotherapy of all patients consisted of 5-6 cycles according their protocol. In all of the 30pts the primary tumor resection was performed after 4-5 cycles. Bone marrow harvest was done after 3-4th cycle. Patient's disease status before BMT: 12pts had CR and 18pts had residual tumors or positive MIBG scan. 28 pts received auto PBSCT and 2pts haplo PBSCT . The conditioning was By\Me in 13pts and Me\eto\carbo in 15pts, By\Me\Flu\Ty in 2pts. Median CD34count was 6x106\kg (range 1x106-37x106\kg), 18 pts after BMT received radiation therapy and treated with 13-cis-retinoic acid.
Results: Four patients (13.3%) had hepatic veno-occlusive disease, but none was severe. Neutrophils (>0.5x109) recovery occurred at day 13(8-38). 3pts (13.3%) had early regimen death. 14\27 (51.8%) had disease relapse\residual tumor after a median time of 6 month (3-19m.) after auto PBSCT. All patients with progression had initial bone metastasis. 15 patients (50%) were alive from 12 to 123m. after diagnosis, 13(48.1%) pts survived disease free.
Conclusion: The results of treatment have demonstrated an improvement in short – term disease survival particularly in those children who did not have initial bone metastasis and who have obtained significant tumor response from conventional induction therapy prior to harvesting and high dose therapy with PBST.

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POC35

Follow-up study of survivors of childhood neuroblastoma - Report from a single institute in Japan

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Background: Neuroblastoma is the most common malignant solid tumor of childhood, but the prognosis of high-risk neuroblastoma (HRNB) is still very poor. Furthermore, children who survive HRNB are also at risk for treatment-related complications. We analyzed these complications in survivors of HRNB in our institute.
Method: From 1992 to 2004, at Nihon University Itabashi Hospital, we treated 25 newly diagnosed HRNB patients with multimodal treatment which consisted of induction chemotherapy (CDDP+THP+VCR+CPA or IFM+CBDCA+VP-16), high-dose chemotherapy (CBDCA+VP-16+L-PAM with or without TBI), primary delayed surgery and local radiotherapy. We investigated many long-term problems, including second neoplasma, physiosocial disturbance, and organ system dysfunction (especially for endocrine, musculoskeletal, neurologic, sensory, cardiac, and pulmonary impairments).
Results: Thirteen patients were still surviving and 10 of these survivors were examined with regard to long-term problems. Five were male and 5 were female. The median age at diagnosis was 4 years old (range, 1y7m to 9y6m) and the median follow-up time was 9 years 10 months (range, 5y8m to 18y0m). None of the patients had a second neoplasma patient and only one showed physiosocial disturbance. Many organ system dysfunctions were noted: 10 high-frequency (>4kHz) hearing loss , 2 cataract, 5 small height (4 growth hormone therapy needed), 2 goiter, 6 hypothyroidism, 2 hypogonadism, and 8 chronic-sinusitis. None of the patients showed cardiac disturbance or pulmonary complication.
Conclusion: There were many long-term problems, especially regarding disturbance in survivors of HRNB children during a median 10-year follow-up. It is still possible that these patients may be at increased risk of cardiac events and/or a second neoplasma over the next 10 years of follow–up. A more precise investigation will be needed.

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POC36

Retrospective analysis of treatment results of high risk neuroblastoma

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Background: The most important risk factors in neuroblastoma are well known: age more than 1 year at the time of diagnosis, advanced stage of disease and MYCN amplification.
Aim: To determine the efficacy of high risk neuroblastoma treatment a retrospective study has been conducted in Pediatric Oncology and Hematology Center, Regional Children's Hospital, Ekaterinburg, Russia.
Methods: Among 140 children with primary neuroblastoma admitted to our clinic since January 1991 till November 2009 aged from 10 days to 15 years (median 18 mo.) were 91 patients (45 girls and 46 boys) with known MYCN status. 41 (43,9%) patients older than 1 year with stage IV or MYCN amplification were stratified to high risk group (HRG) and have been treated according to NB 92, NB 97 and NB 2004 protocols: 9(22%), 19(46,3%) and 13(31,7%) correspondingly. Only 4(9,7%) children underwent high dose chemotherapy and autologous peripheral blood stem cells transplantation (PBSCT). Median of follow up is 48 months.
Results: Patients distribution by stage was as follow: stage I–2(4,9%) patients; stage II–2(4,9%); stage III–4(9,8%); stage IV–33(80,5%). MYCN amplification has been detected in 21(51,2%) of 41 HRG patients. Complete remission and very good partial remission have been achieved in 16(39%); partial remission in 17(41,5%) children. Unfavorable events (relapses, stable disease, progressive disease and therapy related deaths) were observed in 31(75,6%) cases. At present time 15(36,6%) patients are alive; 10(24,4%) are alive without progression; 2(4,9%) patients were lost to follow up. 12-years event free survival (EFS) is 18%±6% and overall survival is 33%±8%. EFS in patients with MYCN amplification (20%±10%) did not differ significantly in comparison with other HRG patients (19%±8%)(p=0,08). Among 4 children after high dose chemotherapy and PBSCT - 1 patient died from progressive disease; local relapse was registered in 1 case and 2 patients are alive in complete remission.
Conclusion: Treatment results of HR neuroblastoma remain unsatisfactory. Our preliminary results suggest auto-PBSCT a curative therapeutic approach in patients with HR neuroblastoma.

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POC37

Comparison of anti-GD2-antibody ch14.18 and 13-cis-retinoic acid as consolidation therapy for high-risk neuroblastoma. Results of the German NB97 trial

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Background: High risk neuroblastoma patients require intensive treatment consisting of induction chemotherapy, high dose chemotherapy (HDCT) followed by autologous stem cell transplant (ASCT) and consolidation therapy. Consolidation therapy has been studied in several trials, however, information on the efficacy of single agents are limited. We thus retrospectively analyzed the patients of trial NB97.
Method/approach: Patients were included in the analysis when they fulfilled all of the following criteria: stage 4 neuroblastoma, ≥1 year at diagnosis, successful induction chemotherapy, HDCT, and ASCT, no combination of antibody ch14.18 and retinoic acid, and at least one cycle of consolidation therapy. Between 1997 and 2002, all trial patients were scheduled for consolidation therapy with the anti-GD2-antibody ch14.18 (six courses consisting of 20 mg/m²/day for 5 days every 2 months; group ch14.18). Between 2002 and 2004, all patients received 13-cis-retinoic acid (nine courses 160 mg/m²/d for 14 days every 28 days with a three-months rest between the 6th and the 7th course; group RA).
Results: A total of 149 consecutive neuroblastoma patients were included, 74 patients received ch14.18, and 75 patients received RA. Both groups were balanced in age (p=.706), MYCN amplification (p=0.718), and remission status prior to ch14.18/RA (p=.541). The median observation time was 7.72 years. The 3-year-event free survival rate from diagnosis was 52.7±5.8% in the ch14.18 group and 50.5±5.8% in the RA group (p=.209). The 3-year overall survival rates were 68.9±5.4 and 65.0±5.5% (p=0.228) for ch14.18 treated and RA treated patients, respectively. Multivariate analysis including the variables age, MYCN status, remission status after ASCT, and type of consolidation also demonstrated no independent impact of consolidation therapy on event free survival and overall survival.
Conclusion: This retrospective analysis of a very homogenous cohort of high-risk neuroblastoma patients demonstrated no difference between 13-cis-RA and ch14.18 as consolidation therapy after high-intensive induction chemotherapy and ASCT.

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POC38

Metachronous neuroblastoma in an infant with constitutional unbalanced translocation t(2;16)(p23;p13.3) involving *ALK*

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Background and Results: The patient, a full-term male born to non-consanguineous parents, had dysmorphic features, hypospadius, inguinal hernia, malrotation, feeding difficulties, left multi-cystic dysplastic kidney, microcytic anemia, and seizures. Chromosomal analysis by G-banding and array comparative genomic hybridization (aCGH) revealed an unbalanced translocation t(2;16)(p23;p13.3), resulting in partial monosomy for 16p13.3-pter and partial trisomy for 2p23-pter. At 3 months, he developed a right adrenal stage I neuroblastoma (NB) and had a complete surgical resection. Six months later, he developed a NB in the contralateral adrenal, which was again treated by resection. Both tumors exhibited favorable histology. Cytogenetics from the first tumor demonstrated the constitutional t(2;16) translocation as well as trisomy 17 and t(1;4)(q12;p11). Analyses by FISH did not detect rearrangement of *ALK*; however, in keeping with the constitutional karyotype, 3 copies of *MYCN* and 3 copies of *ALK* gene were detected. After six months follow-up since the second surgery there is no evidence of recurrence or additional tumors.
Discussion: The *ALK* gene (2p23.2), located ~13 Mb proximal to the *MYCN* gene (2p24.3), has been identified as a major familial NB predisposition gene. Somatic *ALK* mutations and copy number gains have also been reported in sporadic NB. Partial trisomy of 2p has been previously reported to be associated with NB in the context of multiple anomalies (5 case reports, one with bilateral NB). Most of the cases with germline partial trisomy 2p would be expected to have 3 copies of *ALK* and *MYCN*. We propose that *ALK* copy number gain, which drives cellular proliferation in culture, may be one factor that predisposes patients with partial trisomy 2p to develop NB. Utilizing aCGH and other high-resolution genetic platforms, we have recently identified two patients with duplications of 2p (one included *ALK* and *MYCN*; the other only *ALK*). Long-term prospective studies of patients with increased 2p (and *ALK*) copy number will help to determine potential risk for tumors in these patients and need for surveillance screening.

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POC39

Neuroblastomas with non-avid I^{123}-MIBG scan and negative urinary catecholamine secretion: A single institute's experience

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Background: Small studies have reported that non-avid MIBG scans at diagnosis are associated with favorable prognosis. We retrospectively examined clinical characteristics and outcomes for neuroblastoma patients with non-avid MIBG scans and/or negative urine catecholamines at diagnosis.
Methods: We reviewed chart records for all neuroblastoma patients diagnosed from September 1999 to August 2009.
Results: There were a total of 148 patients - 29 INSS stage 1 (20%), 24 stage 2 (16%), 18 stage 3 (12%), 68 stage 4 (47%) and 7 stage 4S(5%); 2 were lost to followup. I^{123}-MIBG scans were performed for 123 patients at diagnosis - 95 (77%) were MIBG-avid, 11 (9%) non-avid and 17 (14%, all low stage) were done post-resection. Among those with non-avid MIBG, 4 had INSS stage 1 disease (36.4%), 1 stage 2 (9%), 3 stage 3 (27.3%) and 3 stage 4 (27.3%); INRG: 5 (45%) low, 3 intermediate and 3 high-risk. Two of the MIBG-negative patients (stage 3 and 4) relapsed. Another patient had stage 4, *MYCN*-amplified disease and is well with no relapse after 9 years. For the MIBG-negative patients, overall and relapse-free survivals were 100% and 82% respectively, compared to 73% and 66% for the entire cohort. In addition, there were 2 patients who relapsed with MIBG-non-avid disease (avid at first diagnosis). Urine homovanillic acid (HVA) and vanillylmandelic acid (VMA) results were available for 145 patients. The levels were negative for 17 (12%), all of whom had low stage disease (1 or 2) and are alive without relapse. Only 2 patients had both normal catecholamines and non-avid MIBG scan at diagnosis. We also identified 9 patients with elevated urine catecholamines at diagnosis who later had normal levels at relapse. All underwent further therapy and 8 are alive (median follow-up 4 years).
Conclusion: Non-avid MIBG scans and negative urine catecholamines are more commonly associated with low stage disease and favorable outcome. Our findings are consistent with two other reports from similar size cohorts. Larger population studies are needed to verify and understand potential mechanisms for this association.

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POC40

Efficacy of tandem high-dose chemotherapy and autologous stem cell rescue in patients with high-risk neuroblastoma: a preliminary report of NB 2004 study at Samsung Medical Center

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Background: Although the strategy using high-dose chemotherapy and autologous stem cell rescue (HDCT/ASCR) has improved the survival of patients with high-risk neuroblastoma, the survival rate after single HDCT/ASCR has been unsatisfactory. In the present study, the efficacy of tandem HDCT/ASCR was investigated to further improve the outcome of patients with high-risk neuroblastoma.
Methods: Patients who were newly diagnosed with high-risk neuroblastoma (stage 4 tumors over 1 year of age or N-myc amplified stage 3, 4 tumors) were enrolled in the NB 2004 study from January 2004 to December 2008. Patients received 9 cycles of chemotherapy (CEDC 5 cycles and ICE 4 cycles in an alternating manner) before first HDCT. CEC regimen (carboplatin + etoposide + cyclophosphamide) was employed for the first HDCT. TM-TBI regimen (thiotepa + melphalan + total body irradiation for stage 4 tumors) or TM regimen (thiotepa + melphalan for stage 3 tumors) were employed for the second HDCT. Local radiotherapy was administered to the primary tumor site following the second HDCT. Differentiation therapy with 13-cis-retinoic acid and immunotherapy with IL-2 were administered after local radiotherapy.
Results: A total of 47 patients were enrolled in the NB 2004 study. Forty-four out of them underwent tandem HDCT/ASCR as assigned at enrollment (1 progression prior to first HDCT/ASCR, 1 life-threatening myocarditis during first HDCT/ASCR and 1 refusal of second HDCT/ASCR). Acute toxicities during tandem HDCT/ASCR were acceptable and no treatment-related death occurred. Thirty-six patients remained event free with a median follow-up of 38 (14-72) months after diagnosis and the probability of 5-year overall survival and event-free survival ± 95% confidence interval were 67.6% ± 19.9% and 66.8% ± 16.0%, respectively.
Conclusions: The survival rates in NB 2004 study were encouraging. However, a longer follow-up will be needed to observe the frequency and severity of long-term side effects in the future.

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POC41

Measurement of tyrosine hydroxylase transcripts in bone marrow using biopsied tissue instead of aspirate for neuroblastoma

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Background: Molecular detection of tyrosine hydroxylase (TH) transcripts by quantitative RT-PCR (qRT-PCR) is a sensitive method to detect neuroblastoma (NB) cells in the bone marrow (BM). However, its clinical utility following chemotherapy has not been thoroughly investigated.
Methods: TH transcripts in the BM were measured by qRT-PCR both at diagnosis and during the course of chemotherapy. The results were analyzed with respect to assay timing, tumor volume and histological findings.
Results: A total of 475 BM specimens from 105 patients were analyzed (66 at diagnosis, 48 after three cycles, 48 after six cycles, 278 after nine or more cycles of chemotherapy, 35 at or after relapse/progression). Of the 475 BM specimens, tumor cells were detected in 63 specimens by conventional histological examination (22 at diagnosis, 9 after three cycles, 6 after six cycles, 15 after nine or more cycles of chemotherapy, 11 at or after relapse/progression). TH transcripts were detected in 100% of BM aspirates at diagnosis in cases with concurrent tumor involvement in the BM section; however, the proportion of TH transcript positive BM aspirates in cases with concurrent tumor involvement in the BM section gradually decreased following chemotherapy (55.5% after three cycles, 28.6% after six cycles and 0% after nine or more cycles of chemotherapy). Decreased proportion of TH transcript positive BM aspirates was associated with reduced tumor volume in the BM and differentiation of tumors into mature forms during chemotherapy. When qRT-PCR was performed with both aspirated and biopsied tissue during chemotherapy, TH transcripts were detected in BM tissue not only in all of the histology-positive cases but also in some of the histology-negative cases, while the proportion of TH transcript positive BM aspirates was low, even in histology-positive cases.
Conclusions: Measurement of TH transcripts in BM aspirate does not appear to be clinically useful during or after chemotherapy. Therefore, molecular monitoring of NB cells during or after chemotherapy using BM tissue is more optimal than testing on BM aspirates.

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POC42

Reduced-intensity allogeneic stem cell transplantation in children with neuroblastoma who have failed a prior tandem autologous stem cell transplantation

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Background: The prognosis of children with neuroblastoma who have failed a prior tandem autologous stem cell transplantation (SCT) is dismal because they can not tolerate further intensive treatment. In this context, investigators evaluated the feasibility and efficacy of reduced-intensity allogeneic stem cell transplantation (RIST) in children who have failed a prior autologous SCT.
Methods: Four to eight cycles of conventional chemotherapy were administered prior to RIST. Surgery and local radiotherapy were done whenever possible prior to RIST. CyFlu (cyclophosphamide 120 mg/kg + fludarabine 150 mg/m2) regimen and CyFlu + ATG (7.5 mg/kg) regimen were used as conditioning regimen for HLA matched SCT and for mismatched related SCT, respectively. Peripheral blood stem cells were transplanted. Cyclosporine (CSA) alone for matched related SCT, and CSA + short-course methotrexate for unrelated or mismatched SCT were used for GVHD prophylaxis. CSA was rapidly tapered from day 30 (matched related SCT), day 60 (matched unrelated SCT) or day 90 (mismatched related SCT) if GVHD was absent or complete donor chimerism was not achieved.
Results: A total of 5 patients were enrolled (1 matched related, 2 matched unrelated and 2 haplo-identical related). Tumor status prior to RIST was VGPR in 1, PR in 3 and SD in 1. Conditioning regimen-related toxicities were mild. Hematologic recovery was rapid and complete donor chimerism was achieved in all patients until day 90. Grade I-II acute GVHD developed in all patients and extensive chronic GVHD developed in all 3 patients who had survived for more than 100 days after RIST. Although CR was achieved on day 90 in 1 patient (VGPR prior to RIST), tumor progressed before day 180 in the remaining 4 patients (PR or SD prior to RIST). The only patient who achieved CR after RIST died from viral myocarditis later.
Conclusion: Although conditioning regimen-related toxicities were mild and complete donor chimerism was achieved, tumor progressed during the early period after RIST in patients who were in PR or SD prior to RIST. More effective treatment to reduce tumor burden prior to RIST might be needed to improve the outcome after RIST.

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POC43

Neuroblastoma detected after ending of mass screening at 6 months of age in Japan

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Purpose: The mass screening (MS) for neuroblastoma (NB) at 6 months of age in Japan was discontinued in 2004. This study assessed the risks and benefits of MS based on an analysis of NB detected before or after discontinuation of MS in Japan.
Methods: The clinical features and Brodeur's genetic type (BGT) based on MYCN, DNA ploidy, and other genetic aberrations were assessed in 116 NB patients (23 cases after and 93 cases (55 MS cases) before the discontinuation of MS) over 6 months of age treated at one institution since 1985.
Results: The 20 NBs detected after MS was discontinued ranged in age from 7 to 67 months, 15 cases were stage 4, and 12 cases would have been detected at 6 months of age if they had undergone MS. The BGT of these 20 patients showed that 26% (6/23) were Type 1 (low risk), 57% (13/23) were Type 2A (intermediate risk), and 17% (4/23) were Type 2B (high risk). While the 93 NB patients detected before MS was discontinued showed 60% (56/93) were Type 1, 18% (17/93) were Type 2A, and 22% (20/93) were Type 2B. Among the Type 2A patients, 85% (11/13) of the patients detected after MS was discontinued showed stage 4, while only 50% (9/18) of those diagnosed before MS was discontinued were stage 4. The genetic analysis using SNP array for Type 2A showed that the pattern of genetic aberration was equivalent in those detected either before or after MS was discontinued.
Conclusion: There was a decrease of Type 1 and an increase of Type 2A NB in patients after MS was discontinued in Japan. These results suggest that the majority of Type 1 detected by MS has regressed, and the majority of Type 2A detected by MS has been appeared sporadically as advanced NB in patients over 1 year of age.

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POC44

Whole-body diffusion-weighted MR imaging is useful to detect bone/bone marrow metastasis of neuroblastoma and monitor response to therapy

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Background: Precise detection of bone or bone marrow metastasis at diagnosis is clinically important in Neuroblastoma. Diffusion-weighted MR imaging (DW-MRI) has been reported to be useful to detect metastasis of solid tumors, such as breast cancer, rectal cancer and prostate cancer. The purpose of this study was to investigate whether DW-MRI is useful to evaluate the staging of neuroblastoma and monitor the tumor response to chemotherapy as compared to conventional methods.
Methods: diagnosis and during the follow-up period using whole-body DW-MRI, 123I-MIBG scintigram, bone scintigram and real-time RT-PCR of tyrosine hydroxylase (TH) in bone marrow aspiration. Four patients had neuroblastoma stage II, one had stage III, nine had stage IV and two had gaglioneuroma. All MR studies were performed with a 1.5T whole body scanner (Magnetom Avanto, Siemens Medical Solutions).
Results: Whole-body DW-MRI could detect bone or bone marrow metastasis in all of the nine stage IV patients, while MIBG detected it in just six (67%) patients, bone scintigram in four patients (33%), and real-time PCR of TH in bone marrow aspiration was detected in six patients (67%). There was no detection of metastasis using whole-body DW-MRI in patients in stage II, III and with ganglioneuroma. After several courses of chemotherapy, some positive spots remained in four patients in stage IV, only detected by DW-MRI. Bone biopsy revealed viable tumor cells in two of the patients, while the others had differentiated tumor or necrosis.
Conclusion: Whole-body DW-MRI may be useful to detect bone or bone metastasis when compared to conventional methods. However, as the sensitivity of DW-MRI is not tumor specific; bone biopsy should be performed to confirm viable tumor when DW-MRI is the only positive result.

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POC45

Identification of a therapy-sensitive subtype and stratification of progressive risk in advanced neuroblastomas

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Background: The aim of this study is to propose a new risk stratification in advanced neuroblsastomas (NBs).
Methods: 196 non-mass NBs (56 in st III , 117 in st IV , 23 in st IVs) were assessed with "MYCN status, INPC findingand" and Ha-ras/Trk A expression. Statistics: Kaplan-Meier method (patient's survival) and Cox proportional hzard model (independence of each factor) were used.
Results :(1)Predictors: Each of *MYCN Amplification, INPC Unfavorable histology and Low Ha-ras/trk A expression* associated significantly with poor patient's outcome. The multivariate analysis could show an independence of each predictors for the outcome. However, when the risk was assessed by a single predictor, a half of the high risk NBs were missed. (2)Stepwise stratification was done in 103 advanced NBs (59 were event-free survivors and 44 died), in which the all three predictors were examined. Total 69 NBs were enrolled stepwise into high risk group (1st; 28 with MYCN Amplification, 2nd; 24 with INPC Unfavorable histology and 3rd; 17 with Low Ha-ras/trk A expression). The 38 patients died in this high risk group and occupied 86% out of total 44 patients died in this study. Moreover, 21NBs with *High Ha-ras/trk A expression, Unamplified MYCN and INPC favorable histology* were classified as a subgroup (*therapy sensitive NB*) with 90% survival rate. (3) Stratification by the pile of the risk: The 69 high risk NBs could be classified into three subgroups according to number of the risk factors. NBs with triple risk were most aggressive and the survival rate was only 10%. Those in the double and single risk subgroups were 29% and 66%, respectively. Including the therapy sensitive group, survivals of the four groups showed significant difference (p<0.0001).
Conclusion; We provided a new means for risk stratification and presented a subgroup *therapy sensitive NBs*in the advanced stages. This is reliable grounds to select an therapeutic intensity at diagnosis for respective patients, which might improve the efficacy of the therapies in high risk NBs and decrease sequelae in the lesser risk NBs.

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POC46

^{18}F-FDOPA PET scan is still useful in the presence of ^{123}I-MIBG and ^{18}F-FDG for neuroblastoma imaging

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Background: In our previous studies using ^{18}F-FDOPA and ^{18}F-FDG PET scans for neuroblastoma imaging, we proved that ^{18}F-FDOPA is very helpful when ^{123}I-MIBG is not available. In this study we added ^{123}I-MIBG for imaging study to re-evaluate the value of ^{18}F-FDOPA in detecting neuroblastoma lesions.
Method/approach: In all 15 cases of neuroblastoma, ^{18}F-FDOPA PET, ^{18}F-FDG PET and ^{123}I-MIBG scans were done in random sequence with each other within 2 weeks. Both ^{18}F-FDOPA and ^{18}F-FDG were produced in our PET Center and ^{123}I-MIBG was produced in INER, Taiwan. ^{18}F-FDOPA PET was done after carbidopa premedication; ^{18}F-FDG PET was done with fasting for 4 hours and ^{123}I-MIBG scan was done with Lugoli's solution for 3 days. All scans were correlated with pathological findings, other imaging studies and clinical follow up for at least 6 months.
Results: The imaging findings can be classified in the following 4 categories. In 3 cases after chemotherapy, all three scans were negative. In a case of multifocal systemic recurrence all three scans were positive and the uptake patterns for 3 agents were similar. In these 4 cases (27%) ^{18}F-FDOPA PET scan showed no advantage over ^{123}I-MIBG scan. In 9 cases (60%) with abnormal ^{18}F-FDOPA uptake, 4 of them were negative on both ^{18}F-FDG and ^{123}I-MIBG scans, 3 showed abnormal ^{18}F-FDG and ^{123}I-MIBG uptake in different sites and 2 showed negative ^{18}F-FDG scan but positive ^{123}I-MIBG scan (less well demonstrated by ^{18}F-FDOPA). The last category included 2 cases (13%) of poorly differentiated tumor at the primary site where ^{18}F-FDOPA showed no uptake while ^{18}F-FDG and ^{123}I-MIBG were positive.
Conclusion: In conclusion, ^{18}F-FDOPA PET was clinically useful in 73% of our cases in: (a) detecting more or different lesions than ^{18}F-FDG/^{123}I-MIBG scans (60%); (b) characterizing a poorly differentiated primary lesion (13%). Only 27% of the cases, ^{18}F-FDOPA scan did not show advantage over ^{123}I-MIBG scans. In a malignancy with variable biological characteristics like neuroblastoma, we recommended adding ^{18}F-FDOPA in the diagnosis and follow up of the tumor after therapy.

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POC47

Molecular imaging with ^{18}F-FDOPA PET in the early detection of new metastatic neuroblastoma in bone marrow

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Background: The biosynthesis of catecholamine in neuroblastomas needs tyrosine hydroxylase(TH) and dopa decarboxylase(DDC). The TH mRNA and DDC mRNA detected by RT-PCR(Reverse transcription-polymerase chain reaction) amplification have been used for detecting minimal residual disease in marrow of neuroblastoma patients since 1991 and 1999 respectively. In this study, we tried to use the ^{18}F-FDOPA PET scan to detect and localize new marrow metastasis of neuroblastoma with DDC as a target.
Method/approach: From 8/1/06 to 7/31/09, we collected 17 cases of childhood neuroblastoma. The mean age of onset was 2y 10m. There were 2 of stage 3, 12 of stage 4 and 3 of stage 4s of disease. All patients were treated with chemotherapy/retinoic acid after surgery. We used ^{99m}Tc MDP bone scans, ^{18}F-FDG and ^{18}F-FDOPA PET scans every 3-6 months (ms) to monitor the progress of the disease and for new bony/bone marrow metastases. Neither ^{131}I-MIBG nor ^{123}I-MIBG was used in this study because governmental regulation did not approve for import or production locally. The final diagnosis is based on tissue proof, correlation with other imaging studies and/or clinical confirmation.
Results: In these 17 cases we detected 8 new bony/marrow metastases, including one of each at skull base, rib, sacrum, radius, femur, calcaneus, and 2 at mandible. In all these cases ^{18}F-FDOPA PET scan detected metastasis earlier than other imaging modalities. They can be detected 0-15 ms, 0-27 ms and 0-20 ms earlier than bone scan, ^{18}F-FDG PET scan and MRI respectively. Four of them are ganglioneuroblastoma and 3 are neuroblastoma.
Conclusion: Uptake of ^{18}F-FDOPA is indicative of the presence of neuroblasoma with DDC. If ^{131}I/^{123}I MIBG is not available, ^{18}F-FDOPA PET scan is highly recommended for early detection of bony metastasis in the follow up of neuroblastoma patients, especially those treated with retinoic acid. We did not intend to use ^{18}F-FDOPA PET scan to replace RT-PCR for DDC mRNA but to provide a convenient, non-invasive imaging method to localize the malignancy. We may miss some lesions that did not contain DDC. We plan to do further study comparing ^{18}F-FDOPA PET with MIBG scan when it is ready.

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POC48

Efficacy of Treosulfan as a single agent in newly diagnosed neuroblastoma stage IV patients

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Treosulfan (Treo) is a structural analogue of Busulfan currently used for high-dose chemotherapy of advanced Ewing sarcomas, neuroblastomas (NB) and high-risk leukemias. There is no clinical data supporting the hypothesis that Treo is active as a single agent in pediatric malignancies. There for, from March 2009 to January 2010, 9 pts (M/F 5/4) with NB st IV, > then 2 years of age at the time of diagnosis were included in our window study. The median age was 8,2 (3-15). Seven pts had stage IV newly diagnosed, and two - relapse of NB (one 3 yrs after haploidentical PBSCT). Treo was applied two times in a dose 10g/m2/ on days 1 and 7. After evaluation of the response high-risk protocol including 4 courses of CT, surgery, and High Dose CT with Auto PBSC rescue, followed by biological phase was delivered. PBSC harvest was performed after the 4 course. MIBG I123 positive lesions retained after transplant were irradiated.
Efficacy of Treo was as follow: bone marrow was morphologicaly clean after 2 courses in 4 out of 9 pts (in 1 pt with relapse). Four pts achieved PR and 4 SD (3 of them without tumor clearance in BM) after 2 courses of Treo. Toxicity was minor with hematological toxicity of stage 1 in 2 pts. Another toxicity was not registered. Four out of 7 newly diagnosed pts completed protocol (2, 2, 5 and 7 mo., correspondingly) : 1 of them in CR and 3 in VGPR before biotherapy. Three pts are still on treatment. One relapsed pt is in VGPR 5 mo. after treatment and one pt died from PD.
In conclusion Treo is an effective agent in newly diagnosed NB pts and further evaluation of the doses, place and schedule are warranted.

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POC49

Irinotecan/Temodal therapy as salvage treatment for children with neuroblastoma - single centre experience

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Aims: In spite of intensive multimodal first line therapy, still most of high risk neuroblastoma patients have relapse or progression of the disease. Unfortunately, so far there is no curative therapy for this group of patients, especially in the case of disseminated relapse. The aim of the study was evaluation of Irinotecan/Temodal as salvage treatment in children with therapy resistant high risk neuroblastoma.
Methods: This is observational study in children with NBL progression/relapse. From 2008-2009, 7 patients with relapse or progression of NBL treated in Hematology/Oncology Department in Krakow received Irinotecan/Temodal therapy (Kushner 2006). The group was heterogenous (1st - 4th relapse, different first line and previous relapse therapy). The end point of the study was survival analysis as well as evaluation of toxicities, quality of life and patient's compliance. Observation was finished on 31.12.2009 r.
Results: In all 7 patients who received Irinotecan/Temodal therapy at last partial response was observed, including 2 with VGPR (residual tumor, no metastases). Median survival time was 31 (24-70,5) months from the first relapse and 61 (37-129) months from diagnosis. Patients received 6-20 chemotherapy cycles. Generally, therapy was well tolerated. The main toxicities were thrombocytopenia and anemia, requiring transfusions after almost every cycle in 4/7 patients as well as elevated transaminase (ALT up to 1500 IU). In 1/7 patients persistent and recurring transaminase increase led to need of decreasing drug dosage and necessity of less frequent chemotherapy employment. Diarrhea was not very severe and was easily controlled with loperamid. Between chemotherapy cycles, as a rule children did not require hospitalization. The quality of life and compliance was satisfactory both for patients and their parents.
Conclusion: Irinotecan/Temodal chemotherapy seems to be reasonable choice for heavy pre-treated children with neuroblastoma, allowing for long-lasting therapy control without unacceptable toxicities, assuring relatively good quality-of-life.

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POC50

Minimal disease detection in non-metastatic neuroblastoma patients

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Background: In non-metastatic Neuroblastoma (NB), the identification of cases that require more intensive treatment is still difficult. Minimal disease (MD) and minimal residual disease detection in outcome prediction seems to be important in advanced Neuroblastoma, but there are not many studies focused on patients with non-metastatic disease.
Aim: The aim of this study was to determine whether the presence of Minimal Disease detected at diagnosis in peripheral blood (PB) and bone marrow (BM) samples from patients with non-metastatic NB could be associated with bad prognosis.
Methods: 102 non-metatastatic NB patients from 34 cooperative Spanish hospitals were included in the study. All of them were also included in SEHOP or SIOPEN cooperative controlled studies. NB staging was established according to INSS. Quantitative reverse transcriptase polymerase chain reaction (QRT-PCR) was performed on PB and BM samples from patients at diagnosis for tyrosine hydroxylase (TH) and doublecortin (DCX) mRNAs detection. Survival analysis was performed to compare Overall Survival (OS) and Events Free Survival (EFS) between groups using the Kaplan-Meier method and differences between groups were tested by using log-rank tests (SPSS 12.0).
Results: The frequency of detecting MD biomarkers in PB was 27% (8/30) of stage 1 PB, 12% (1/8) of stage 2 patients and the 23% (10/43) of stage 3 patients. Positive BM samples were observed in 18% (6/34) of stage 1, 18% (2/11) of stage 2 and 33% (16/49) of stage 3 patients. OS and EFS were similar for patients who expressed or not the MD biomarkers in PB and/or BM. Detection of MD in PB from stage 3 patients younger than 18 months could indicate lower OS. (p.value= 0.0517)
Conclusion: No significant association was found between MD detection in PB and BM and survival of all non-metastatic Neuroblastoma patients. MD detection by QRT-PCR in PB from stage 3 NB patients could be useful for predicting outcome and for early and sensitive relapsing disease detection. Stage 1 and 2 Neuroblastoma patients showed high survival rates and minimal disease was detected in a small number of patients being non-contributory for predicting patient outcome

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POC51

Clinical report on the treatment of children in the late stage of neuroblastoma using chemotherapy combined with Zhongluo 3

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Objective: To evaluate the effect, toxicity and lifecycle of small-dose chemotherapy combined with traditional Chinese decoction-Zhongluo3(L3) in treating children in the late stage of neuroblastoma (NB).
Methods: Forty-four children with NB in the 3rd or 4th stage were treated with VP, VCP, or VAP chemotherapy combined with L3.
Results: Twenty-five children got complete remission (CR), 7 got partial remission (PR). The total efficiency was 72.72% in this group, and no side effect related to death was found. With follow-up of 3-24 years, free-survival rate was found to be 51.3% within 3 years, 41.0% within 5 years, and 30.8% within 10 years.
Conclusion: The combination of small-dose chemotherapy with L3 is safe and effective in the treatment of children with NB in the 3rd or 4th stage, and the survival rate was found to increase greatly.

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Posters – Late breakers
POLB1–POLB13

POLB1

Effect of retinoic acid and chemotherapeutic agents on ultrastructural localization of Myc-N in neuroblastoma

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Purpose: Neuroblastoma is an important pediatric tumor that myc-n amplification is a well known poor prognostic indicator. The effect mechanism of pharmacological agents used in neuroblastoma treatment on myc-N expression is still unclear. Myc-N amplification does not change with any agent. The aim of this study is to investigate the effect of chemotherapeutic agents and cisplatin on ultrastructural localization of myc-N in neuroblastoma.
Method: We analyzed ultrastructural localization changes of n-myc by immunoelectron microscopy in n-myc positive, Kelly human neuroblastoma cell line using retinoic acid and cytotoxic drugs (cisplatin, vincristine, cyclophosphamide, etoposide, doxorubicin) and their combinations incubated for 24 hours in preoptimised LD50 doses in cell culture compared with control conditions. Cells were fixed in gluteraldehide fixative and n-myc was applied by immunoelectron microscopy method using colloidal gold for visualization. Results were scored semiquantitatively as negative, mild, moderate, or high positive in nucleus, ribosome and cell membrane.
Results: Immunogold particles labeling myc-N was high in nucleus, ribosomes and low in cell membrane in control without any drug. It was moderate in nucleus in retinoic acid, cyclophosphamide, etoposide, cisplatin and their combinations groups. The nuclear expression was mild in, vincristine, doxorubicin and their combinations groups. N-myc expression was negative in cell membrane in all drugs. It was negative in ribosomes in all combination groups and doxorubicin and retinoic acid combined with vincristine group. Immunoelectron microscopic results showed that chemotherapeutic agents and their combinations caused a prominent decrease in myc-N expression in cell membrane, a medium level decrease in ribosomal level and a low decrease in nuclear ultra structural localization.

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POLB2

Betulinic acid affects metastasis related genes in neuroblastoma cells

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Purpose: Betulinic acid is a pentacyclic triterpene found in many plant species. BA was reported to display a wide range of biological effects, including antibacterial and anti-inflammatory activities, and in particular to inhibit growth of cancer cells. The aim of this study is to explore effect of cisplatin, betulinic acide and their combination on metastasis related genes in neuroblastoma.
Methods: Kelly (N-Myc positive) and SHSY5Y (N-Myc negative) neuroblastoma cell lines were cultured and the agents and their combinations were applied for 24 hour in pre-optimized doses. After RNA isolation and cDNA converting, expression of 84 custom array genes of tumor metastasis (SABiosciences, PATS028A) was determined by Real Time PCR for each condition. Kelly and SHSY5Y neuroblastoma cell lines without any agents were used as control. Fold changes according to control group of each three condition were calculated at manufacturer's online free data PCR expression analysis page.
Results: High expressed genes after betulinic acide application are only PNN in SHSY5Y cells and most of the metastatic genes in Kelly cells. Betulinic acide, cisplatin or the combinations showed in low expression most of the metastatic genes in SHSY5Y cells.Cisplatin and betulinic acide combination was showed same as betulinic acide metastatic gene expression pattern in each cell line. Cisplatin caused increases only in adhesion associated FXYD5 in Kelly while DENR, SMAD and PNN in SHSY5Y cells. In Kelly cells, cisplatin decreased some metastatic gene expressions but betulinic acide or betulinic acide-cisplatin combination reduced only in DENR and RB1 gene expressions.
Conclusion: Betulinic acide showed prominent effect of gene expression related with tumor metastasis after application to neuroblastoma cells alone and combination with cisplatin in particularly N-myc positive Kelly neuroblastoma cells. Contrarily, betulinic acide and combinations decreased the metastatic gene expressions like as cisplatin in N-myc negative SHSY5Y neuroblastoma cells. These results suggesting that cisplatin is the most relevant agent in especially aggressive neuroblastoma treatment.

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POLB3

Human neuroblastoma microenvironment supports T-cell activation in tumor associated lymphocytes

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Background: Although lymphocytic infiltration has been previously linked to increased survival of patients with neuroblastoma (NB), the development of these tumors was not prevented by the presence of T cells in their microenviroment, suggesting that either the tumor milieu is not permissive for T cell activation, or lymphocytic NB infiltrates are devoid of T-cells, able to efficiently control tumor progression. Here, we report systematic analysis of T-cell subsets present in the peripheral blood and tumor samples from NB patients, representing all clinical and genetic forms of the tumor. We also characterize the pattern of cytokine production by T-cells in both compartments.
Method/approach: Analysis of T-cell phenotype was done by immunostaining and flow cytometry. Neuroblastoma-infiltrating T-cells were visualised by immunohistochemistry. In vitro cytokine production was measured using multiplex analysis and ELISA. All patients followed for >3 years.
Results: This study shows that tumor-associated lymphocytes (TALs) from NB lesions and peripheral blood lymphocytes (PBLs) analyzed on the day of tumor excision differ in their subset composition, phenotype and functional characteristics. The NB microenvironment promoted accumulation of CD3+CD8+ T-cells with activated effector memory phenotype. Ex vivo stimulation with autologous tumors increased the proportion of effector memory T-cells and led to upregulation of CD25 and downregulation of TGFƒÒƒn and FoxP3 expression in short term cultures of PBLs. In situ proliferation and characteristic pattern of T-cell receptor aggregation at the contacts with tumor cells were revealed by immunostaining of TALs in tumor biopsies.
Conclusion: Our results are consistent with ongoing specific activation of CD8+ T-cells in NB microenvironment, which appears to be permissive for effector memory T-cell differentiation and suppress the development of regulatory T-cells.

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POLB4

Expectant management of congenital adrenal neuroblastoma

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Background: There are no established guidelines for the management of congenital adrenal neuroblastoma. We describe our single institution experience on managing this tumour with a wait-and-see policy.
Methods: Thirteen consecutive patients with adrenal mass detected antenatally or within the first 3 months of life were enrolled between February 2002 and March 2010. Diagnostic work-up included a combination of the following investigations: urinary catecholamine metabolites, imaging studies (US, MRI or CT scan), MIBG scan or trucut needle biopsy.
Results: Male to female ratio was 1.6 to 1. Median tumour size at presentation was 29 mm (range, 10 to 50). Overall, 7 of 13 lesions were antenatally detected. Ten lesions were diagnosed as localised neuroblastoma (by urinary catecholamines, MIBG or needle biopsy). Four of these were excised because of parental preference (n=2), tumour persistence over 10 mos (n=1) or enlargement (n=1). The remaining 6 patients uneventfully underwent watchful clinical observation, which showed progressive tumour shrinking and complete regression within a period ranging from 8 to 39 months (median 12). Regression started at a median time of 3.5 months (range 2-5). Furthermore, 3 small adrenal lesions without clear-cut diagnosis were managed non invasively. All 13 patients are alive and disease-free at mean follow-up of 59 months (range 2-101, median 73). One of the 10 localised neuroblastoma progressed to stage 4 despite early surgery performed at 28 days of age because of tumor enlargement. The child received high-dose chemotherapy with stem cell rescue and is disease-free 2 months after transplantation.
Conclusions: Congenital adrenal neuroblastoma carries a good outcome in most cases. A wait-and-see strategy seems a safe and effective approach that reduces unnecessary surgical procedures. In cases with a large tumour and/or tumour enlargement early surgery can be recommended, although in our patient this approach did not prevent tumor progression. Prolonged expectant management of stable and persistent lesions for more than several months may not to be cost-effective.

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POLB5

Allicin increases metastasis related genes in neuroblastoma

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Purpose: Overcoming of toxic effects of cisplatin in neuroblastoma treatment is a current issue. The try of discovering new agents to decrease toxicity of chemotherapeutic agents needs very much careful assessment not to cause tumor progression. The aim of this study is to explore effect of cisplatin, allicin and their combination on metastasis related genes in neuroblastoma.
Method: Kelly cell line was cultured and the agents and their combinations were applied for 24 hour in pre-optimized doses. After RNA isolation and cDNA converting, expression of 84 custom array genes of tumor metastasis (SABiosciences, PATS028A) was determined by Real Time PCR for each condition. Kelly neuroblastoma cell line without any agent was used as control. Fold changes according to control group of each three condition were calculated at manufacturer's online free data PCR expression analysis page.
Results: High expressed genes after allicin application are ITGB3, TNFSF10, HGDC, CCL7, CTSL1, ETV4, KISS1R, HTATIP2, IL1B, IL8RB, ITGA7, KISS1, MMP10, MMP3, MMP7, MYC, MYCL1, SYK, TIMP4, MMP13, TRPM1, CDH1, and FXYD5. The high expressed genes are related with cell growth and proliferation, extracellular matrix proteins, transcription factors. This high expression was not observed alone with cisplatin, but also observed in cisplatin+allicin combination. Cisplatin alone caused decrease in expression of EPHB2, TIMP3, CDH6, RORB, COL4A2, IL18, CDH11, MGAT5, CTNNA1, RB1, CD44, MTA1, VEGFA; genes related with extracellular matrix proteins, cell growth and proliferation but also cell adhesion molecules.
Conclusion: Allicin showed prominent effect of gene expression related with tumor metastasis after application to neuroblastoma cells alone and in combination with cisplatin application. Our data showing increase effect of allicin on metastasis related genes indicates that allicin additive treatment as a protective agent of chemotherapy toxicity should be very well questioned in neuroblastoma.

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POLB6

Diagnostics and treatment of children with localized and locally advanced thoracoabdominal neuroblastoma

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Aim: to improve the results of treatment of children with localized and locally advanced thoracoabdominal neuroblastoma.
Methods: 54 patients were enrolled between 2008 and 2010. There were 14 stage I patients, 25 stage II patients and 15 stage III patients. After evaluation included imaging, MIBG, bone marrow morphology, molecular studies, immunohistochemistry all patients proceeded to surgery/ biopsy. After patholgy report INSS and risk group was assigned which directed further radio and chemotherapy with VP-16, CBCDA, CPM and doxorubicin, according to COG Ð9641, À3961 protocols.
Results: all 54 patiens are alive without evidence of metastasis and/or progression of the disease with a followup of 2 to 24 months.
Conclusion: decrease of intensity and length of treatment does no compromise survival of patients with localized and locally advanced neuroblastoma.

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POLB7

Modeling the p53-Mdm2 core module in neuroblastoma

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Background: The p53-MDM2 regulatory unit controls the cellular decision to undergo apoptosis or cell-cycle arrest upon DNA damage. This p53-MDM2 core module is frequently altered in neuroblastoma leading to impaired cell-cycle arrest and DNA-damage response. Unlike in other tumor entities, the aberrant function is rarely attributed to genetic mutations. It appears to be due to an imbalance in the expression and function of p53 and MDM2, which is influenced by deregulated N-MYC.
Aims: To rationalize how deregulated N-MYC perturbs the p53-MDM2 core module (upon DNA-damage) using a systems biology approach.
Method: To describe N-MYC-dependent perturbations of the p53-MDM2 module, we used cell systems that allow targeted overexpression or knock down of N-MYC and developed a data-based kinetic model. By means of ordinary differential equations the dynamics of p53, MDM2 as well as cell-cycle (e.g. p21) and apoptosis-related (e.g. PUMA) p53 targets are simulated.
Results: Our model simulations qualitatively reproduce the observed changes of the mRNA and protein measurements. First results indicate that the interplay of p53, MDM2, E2F1 and N-MYC can account for the experimentally observed dynamics upon induction of DNA-damage in *N-MYC* single-copy versus amplified tumor cells. Simulations suggest a dominant role of MDM2 in disabling the DNA-damage response by directly inhibiting both p53 and p21.
Conclusion: The mathematical model is capable of qualitatively reproducing the observed protein kinetics. Although in *N-MYC* amplified cells both p53 and MDM2 are overexpressed, MDM2 appears to be dominant for the p21 response upon DNA-damage.

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POLB8

Biological characteristics of neuroblastoma in children of Belarus.

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Background: To evaluate the biological characteristics of neuroblastoma in Belarusian children and to estimate there prognostic value.
Method/approach: 101 patients (pts) received treatment from October 1997 till October 2009 were included into the study. N-MYC amplification, 1p aberration and ploidy of tumor cells were assessed by FISH method.
Results: N-MYC amplification was evaluated in 101 pts: negative n=70(69.3%); less than 10 copies n=8(7.9%); 10 copies and more n=23(22.7%). N-MYC amplification was detected in 2/18 pts with stage 1; 2/8 pts with stage 2; 5/32 pts with stage 3; 14/38 pts with stage 4; 0/5 pts with stage 4S. N-MYC was detected in 10/32 pts with localization of primary tumor in adrenal gland. Aberration 1p was evaluated in 52 pts: positive n=9(17.3%); negative 43(82.7%). 5/9 pts with 1p had stage 4. Combination of N-MYC amplification and 1p aberration was found in 7/9 pts. 5 years PFS in N-MYC negative pts (n=70) is 72%+ 5%. It was better (p<0,001) than PFS in pts with N-MYC. 5-years PFS in pts with N-MYC (n=23) is 29%+9%. Ploidy of tumor cells was evaluated in 63 pts. Pts were divided into 3 groups depending on tumor ploidy: near-diploidy and near-tertraploidy n=51pts (80.9%); near-triploidy n=8(12.6%) and group of heterogeneous tumor n=4(6.3%). Local form and younger age were observed more often in group with near-triplody tumor (p=0.025). Adverse prognostic markers as N-MYC amplification and 1p aberration have not been found in this group.
Conclusion: In children of Belarus with neuroblastoma adverse prognostic tumor markers (N-MYC amplifications and 1ð deletion) observed in 25.9% that correlate with the data in the world. Occurrence of N-MYC amplification associates with disease stages increasing at the metastatic form. Patients with N-MYC amplification in a tumor and/or a bone marrow have significantly poorer prognosis and should receive high risk treatment. Near-diploidy and near-tetraploidy are prevalent in children with neuroblastoma. Near-triploidy is correlated with better prognosis and favorable prognostic factors (younger age, local stage of disease, absence of N-MYC and 1p).

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POLB9

Treatment results for neuroblastoma in children of Belarus

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Background: To evaluate the treatment results for neuroblastoma in children.
Method/approach: 155 patients (pts) were included since October 1997 till October 2009. Age median was 1,72 years (from 12 days till 12 years), pts under 1 year of age n=55 (35,4 %). Pts enrolled and observed n=137 (88.4%), pts excluded from the protocols n=18 (11.6%). Treatment protocols were the following: NB 1999 02, UK Trial: ENSG 9, STAGE 2B\3 (NB 9502) (for patients with III and II stages at progression); protocol EINS (for children under 1 year). They were used till 2008 year. PFS and OS were calculated by Kaplan-Meier method.
Results: PFS depending on a stage of disease was the following: I, II, IVs stages (group1, n=40) is 90%, median follow up (Mf)=78,13mo; III stage (group2, n=29) is 64%, Mf=48,40mo; IV stage (group 3, n=31) is 36%, Mf=19,60mo; p(1-2)=0.007, p(1-3)<0.001. PFS depending on a stage of disease in children under 1 year old was the following: group 1 (n=19) is 95%, Mf=71.62mo; group 2 (n=9) is 78%, Mf=73.77mo; group 3 (n=6) is 63%, Mf=22, 60mo; p(1-2)=0.031. PFS depending on a stage of disease in children older than 1 year was the following: group 1 (n=20) is 85%, Mf=90.9mo; group 2 (n=20) is 57%, Mf= 34.8mo; group 3 (n=25) is 30%, Mf=19,60mo; p(1-2)=0.045, p(1-3)=0.002. 5-year PFS for patients with stage IV depending on the tumor volume resection: total resection (n=16) is 45%, Mf 41.92mo; subtotal resection and biopsy (n=14) is 21%, Mf=7.67mo (p is n.s.). The same tendency founded for N-MYC positive patients. PFS for stage IV depending on the performed AutoSCT: with AutoSCT (n=18) is 48%, Mf=44,32mo; without AutoSCT (n=13) is 12%, Mf=20,83mo (p=0.01).
Conclusion: Patients with neuroblastoma with I, II, IVs disease stages have the best results of treatment. 12-year PFS in stages III and IV is unsatisfactory that is correlated with the world results and demand further improvement of therapy approaches. Total resection of tumor seems to improve PFS in pts with neuroblastoma including pts with N-MYC amplification. AutoSCT significantly improves PFS in pts with IV stage.

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POLB10

Autochthonous TNF alpha as an inducer of immune resistance and survival of neuroblastoma

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Background: Tumor necrosis factor (TNF) alpha, originally described as a death-inducer in tumor cells, is also known as an effector molecule of NK-cells and cytotoxic T-lymphocytes (CTL) involved in tumor rejection. However, accumulating evidence suggests that TNF can also be a tumor promoting factor. We observed expression of membrane-associated TNF on the surface of neuroblastoma (NB) cells derived from humans or mice.
Method/approach: Expression of TNFalpha, TNF-R1 and -2 in human NB lines and mouse short-term NB cell lines derived from TH N-MYC mice was monitored by immunostaining and flow cytometry. Inhibition of TNF expression and/or function was achieved by specific shRNAs, blocking antibody and soluble TNF-R2. Sensitivity of human neuroblastoma cells to CTLs and natural killer (NK) cells was determined in cytotoxicity assays. Viability and proliferation of human and mouse neuroblastoma cells were determined by flow cytometry-based methods.
Results: The majority of established human NB lines and spontaneous NB tumors from N-MYC transgenic mice express TNF at the cell surface. TNF is expressed on the surface of NB cells in the membrane-bound form and continuously occupies a large proportion of TNF-receptors expressed on the surface of NB cells. TNF blockade results in inhibition of proliferation, increased rate of apoptosis and increased sensitivity of NB cells to lysis by the cytotoxic lymphocytes.
Conclusion: TNF alpha acting in an autochthonous fashion initiates intracellular signaling, which promotes tumor growth and/or survival and results in decreased susceptibility of neuroblastoma cells as targets for recognition by the immune system.

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POLB11

Identifying lesions in translational control of gene expression in neuroblastoma by mRNA polysomal profiling and information-intensive computational integration

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In neuroblastoma, as well in other tumor types, microarray-based genome-wide measurements of copy number alterations and of transcriptome variations lack to provide clues on a neglected layer of systems-level changes, the “translatome”, whose variation is defined by the activity of the translational regulation machinery. Our study extends the conventional omics approaches to translatome profiling in neuroblastoma, by means of polysomal sucrose gradient separation and microarray analysis (POL-CHIP method).
A panel of 16 parental (not subcloned) neuroblastoma cell lines was assayed at the levels of genome, transcriptome, translatome and miRome analysis. In order to initially explore the differences in gene expression changes between transcriptome and translatome, we applied a hierarchical clustering algorithm to the resulting dataset, followed by cluster similarity measurements. Gene expression variations between the two levels resulted generally coupled, but large deviations from this trend were captured by principal component analysis residuals, defined as the differences between observed polysomal mRNA signals and the expected ones on the basis of the corresponding total mRNA signals. Uncoupled genes (genes bearing large deviations from the general trend) are thus likely targets of disrupted translational regulation mechanisms. These genes were subjected to feature selection in order to identify putative upstream common lesions, and were correlated with copy number alterations in order to establish whether these lesions could be related to allelic gains or losses. We finally integrated the prioritarized uncoupled genes with miR variations, to assess the relative role of these non-coding RNAs on the process.
Profound differences in clustering between polysomal and total mRNA profiles, and strong correlation patterns of uncoupled genes with copy number alterations and miRs clearly indicate that the impact of genomic aberrations on translational control should not be underestimated. These findings indeed suggest that translatome profiling would greatly increase informativity of gene expression signatures in neuroblastoma.

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POLB12

An integrative bioinformatics approach in neuroblastoma identifies converging alterations in protein networks related to mitotic spindle assembly and splicing

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Genome-wide studies, profiling either DNA copy number or gene expression, are importantly proposed for neuroblastoma prognosis and possible treatment choice. Nonetheless, it is still largely unclear how to integrate these systems-level molecular portrait types of tumor genetic instability in order to obtain the maximal informative power.
We suggest a novel unbiased way to combine matching aCGH and microarray transcriptome analysis from single neuroblastoma tumor samples, initially applied to public domain data. At the core of our approach is the ability to use both data types in an unbiased and unsupervised fashion, identifying statistically significant high correlation modules. Each module associates copy number alterations and genes whose expression follows a common profile in different tumors, therefore representing putative cause-effect genome-transcriptome maps. Enrichment analysis through gene ontology categories was conducted on the significant modules and the derived results were used to isolate the maximally informative genes. Remarkably, increased expression of each of these genes significantly correlated with the clinical outcome of the profiled patients, and the corresponding proteins were all interacting in a functional network. A panel of parental neuroblastoma cell lines profiled at high resolution for copy number alterations allowed us to more precisely define the genomic lesions highly related to the informative genes, and to visualize the corresponding binary network.
This integrative analysis led to the formulation of an unanticipated hypothesis on converging genomic alterations in neuroblastoma, polarized on the upregulation of several genes active in the spindle assembly process and in the spliceosome complex. We also report a phenotypic analysis in neuroblastoma cells confirming functionally impacting alterations in both cell machineries, therefore highlighting the power of an unbiased integration of genome and transcriptome data.

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POLB13

Induction of human embryonic stem cells into sympathoadrenal cells

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Background/Aims: Neuroblastoma originates from sympathoadrenal precursor cells, and has a broad spectrum of clinical presentation associated with the degree of tumor differentiation. Amplification of MYCN, a proto-oncogene involved in SNS differentiation, is observed in a third of children with aggressive neuroblastomas, characterized by poorly differentiated neuroblasts. Despite the prominence of MYCN in developmental pathways and neuroblastoma, mechanisms of its pathogenesis in human developmental models are poorly understood, and MYCN oncogenesis studies have been limited to primary tumors, cell lines or MYCN driven mouse models. Utilization of human embryonic stem cells (hESCs) for studying neuroblastoma development is advantageous by directly addressing the effects of oncogene deregulation in cell types believed to be the origins of neuroblastoma. Use of such a model, however, is limited by the lack of established protocols for inducing sympathoadrenal differentiation in hESCs. We aim to improve protocols for hESCs into sympathoadrenal cells and plan to genetically manipulate these cells to investigate the role of oncogenes such as MYCN in normal sympathoadrenal cell development and neuroblastoma induction.
Methods: We induced neural crest precursor cells from H9 hESCs, using established protocols. These cells were treated with combinations of BMP4, BMP2 and NGF to induce sympathoadrenal differentiation. Combinations were compared in their ability to induce a sympathoadrenal cell phenotype by qRTPCR, flow cytometry and Cellomics high-content screening.
Results: Treatment with BMP4 and BMP2 significantly increased the median fluorescent intensity of Tyrosine Hydroxylase (TH) staining by flow, 2.2 +/- 0.3 times. Expression of TH along with peripherin increased 2.6 times as measured by high-content screening. Similarly the combination increased neural crest markers ASCL1 and Phox2b and sympathoadrenal markers chromogranin A and dopa decarboxylase by qRTPCR.
Conclusions: Recovery of sympathoadrenal cell phenotypes is improved by treatment with BMPs 4 and 2. This improved recovery will facilitate study of neuroblastoma development.

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