WELCOME TO TORONTO!

The Local Organizing Committee is thrilled to be hosting you here in our great city and has worked tirelessly to put together what we hope you find is a solid and thought provoking conference program.

We have brought together more than 500 delegates made up of scientists, clinician scientists, clinician investigators, nurses, allied health professionals, students and parents from 32 countries to this Conference to share their research and experiences in our battle against Neuroblastoma.

I look forward to meeting with you over the course of the Conference and that wherever you are coming from, you feel at home.

On behalf of the local organizing committee, and with special thanks to Meredith Irwin, David Kaplan, Denise Mills, and Shelley Anderson for their tremendous work in helping develop the program,

Sylvain Baruchel
Chair, ANR 2012

CONGRATULATIONS TORONTO! FOR HOSTING ANR 2012

ANR Advances in Neuroblastoma Research Association

THE ANR 2012 CONFERENCE WOULD LIKE TO THANK THE FOLLOWING ABSTRACT REVIEWERS FOR THEIR TIME & EFFORT.

THANK YOU!

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The Garron Family Cancer Centre is pleased to support the Advances in Neuroblastoma Research 2012 conference in Toronto. The Centre is designed to enhance and integrate all aspects of clinical, research and educational activities in the discipline of oncology at The Hospital for Sick Children (SickKids). The Centre's focus is to facilitate and catalyze innovative, multidisciplinary research in order to transform clinical care and dramatically improve clinical outcomes for children with cancer. SickKids currently treats more than 25 per cent of all children diagnosed with cancer in Canada including the majority of neuroblastoma patients. The Garron Family Cancer Centre is proud to sponsor an Experimental Therapies session as well as co--sponsor the Nursing Symposium at ANR 2012.

SickKids® | Garron Family Cancer Centre

Proud Supporter of ANR 2012 Conference

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PROGRAMME BOOK

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COMMITTEES*

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*as at spring 2012.

ACKNOWLEDGEMENTS & **SPONSORS**

THE ANR 2012 CONFERENCE WOULD LIKE TO THANK THE FOLLOWING SPONSORS FOR THEIR GENEROUS SUPPORT AND CONTRIBUTIONS:

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Dr. David Kaplan

SOCIAL PROGRAMME

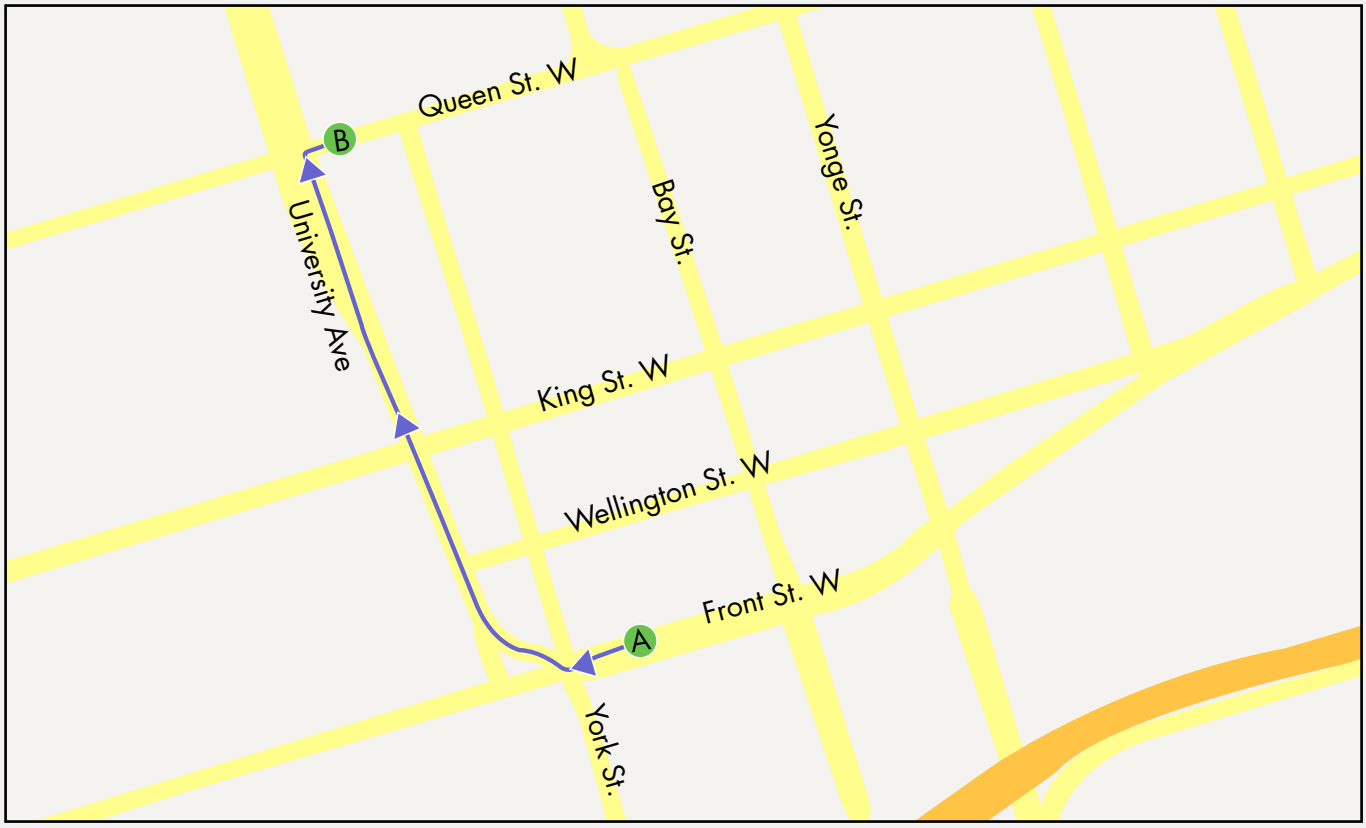
WELCOME RECEPTION

The Welcome Reception will be taking place at the Four Seasons Centre for the Performing Arts on Monday, June 18th at 6:45 p.m. and will include a cocktail-style reception and entertainment by Sistema-Toronto and Tafelmusik Baroque Orchestra.

The Four Seasons Centre for the Performing Arts is located at 145 Queen St. W., on a full city block bordered by University Avenue, Queen, Richmond and York streets. The main entrance is at the southeast corner of Queen and University. The Four Seasons Centre for the Performing Arts is a 10 minute walk from the Fairmont Royal York Hotel.

WALKING DIRECTIONS FROM THE FAIRMONT ROYAL YORK:

- Head west on Front Street West to University Ave.
- Turn north (right) onto University Ave until you arrive to Queen Street West
- The reception is open to registered participants and registered accompanying guests, only if selected on the registration form. Please bring your name badge.



Ⓐ The Fairmont Royal York Hotel Ⓑ The Four Seasons Centre for the Performing Arts

GALA DINNER

The Gala Dinner will take place at The Fairmont Royal York in the Concert Hall on Wednesday, June 20th at 7:00pm. The evening will feature a scrumptious Canadiana dinner paired with local wines along with interactive entertainment.

REGISTRATION DETAILS

DELEGATE* FULL CONFERENCE RATE INCLUDES:

- Admission to the Conference, commercial exhibition, and poster exhibition
- Conference documentation
- Daily breakfast, coffee/tea breaks and lunches
- Welcome Reception at Four Seasons Centre for the Performing Arts on Monday, June 18, 2012
- Gala Dinner at The Fairmont Royal York on Wednesday, June 20, 2012

DELEGATE* CONFERENCE ONLY RATE INCLUDES:

- Admission to the Conference, commercial exhibition, and poster exhibition
- Conference documentation
- Daily breakfast, coffee/tea breaks and lunches
- Welcome Reception at Four Seasons Centre for the Performing Arts on Monday, June 18, 2012

TRAINEE/NURSE/ALLIED HEALTH PROFESSIONALS** CONFERENCE RATE INCLUDES:

- Admission to the Conference, commercial exhibition, and poster exhibition
- Conference documentation
- Daily breakfast, coffee/tea breaks and lunches
- Welcome Reception at Four Seasons Centre for the Performing Arts on Monday, June 18, 2012
- Does not include admission to the Gala Dinner at The Fairmont Royal York on Wednesday, June 20, 2012. Tickets can be purchased for $150 CDN at time of registration.

NURSING SYMPOSIUM INCLUDES:

- Admission to the Nursing Symposium only taking place on Tuesday, June 19, 2012 from 1:00pm to 5:00pm

PARENT SYMPOSIUM INCLUDES:

- Admission to the Parent Symposium only taking place on Tuesday, June 19, 2012 from 5:30pm to 7:00pm
- A Reception will follow after the Parent Symposium. The Symposium and Reception are free to attend for neuroblastoma parents and families.
- For admission to the Conference, please register under the Trainee/Nurse/Allied Health Professionals** Conference Rate

GALA DINNER TICKET*** INCLUDES:

- Welcome Reception at Four Seasons Centre for the Performing Arts on Monday, June 18, 2012
- Gala Dinner at The Fairmont Royal York on Wednesday, June 20, 2012

PLEASE NOTE
Accompanying Guests must purchase a Gala Dinner ticket in order to have access to the Welcome Reception.

**scientists, research associates, and physicians*

***graduate students, post-doctoral fellows, nurses, pharmacists, nutritionists, and social workers*

****Accompanying Guests must purchase a Gala Dinner ticket in order to have access to the Welcome Reception.*

GENERAL INFORMATION

BADGES
Each participant will receive a name badge upon registration onsite at the ANR Conference registration desk at the Fairmont Royal York Hotel. For security reasons all participants are requested to wear their badge for the duration of the conference including all activities and social events.

BANKS, CREDIT CARDS AND CURRENCY EXCHANGE
Most banks in downtown Toronto are open between 8.00 and 17.00 on weekdays. Some banks in downtown Toronto are open on Saturdays. Major credit cards are accepted in hotels, restaurants, and shops. It is advised to carry a form of photo identification.

The official currency is the Canadian Dollar (CAD). For denominations under five dollars, coins are used. USD 1 = CAD 0.99, EUR 1 = CAD 1.30 (April, 2012). Cash machines/ATMs can be found in airports, most banks, hotels, and shopping centres. Currency exchange is available at banks and kiosks throughout the city and at the airport.

CLIMATE AND DRESS
The weather in Toronto at this time of the year is usually warm and sunny with temperatures of approximately 15-20 degrees Celcius; showers may occur. The conference attire is business casual.

DISCLAIMER/LIABILITY
The ANR 2012 Organizing Committee and One to One Communications 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 Canada is 110 V/60 Hz. Appliances designed to operate on 120/220 Volts need a voltage converter and a plug adapter.

SPONSOR/EXHIBITION OPPORTUNITIES
More information on sponsorship/exhibition opportunities can be obtained from the ANR 2012 Sponsorship Brochure by visiting www.anr2012.com or from ANR2012@onetoonecommunications.ca.

INTERNET
Guests of the Fairmont Royal York will enjoy complimentary high speed internet in all guestrooms as well as wireless internet access in public areas such as lobbies and lounges.

OFFICIAL CONFERENCE ORGANIZER
One to One Communications has been appointed the official conference organizer for this conference. One to One Communications offers full conference and event management services for associations and corporations across Canada as well as in the US and Europe. For more information, please visit www.onetoonecommunications.ca

GENERAL INFORMATION

ON-SITE REGISTRATION AT THE FAIRMONT ROYAL YORK

On-site registration will start on Sunday, June 17th at 12:00 p.m. The registration desk will be open as follows:

Sunday, June 17:	12:00 p.m. - 7:00 p.m.
Monday, June 18:	7:00 a.m. - 7:30 p.m.
Tuesday, June 19:	7:00 a.m. - 5:00 p.m.
Wednesday, June 20:	7:00 a.m. - 5:00 p.m.
Thursday, June 21:	7:00 a.m. - 4:00 p.m.

TIME ZONE

The time zone in Toronto is GMT - 5 hours. Daylight Saving Time is used during the summer.

TIPPING

It is common to leave a tip of around 15-20% on the pre-tax bill when visiting restaurants, travelling by taxi, and other services such as haircuts.

TOURIST INFORMATION

One to One Communications will be available to give you more information about Toronto.
For additional information please contact Tourism Toronto, the official tourist guide of Toronto, at:
Web site: www.seetorontonow.com
E-mail: toronto@torsvb.com
Phone: +416 203 2500

TRANSPORTATION - ARRIVAL AT LESTER B. PEARSON INTERNATIONAL AIRPORT (YYZ)

Lester B. Pearson International Airport is located 25 km northwest of downtown Toronto. The airport express bus, Pacific Western Airport Express Bus, is the most economical way of travelling between Lester B. Pearson International Airport and downtown Toronto. The journey takes between 45 to 90 minutes and buses depart every 20 to 30 minutes, every day of the year. The bus stops at The Fairmont Royal York Hotel and the cost is $23.95 CAD per adult for one way fare and $39.95 CAD per adult for round trip fare.
For more information, visit www.torontoairportexpress.com

Airport taxis and limos are available outside the arrival platform of Lester B. Pearson International Airport. Prices may vary so you are advised to ask for the price before entering the vehicle. Many taxi/limo companies offer a fixed price or flat rate fare of $53 to $56 + tip for Taxi and $58 to $62 + tip for Limo from Lester B. Pearson International Airport to downtown Toronto and the driver can provide a receipt upon request (April, 2012). Debit cards are not accepted, and if paying by credit card, check with the driver in advance. When you are ready to leave the airport, follow the signs to the taxi queue or limo queue. Advance reservations are not required and the wait is typically very short.

GENERAL INFORMATION

TRANSPORTATION - ARRIVAL AT TORONTO CITY CENTRE AIRPORT (YTZ)

Toronto City Centre Airport is located 3 km southwest of downtown Toronto's financial district. To get between the island airport and the mainland, use the airport's complimentary ferry that makes its short run every 15 minutes. Note that this is not the same ferry as the city-operated Toronto Island ferries – you cannot walk between the City Centre Airport and the rest of the Toronto Islands. Once on the mainland, take the Porter Airlines Shuttle Bus directly to the Fairmont Royal York. There is no charge for this shuttle that runs every 10 minutes and the ride is less than 10 minutes.

Taxis are also available outside the mainland ferry terminal at the taxi queue. Prices range from $8 to $10 + tip from Toronto City Centre Airport to the Fairmont Royal York and the driver can provide a receipt upon request (April, 2012). Debit cards are not accepted, and if paying by credit card, check with the driver in advance. Advance reservations are not required and the wait is typically very short.

VISA AND INVITATION LETTER

Participants are advised to make their own arrangements with respect to entering Canada. Individuals requiring an official letter of invitation in order to obtain a visa and authorization to attend the Conference should contact ANR2012@onetoonecommunications.ca. The invitation letter will be issued once the registration form and payment has been received by ANR 2012 Inc.

SCIENTIFIC INFORMATION

POSTER SESSIONS

Posters will be displayed in the Canadian Ballroom on either Tuesday, June 19th or Wednesday, June 20th. Please see the abstract book for details on which day specific posters will be displayed. Time is set aside for poster viewing only and presenting authors of the abstracts are asked to stand by their posters at this time.

POSTER MOUNTING AND DISMANTLING

	Tuesday, June 19th	**Wednesday, June 20th**
Mounting:	7:00 a.m. – 12:00 p.m.	7:00 a.m. – 12:00 p.m.
Dismantling:	6:30 p.m. – 8:00 p.m.	6:30 p.m. – 8:00 p.m.

SPEAKERS ROOM AND AV INFORMATION

The Speakers Room is located in Salon B and will open as follows:

Sunday, June 17, 2012	1:00 p.m. – 7:00 p.m. (satellite Speakers Room by Registration Desk)
Monday, June 18, 2012	8:00 a.m. – 5:00 p.m.
Tuesday, June 19, 2012	8:00 a.m. – 5:00 p.m.
Wednesday, June 20, 2012	8:00 a.m. – 5:00 p.m.
Thursday, June 21, 2012	8:00 a.m. – 1:00 p.m. (satellite Speakers Room by Registration Desk)

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 24 hours before the start of their particular session.

Speakers are asked to come with their presentation on a USB stick in Microsoft PowerPoint (PPT) format.

Speakers presenting on Monday, June 18, 2012 from 09:00 - 11:30:
If you are coming from out of town and arrive after your scheduled time, be sure to visit the Speakers' Room at 08:00 on Monday, June 18th to hand over your presentation.

Speakers presenting on Monday, June 18, 2012 from 13:00 - 17:00:
If you are coming from out of town and arrive after your scheduled time, be sure to visit the Speakers' Room by 11:00 on Monday, June 18th to hand over your presentation.

AWARDS

Awards will be issued to the top oral presentation and to the top 3 posters as selected by the scientific committee. All awards will be announced during the Gala Dinner on Wednesday, June 20, 2012, and at the closing ceremony on Thursday, June 21, 2012.

FAIRMONT ROYAL YORK
MEETING ROOM MAPS

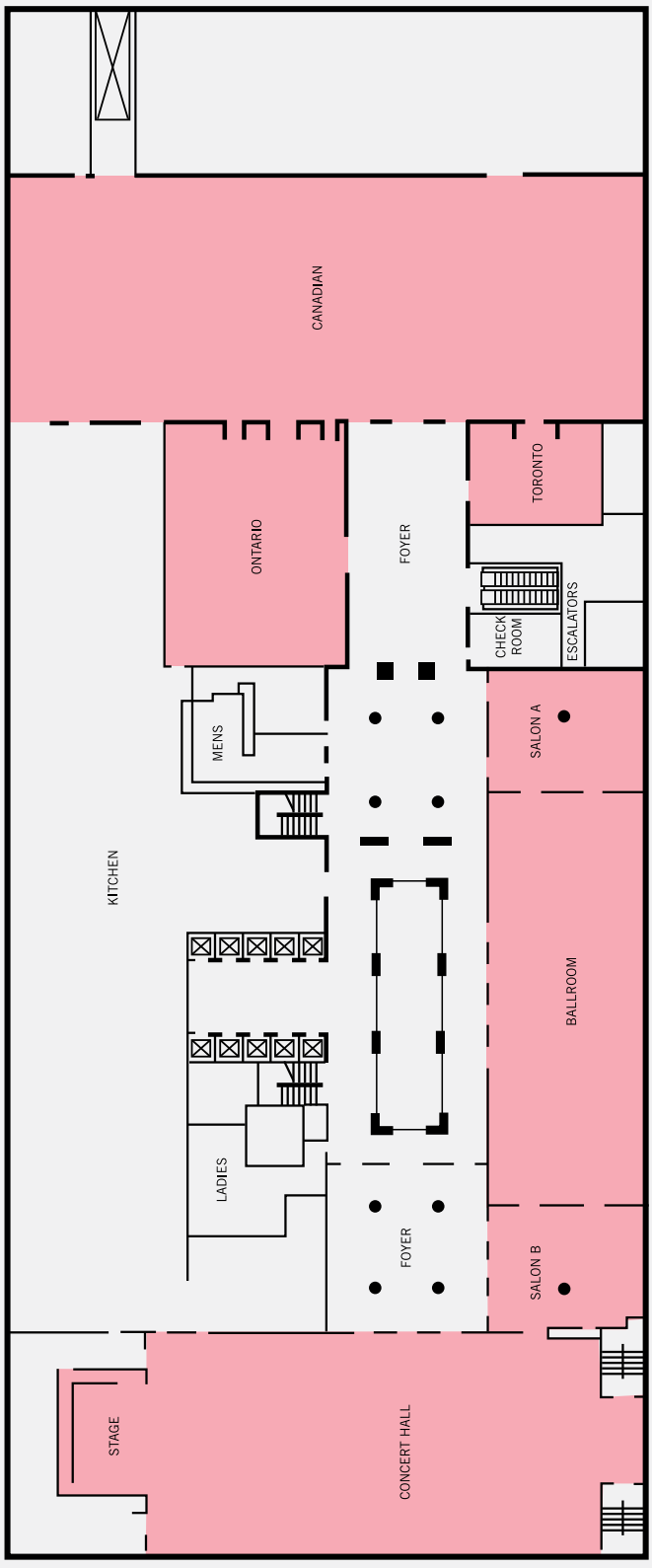
The meeting space for the ANR 2012 Conference is conveniently located on two adjacent floors at The Fairmont Royal York.

The Larger rooms for Plenary Sessions, some Parallel Sessions and Poster Display are located on the Convention Floor.

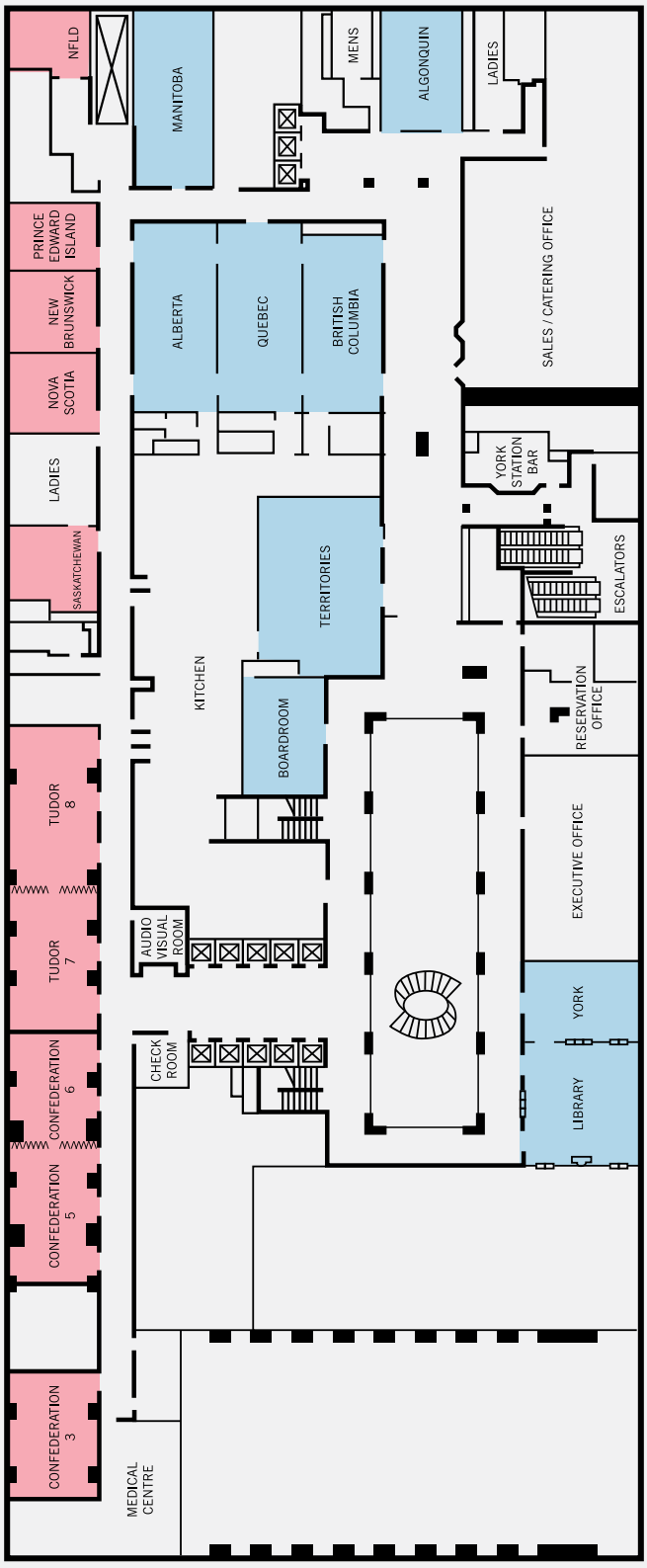
One floor below, on the Main Mezzanine Floor, you will find the rooms for some of the Parallel Sessions.

Please see the main Program for listings of the designated rooms for each session.

CONVENTION FLOOR



FAIRMONT ROYAL YORK
MEETING ROOM MAPS



MAIN MEZZANINE FLOOR

PROGRAMME

MONDAY, JUNE 18, 2012
09:00 – 11:30
IMPERIAL ROOM

Workshop 1: Precision Medicine: A Newly Revised INRC (3)
Organizer: Julie Park

09:00 - 09:10		**Introduction** Rochelle Bagatell
09:10 - 09:25		**Primary Site** Kieran McHugh and Jed Nuchtern
09:25 - 09:40		**Metastatic Site** Katherine Matthay
09:40 - 09:55		**Bone Marrow** Frank Berthold
09:55 - 10:15	**WS11**	**Consensus Statement on the Revised International Neuroblastoma Response Criteria** Julie Park, United States *Abstract: Page 71*
10:15 - 10:45		**Discussion**
10:45 - 11:00		**Phase 2** Julie Park and Dominique Valteau-Couanet
11:00 - 11:15		**Ultra High Risk** John Maris and Gudrun Schleiermacher
11:15 - 11:30		**Discussion**

MONDAY, JUNE 18, 2012
13:00 – 15:30
IMPERIAL ROOM

Workshop 2: Targeted Radiopharmaceutical Therapy of Neuroblastoma
Organizers: Sylvain Baruchel and Katherine Matthay
Chairs: Gregory Yanik and Katherine Matthay

13:00 - 13:20	**WS21**	**Targeting the norepinephrine transporter for imaging and therapy of high risk neuroblastoma** Katherine Matthay, United States *Abstract: Page 71*
13:20 - 13:35	**WS22**	**Pretherapy dosimetry with I-124 MIBG PET/CT** Youngho Seo, United States *Abstract: Page71*
13:35 - 13:50	**WS23**	**Pre-clinical testing of radiosensitizers with MIBG** Robert Mairs, United Kingdom *Abstract: Page 72*
13:50 - 14:05	**WS24**	**Clinical Trials of 131I-metaiodobenzylguanidine (MIBG) with Concomitant Radiation Sensitizers for the Treatment of Neuroblastoma** Steven DuBois, United States *Abstract: Page 72*
14:05 - 14:20	**WS25**	**Lu-177-DOTATATE for targeted therapy of neuroblastoma** Mark Gaze, United Kingdom *Abstract: Page 72*
14:20 - 14:35	**WS26**	**Development of α-particle emitting meta-[211At]-astatobenzylguandidine for neuroblastoma therapy.** John Maris, United States *Abstract: Page 73*
14:35 - 14:50	**WS27**	**Comparison among 18F-FDOPA PET, 18F-FDG PET, and 123I-MIBG scan in neuroblastic tumors** Yen-Lin Liu, Taiwan *Abstract: Page 73*
14:50 - 15:05	**WS28**	**Development of a MIBG treatment facility, nuts and bolts** Suzanne Shusterman, United States *Abstract: Page 73*
15:05 - 15:30		**Panel Discussion**

MONDAY, JUNE 18, 2012
13:00 – 15:30
CONFEDERATION 5/6

Workshop 3: Cellular and Animal Models of Neuroblastoma: Searching For The Most Predictive Pre-clinical Models
Organizers: Meredith Irwin, David Kaplan, Michael Hogarty, and Patrick Reynolds

13:00 - 13:10		**Introduction** Meredith Irwin

Session 1: Cellular Models
Chairs: Michael Hogarty and David Kaplan

13:10 - 13:25		**Biological and methodological parameters to consider for implementing models to study preclinical therapeutics of neuroblastoma** Patrick Reynolds, United States
13:25 - 13:35	**WS31**	**Establishing validated neuroblastoma cell lines and xenografts from post-mortem blood samples** Tito Woodburn, United States *Abstract: Page 74*
13:35 - 13:45		**TIC and cancer stem cell models: do we need new models for neuroblastoma?** David Kaplan, Canada
13:45 - 13:55	**WS32**	**Establishment of a condition for tumor sphere formation from primary neuroblastoma tissues** Cao Dongliang, Japan *Abstract: Page 74*
13:55 - 14:05	**WS33**	**Functional Profiling of Neuroblastoma Tumor-Initiating Cells** Nicole Gross, Switzerland *Abstract: Page 74*
14:05 - 14:15		**Overview of cell model issues and discussion** Michael Hogarty and David Kaplan, Session Chairs

Session 2: Animal Models
Chairs: Louis Chesler and Carol Thiele

14:20 - 14:35		**Activated ALK Collaborates with MYCN in Neuroblastoma Pathogenesis in zebrafish** Tom Look, United States
14:35 - 14:45		**Murine modeling strategies for neuroblastoma biology and therapeutics** Louis Chesler, United Kingdom
14:45 - 14:55	**WS34**	**Genomic profiling of ALKF1174L and TH-MYCN transgenic neuroblastoma mouse models provides insights into the dynamic process of tumor formation** Katleen De Preter, Belgium *Abstract: Page 75*
14:55 - 15:05	**WS35**	**A new MYCN-driven neuroblastoma mouse model using Cre-driven conditional expression of MYCN** Johannes Schulte, Germany *Abstract: Page 75*
15:05 - 15:15	**WS36**	**Caspase-8 deficiency enhances neuroblastoma metastasis in vivo - a new mouse model for metastatic neuroblastoma** Tal Teitz, United States *Abstract: Page 75*
15:15 - 15:30		**Overview, discussion, and future directions** Louis Chesler and Carol Thiele, Session Chairs

MONDAY, JUNE 18, 2012
16:00 – 17:00
CONCERT HALL

Opening Lecture

16:00 - 17:00 **PL00** **Personalized Treatment for Children with Neuroblastoma: An Old Paradigm with New Tools**
Susan L. Cohn, United States
Abstract: Page 78

Dr. Susan L. Cohn received her MD from the University of Illinois Medical School in Chicago and completed a residency in pediatrics at Michael Reese Hospital & Medical Center. She then completed a fellowship in pediatric hematology/oncology at Children's Memorial Hospital and Northwestern University. Dr. Cohn joined the faculty at Northwestern University in 1987 and remained at that institution until 2007, when she joined the faculty at the University of Chicago as a Professor of Pediatrics and Director of Pediatric Clinical Sciences. Dr. Cohn has devoted her career to the care of children with neuroblastoma, and to understanding the molecular pathogenesis of the disease through the investigations of her laboratory. The overall goal of Dr. Cohn's research is to develop individualized care for children with neuroblastoma using precise risk stratification and by identifying novel targets for therapy. Most recently, Dr. Cohn has been investigating pharmacogenetic factors that contribute to racial and ethnic disparities in outcome in children with neuroblastoma. Dr. Cohn is a member of the Scientific and Executive Committees in the Children's Oncology Group (COG) and the co-Chair of the International Neuroblastoma Risk Group (INRG) task force. Dr. Cohn is also a member of the American Society of Clinical Oncology Board of Directors and is the Treasurer-elect of the Society.

MONDAY, JUNE 18, 2012
18:45 – 21:00
FOUR SEASONS CENTRE FOR THE PERFORMING ARTS

Welcome Reception

To welcome you to Toronto, the Local Organizing Committee has put together a fantastic opening reception at one of Canada's best cultural centres for the Performing Arts.

The evening will feature performances by two Toronto based organizations.

SISTEMA - TORONTO
A program offering free, intensive music education to children in a culturally diverse neighbourhood of Toronto. The students work with professional artists and teachers for two hours at the end of every school day, and after three years each child will have received the equivalent of over ten years' musical experience.

TAFELMUSIK BAROQUE ORCHESTRA, JEANNE LAMON, MUSIC DIRECTOR
Tafelmusik, Canada's award-winning period instrument orchestra, was founded in 1979 and has long been renowned in North America and internationally for its distinct, exhilarating and soulful performances. Under the outstanding leadership of Music Director Jeanne Lamon, C.M., it has excelled equally in music ranging from the baroque and classical eras and beyond, including adventurous cross-cultural reinventions of baroque classics.

You will be treated to the culinary stylings of Chef Jean-Pierre Challet. JP was born and raised in Lyon, the gastronomic heart of France. He studied culinary arts at the Ecole Hoteliere in Nice before immigrating to Canada, to become one of this country's top chefs.

TUESDAY, JUNE 19, 2012
07:30 – 09:25
CONCERT HALL

Opening Ceremony

07:30 - 08:30		**CONTINENTAL BREAKFAST (Canadian Ballroom)**
08:30 - 08:35		Dr. Sylvain Baruchel, Chair ANR 2012 Conference
08:35 - 08:40		Denis Daneman, Paediatrician-in-Chief, The Hospital for Sick Children; Professor, Department of Paediatrics, University of Toronto; Canada
08:40 - 09:25	**PL01**	**Future Anti-Cancer Therapeutic Targets: Putting the Carts Before the Horses?** Tak Mak, Canada *Abstract: Page 78*

Tak W. Mak is the Director of the Campbell Family Institute for Breast Cancer Research at the Princess Margaret Hospital, and Professor in the Departments of Medical Biophysics and Immunology at the University of Toronto. Dr. Mak's research interests center on immune cell recognition/regulation and molecular mechanisms underlying the survival and death of normal and malignant cells. He is best known as the lead scientist of the group that first cloned the genes of the human T cell antigen receptor, a discovery that provided essential insights into the molecular basis of cellular immunity. Subsequently, he became one of the most prolific contributors to "knockout" mouse models created by homologous recombination technology. His laboratory has generated hundreds of genetically modified mouse strains that have proven critical to unravelling the intracellular programs governing the development and function of the immune system, as well as various cell survival and apoptotic pathways, including CTLA4, bcl-10, and MALT1.

In the last dozen years, Dr. Mak has devoted a large portion of his research to investigating the pathogenesis of cancer. His group's analyses of many cancer-related knockout mice has led to novel discoveries including the finding that the breast cancer susceptibility genes BRCA1 and BRCA2 function in DNA repair, and that the cell cycle checkpoint kinase Chk2 activates the tumour suppressor gene p53. Other high impact discoveries include the demonstration that RhoC is involved in the metastasis of breast cancers, and that only the transactivatory isoforms of the p73 protein are tumour suppressors. Dr. Mak has recently combined his interests in cancer pathogenesis and immunology, with the demonstration that the cytokine IL-7 enhances immunotherapeutic strategies against tumours.

Dr. Mak has co-authored over 740 scientific papers, holds 21 U.S. patents, and is one of the most highly cited scientists in basic biomedical research (>60,000 citations to date). His accomplishments have been recognized through numerous prestigious awards and honours, including Germany's Emil Von Behring Prize Canada's Gairdner Foundation International Award for outstanding contributions in the field of medical science, the King Faisal International Prize for Medicine, the Alfred E. Sloan Prize of General Motors Cancer Foundation, and the Novartis Immunology Prize, and in 2002 he was elected as a Foreign Associate of the U.S. National Academy of Sciences. In addition, he serves on numerous advisory boards of scientific journals (PNAS, Oncogene, Cancer Cell) and medical centers (Immune Diseases Institute, Harvard Medical School, MD Anderson Hospital, Mayo Clinics, Ohio State University). Dr Mak is also an effective mentor, and has trained (since 1992) over 93 graduate students and post-doctoral fellows, many of whom now are directors of research institutes.

PROGRAMME

TUESDAY, JUNE 19, 2012
09:25 – 10:25
CONCERT HALL

Plenary Session 1: ALK "From Fish to Bedside"
Session Chairs: Louis Chesler and Michelle Haber

09:25 - 09:45	**PL02**	**A New Model of Neuroblastoma in Zebrafish to Evaluate Candidate Genes involved in Neuroblastoma Pathogenesis** Tom Look, United States *Abstract: Page 78*
09:45 - 10:00	**PL03a**	**ALKF1174L is a driving oncogene of neuroblastoma in transgenic mice** Johannes Schulte, Germany *Abstract: Page 78*
10:00 - 10:10	**PL03b**	**The ALKF1174L mutation potentiates the oncogenic activity of MYCN in a mouse model of neuroblastoma.** Rani E. George, United States *Abstract: Page 79*
10:10 - 10:25	**PL04**	**Efficacy of crizotinib in children with relapsed/refractory ALK-driven tumors including neuroblastoma: A Children's Oncology Group Phase 1 Consortium Study** Yael Mosse, United States *Abstract: Page 79*
10:25 - 10:45		**BREAK**

TUESDAY, JUNE 19, 2012
10:45 – 12:30
CONCERT HALL

Plenary Session 2: High Risk Neuroblastoma "From Genetics to Therapeutics"
Session Chairs: John Maris and Barbara Hero

10:45 - 11:00 **PL05**
NCYM, a novel MYCN cis-antisense gene product, stabilizes MYCN and contributes to aggressiveness of human neuroblastoma
Yusuke Suenaga, Japan
Abstract: Page 79

11:00 - 11:15 **PL06**
Whole Genome Sequencing of Neuroblastoma identifies frequent Chromothripsis and Neuritogenesis Gene Defects.
Rogier Versteeg, Netherlands
Abstract: Page 80

11:15 - 11:30 **PL07**
Prognostic Subgroups of High-Risk (HR) Neuroblastoma (NB) Patients Are Identified by Analysis of Peripheral Blood Stem Cells (PBSC) with a Highly Sensitive TaqMan® Low Density Array (TLDA) Assay for Five Neuroblastoma-Associated Genes: Children's Oncology Group (COG) Study A3973
Judith Villablanca, United States
Abstract: Page 80

11:30 - 11:55 **PL08**
Final Results from the HR-NBL1/SIOPEN Trial favour Busulphan-Melphalan as Superior Myeloablative Therapy (MAT) for High Risk Neuroblastoma.
Ruth Ladenstein, Austria
Abstract: Page 80

Ruth Ladenstein, MD, MBA, cPM is Head of S2IRP Studies and Statistics on Integrated Research and Projects/ Children's Cancer Research Institute (CCRI) of the St. Kinderkrebsforschung e.V., Vienna since 1996 as well as Head of the department for paediatric solid tumours St. Anna Children's Hospital, Vienna since 1998. Assoc. Prof. Ladenstein has considerable experience in the management of academic concerted actions, she is:

- President of SIOP EUROPE since September 2009 (board member since September 2006)
- Project coordinator of the EU FP7 funded Network of Excellence: "EUROPEAN NETWORK for CANCER research in CHILDREN and ADOLESCENCE"
- Advisory board member of SIOPEN (SIOP Europe Neuroblastoma Group) since May 2011 (president from May 2007 – May 2011)

SIOP European Neuroblastoma Research Network (SIOPEN-R-NET) is the European Association for clinical research in neuroblastoma established with the initial support of a European Union 5th Framework grant (EC grant no. QLRI-CT-2002-01768) and was coordinated by Dr. Ladenstein. It now involves more than 200 clinics from more than 20 countries. Currently Dr. Ladenstein is co-ordinating various neuroblastoma, Ewing sarcoma and soft tissue sarcoma trials on international and national level.

11:55 - 12:15 **PL09**
HSCT improves outcome for high risk neuroblastoma: What's next?
Julie Park, United States
Abstract: Page 80

12:15 - 12:30 **Q&A with Ruth Ladenstein and Julie Park**

12:30 - 14:30 **LUNCH & POSTER VIEWING (Canadian Ballroom)**
Refer to page 30 for Posters

PROGRAMME

TUESDAY, JUNE 19, 2012
13:00 – 16:30
TUDOR 7

Nursing Symposium
Session Chairs: Denise Mills and Eleanor Hendershot

13:00 - 13:10		**Welcome & Introductions**
13:10 - 13:45		**Immunotherapy** Alice Yu, United States
13:45 - 14:20		**The INRG – the Past, Present and Future** Andrew Pearson, United Kingdom
14:20 - 14:55	**NS0**	**I-131 Metaiodobenzylguanidine therapy for Neuroblastoma** Jennifer Saggio, United States *Abstract: Page 76*
14:55 - 15:05		**BREAK**
15:05 - 15:15	**NS1**	**The role and function of a case manager for children with neuroblastoma and their families** Ya-Ling Lee, Taiwan *Abstract: Page 76*
15:15 - 15:25	**NS2**	**Administering Chimeric Antibody,what it means for Bedside Nurses** Tara McKeown, Canada *Abstract: Page 76*
15:25 - 15:35	**NS3**	**Study of Carrier Use and Administration of LXS Fenretinide Powder in Patients with Recurrent/Refractory Neuroblastoma within the New Approaches to Neuroblastoma Therapy (NANT) Consortium Study: NANT 2004-04** Scarlett Czarnecki, United States *Abstract: Page 76*
15:35 - 15:45	**NS4**	**The Road to a Canadian 131 I-MIBG Program** Denise Mills, Canada *Abstract: Page 77*
15:45 - 16:20	**NS5**	**Late effects in survivors of childhood neuroblastoma** Paul Nathan, Canada *Abstract: Page 77*
16:20 - 16:30		**Concluding Remarks**

TUESDAY, JUNE 19, 2012
14:30 – 15:40
CONCERT HALL

Parallel Session 1: ALK
Session Chairs: Yael Mosse and Bengt Hallberg

14:30 - 14:40	**OR01**	**ALK-based therapeutic stratification of patients with neuroblastoma** Daniel Weiser, United States *Abstract: Page 86*
14:40 - 14:50	**OR02**	**The growth factor Midkine plays a critical role in the tumorigenesis of neuroblastoma via Notch2 signaling** Satoshi Kishida, Japan *Abstract: Page 86*
14:50 - 15:00	**OR03**	**MDK promotes survival of neuroblastoma cells by signaling through ALK and other receptors** Gilles Vassal, France *Abstract: Page 86*
15:00 - 15:10	**OR04**	**Anaplastic Lymphoma Kinase (ALK) expression is an independent prognostic factor in neuroblastoma patients and correlates well with ALK inhibitor response in vitro.** Max van Noesel, Netherlands *Abstract: Page 87*
15:10 - 15:20	**OR05**	**Mechanisms of acquired resistance in crizotinib-treated neuroblastoma** Erica Carpenter, United States *Abstract: Page 87*
15:20 - 15:30	**OR06**	**Combined inhibition of ALKF1174L and downstream signaling leads to tumor regression and prolongation of survival in transgenic mice with ALKF1174L/MYCN tumors.** Teeara Berry, United Kingdom *Abstract: Page 87*
15:30 - 15:40	**OR07**	**Association of ALKF1174L expression with altered vasculature in transgenic murine models of neuroblastoma** Laura Glass, United Kingdom *Abstract: Page 88*
15:40 - 16:10		**BREAK (Canadian Ballroom)**

PROGRAMME

TUESDAY, JUNE 19, 2012
14:30 – 15:40
ONTARIO ROOM

Parallel Session 2: miRNA
Session Chairs: Frank Speleman and Jason Shohet

14:30 - 14:40	**OR08**	**MYCN directly regulates long non-coding RNA expression in neuroblastoma** Pieter Mestdagh, Belgium *Abstract: Page 88*
14:40 - 14:50	**OR09**	**miR-542-3p exerts tumor suppressive functions in neuroblastoma by downregulating survivin** Kristina Kieckbusch, Germany *Abstract: Page 89*
14:50 - 15:00	**OR10**	**Enrichment analysis for MYCN pathway genes in focal genomic gains and losses identifies new components of the MYCN-miRNA regulatory network in neuroblastoma** Annelies Fieuw, Belgium *Abstract: Page 89*
15:00 - 15:10	**OR11**	**MYCN and HDAC2 cooperate to repress miR-183 signaling in neuroblastoma** Marco Lodrini, Germany *Abstract: Page 89*
15:10 - 15:20	**OR12**	**MiR-137 is epigenetically silenced in MYCN amplified neuroblastomas and targets the polycomb repressive complex 2 (PRC2) component EZH2** Anneleen Beckers, Belgium *Abstract: Page 90*
15:20 - 15:30	**OR13**	**Establishment of a miRNA/mRNA regulatory network through integrated analysis of neuroblast and neuroblastoma expression profiles** Sara De Brouwer, Belgium *Abstract: Page 90*
15:30 - 15:40	**OR14**	**Outcome prediction of neuroblastoma patients using microRNA gene expression profiling in both fresh frozen and archived tumor samples** Fjoralba Zeka, Belgium *Abstract: Page 90*
15:40 - 16:10		**BREAK (Canadian Ballroom)**

TUESDAY, JUNE 19, 2012
16:10 – 17:30
ONTARIO ROOM

Parallel Session 3: MYC-N
Session Chairs: William Weiss and Akira Nakagawara

16:10 - 16:20	**OR15**	**Anaplastic Lymphoma Kinase (ALK) regulates initiation of transcription of MYCN in neuroblastoma cells.** Bengt Hallberg, Sweden *Abstract: Page 91*
16:20 - 16:30	**OR16**	**Direct effects of Bmi1 on p53 protein stability inactivates oncoprotein stress responses in neuroblastoma precursor cells at tumor initiation** Glenn Marshall, Australia *Abstract: Page 91*
16:30 - 16:40	**OR17**	**Targeting the MYCN Signaling Pathway by Inhibiting the Histone Demethylase JMJD2B** JUN YANG, United States *Abstract: Page 91*
16:40 - 16:50	**OR18**	**The class III histone deacetylase SIRT2 stabilizes N-Myc oncoprotein by transcriptional repression of the E3 ubiquitin-protein ligase NEDD4** Tao Liu, Australia *Abstract: Page 92*
16:50 - 17:00	**OR19**	**Suppression of neuroblastoma tumorigenesis using ENU mutagenesis in the TH-MYCN mouse model of neuroblastoma** Jayne Murray, Australia *Abstract: Page 92*
17:00 - 17:10	**OR20**	**Identification and partial characterization of novel Aurora kinase inhibitors to target MYCN destabilization** William Clay Gustafson, United States *Abstract: Page 92*
17:10 - 17:20	**OR21**	**Paucity of early tumor-driving gene mutations in MYCN amplified neuroblastoma** Jan Molenaar, Netherlands *Abstract: Page 93*
17:20 - 17:30	**OR22**	**NLRR1, a direct target of MYCN, regulates cell growth both in vitro and in vivo and can be a therapeutic target against high-risk neuroblastoma** Atsushi Takatori, Japan *Abstract: Page 93*

PROGRAMME

TUESDAY, JUNE 19, 2012
16:10 – 17:30
CONCERT HALL

Parallel Session 4: HIGH RISK NEUROBLASTOMA BIOMARKERS LEADING TO THERAPY
Session Chairs: Dominique Valteau-Couanet and Julie Park

16:10 - 16:20	**OR23**	**Bone marrow monitoring by AIPF – a prognostic tool for high risk patients over 18 months of age at diagnosis** Peter Ambros, Austria *Abstract: Page 93*
16:20 - 16:30	**OR24**	**Identification of ultra-high risk neuroblastoma by gene expression-based classification** Frederik Roels, Germany *Abstract: Page 94*
16:30 - 16:40	**OR25**	**Prominent Nucleolar Formation and N-myc/C-myc Protein Expression in Undifferentiated Neuroblastoma: Immunohistochemical study indicates the worst prognosis for the patients with C-myc positive tumors – A Report from the Children's Oncology Group** Larry L. Wang, United States *Abstract: Page 94*
16:40 - 16:50	**OR26**	**Tandem high-dose chemotherapy (HDC) with Thiotepa and Mel-Bu and autologous stem cell transplantation (ASCT): the way to improve very high risk neuroblastoma patients prognosis ?** Dominique Valteau-Couanet, France *Abstract: Page 94*
16:50 - 17:00	**OR27**	**Haploidentical Natural Killer Cells plus Monoclonal Antibody 3F8 for Resistant High-Risk Neuroblastoma: Preliminary Results of an Ongoing Phase I study** Shakeel Modak, United States *Abstract: Page 95*
17:00 - 17:10	**OR28**	**Dose individualisation of 13-cis-retinoic acid in high-risk neuroblastoma patients based on pharmacological exposure – a national UK study** Gareth Veal, United Kingdom *Abstract: Page 95*
17:10 - 17:20	**OR29**	**High-dose 131I-MIBG treatment incorporated into tandem HDCT/autoSCT for high-risk neuroblastoma: Preliminary results of SMC NB-2009 study** Ki Woong Sung, Republic of Korea *Abstract: Page 95*
17:20 - 17:30	**OR30**	**The role of surgery in the treatment of stage 4 neuroblastoma patients older than 18 months of age** Thorsten Simon, Germany *Abstract: Page 95*

PROGRAMME

TUESDAY, JUNE 19, 2012
17:30 – 18:30
CANADIAN BALLROOM

Poster Viewing

BASIC

POB001 **Midkine and Alk signaling in sympathetic neuron proliferation and neuroblastoma predisposition**
Hermann Rohrer, Germany
Abstract: Page 116

POB002 **Internalization and down-regulation of the ALK receptor in neuroblastoma cell lines upon monoclonal antibodies treatment.**
Marc Vigny, France
Abstract: Page 116

POB003 **A novel mechanism of cell migration by NMyc through a direct transactivation of ALK gene in neuroblastoma**
Md. Kamrul Hasan, Japan
Abstract: Page 116

POB004 **Complex genomic rearrangements within the ALK gene may lead to ALK activation in a subset of neuroblastoma samples**
Alex Cazes, France
Abstract: Page 116

POB005 **Flotillin-1 is a novel ALK binding protein which regulates ALK singnaling through receptor endocytosis.**
Arata Tomiyama, Japan
Abstract: Page 117

POB006 **NLRR1 binds to ALK and regulates its function in cell proliferation**
Shunpei Satoh, Japan
Abstract: Page 117

POB007 **SH2 domain containing adaptor protein Shf, a favorable prognostic factor of neuroblastoma, inhibits ALK-induced oncogenic signals in neuroblastoma**
Yasutoshi Tatsumi, Japan
Abstract: Page 117

POB008 **The ALK-F1174L activating mutation is tumorigenic in MONC-1 neural crest stem cells in an orthotopic murine model of neuroblastoma**
Annick Muhlethaler-Mottet, Switzerland
Abstract: Page 118

POB009 **ETV5 regulates cell proliferation downstream of the ALK signaling pathway**
Candy Kumps, Belgium
Abstract: Page 118

POB010 **Screening of a natural resource library for antitumor activities using midkine as an indicator**
Rie Sonamoto, Japan
Abstract: Page 118

POB011 **Enhanced Tumorigenicity of Neuroblastoma Cells after Retinoic Acid Treatment is Reversed by Telomerase Inhibition.**
Tatiana Lipman, Canada
Abstract: Page 119

POB012 **Mitochondrial apoptosis is the preferential pathway in neuroblastoma by effect of Casiopeínas®.**
Carmen Mejía, Mexico
Abstract: Page 119

POB013 **Determination of apoptosis and autophagy in neuroblastoma by Casiopeínas®.**
Carmen Mejía, Mexico
Abstract: Page 119

POB014 **Identification of novel candidate compounds targeting TrkB to induce apoptosis in neuroblastoma: in silico screening utilizing a grid computing technology**
Yohko Nakamura, Japan
Abstract: Page 119

POB015 **An investigation into the potential therapeutic benefit of targeting Skp2 in Neuroblastoma.**
Laura Evans, United Kingdom
Abstract: Page 120

POB016 **A large tenascin C isoform promotes micro-vessel remodeling after treatment with anti-endothelial cell monoclonal antibodies in an orthotopic model of human neuroblastoma**
Annalisa Pezzolo, Italy
Abstract: Page 120

POB017 **Whole-exome sequencing reveals second driver mutations in MYCN-amplified neuroblastoma**
Matthias Fischer, Germany
Abstract: Page 120

POB018 **Genomic Heterogeneity within Neuroblastomas**
Gisela Lundberg, Sweden
Abstract: Page 121

POB020 **No evidence for viral replication in deep transcriptome sequencing data of metastatic neuroblastoma with progressive stage 4 and regressive stage 4S**
Frank Berthold, Germany
Abstract: Page 121

POB021 **Exome sequencing reveals few recurrent somatic mutations in neuroblastoma**
Susanne Fransson, Sweden
Abstract: Page 121

POB022 **Whole-exome sequencing of advanced neuroblastomas**
Riki Nishimura, Japan
Abstract: Page 121

POB023 **Detection of genes and pathways involved in the development of aggressive neuroblastoma using genome copy number data**
Valentina Boeva, France
Abstract: Page 122

POB024 **Identification of familial neuroblastoma associated genes by whole exome sequencing**
Luca Longo, Italy
Abstract: Page 122

POB025 **R2: A Public User-friendly Website For integrated Analysis Of Expression Data And Associated Clinical Parameters In Neuroblastoma.**
Jan Koster, Netherlands
Abstract: Page 122

POB026 **Identification of molecular subgroups and pathways in pheochromocytomas and paragangliomas**
Annica Wilzen, Sweden
Abstract: Page 122

POB027 **Impact of MDM2 SNP309 on the survival of neuroblastoma patients**
Ali Rihani, Belgium
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POB028 **miR-137 exerts tumor suppressive functions mediated via downregulation of the epigenetic target enzyme LSD1 in neuroblastoma cells**
Kristina Kieckbusch, Germany
Abstract: Page 123

POB029 **MicroRNA-125b regulates proliferation and differentiation of Neuroblastoma Cells by targeting Lin28b**
Sheng-Kai Chang, Taiwan
Abstract: Page 123

POB030 **Identification of prognosis-related miRNA expression profiles in neuroblastoma**
Miki Ohira, Japan
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POB031 **Genome-wide profiles reveal candidate miRNAs potentially involved in the regulation of ALK expression in neuroblastoma**
Marilena De Mariano, Italy
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POB032 **miR-18a inhibits differentiation of MYCN-amplified neuroblastoma by deregulation of estrogen and NGF signaling**
Marie Arsenian Henriksson, Sweden
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POB033 **An unbiased high-throughput microRNA library screen identifies microRNA-interactomes of key neuroblastoma genes**
Gert Van Peer, Belgium
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POB034 **Micro RNA-21 regulates the cisplatin resistance in human neuroblastoma cells**
Ya-Hui Tsai, Taiwan
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POB035 **p53 expression and functional status in a TH-MYCN transgenic murine neuroblastoma cell line**
Lindi Chen, United Kingdom
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POB036 **Characterization of tumor development, growth characteristics and spectrum of genetic aberrations in the TH-MYCN mouse model of neuroblastoma**
Agnes Rasmuson, Sweden
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POB037 **Identifying genes underlying bone marrow metastasis in neuroblastoma**
Jamie I. Fletcher, Australia
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POB038 **The development of metastatic mouse models of neuroblastoma to bone and brain to identify the molecular mechanisms governing metastasis.**
Kelly Fathers, Canada
Abstract: Page 126

POB039 **Molecular-Genetic Detection Of Minimal Disease In Neuroblastoma.**
Anatoli Kustanovich, Belarus
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POB040 **MINA expression in neuroblastoma correlates with unfavorable patient outcome**
Andrea Odersky, Germany
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POB041 **The role of NEDD9 in neuroblastoma cell migration**
Thi Thu Cuc Bach, Australia
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POB042 **A constitutively active TrkB receptor is sufficient to confer a highly aggressive and transformed phenotype in a rat neural crest derived cell line**
John DeWitt, United States
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POB043 **Regulation of the wnt inhibitor DKK-1 in neuroblastoma**
Bjørn Helge Haug, Norway
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POB044 **Individual caspase-10 isoforms play distinct and opposing roles in the initiation of death receptor-mediated tumour cell apoptosis.**
Annick Muhlethaler-Mottet, Switzerland
Abstract: Page 127

POB045 **CHD5, A Neuroblastoma (NB) Tumor Suppressor Gene (TSG), Also Regulates Spermiogenesis in Mice**
Tiangang Zhuang, United States
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POB046 **CHD5 Nucleosome Remodeling and Deacetylation (NuRD) Complex in Neuroblastoma (NB) Cell Lines**
Venkatadri Kolla, United States
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POB047 **Identification and characterization of FOXP1 as a candidate tumor suppressor gene in neuroblastoma**
Sandra Ackermann, Germany
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POB048 **Functional analysis of a novel 1p tumor suppressor DMAP1**
Yohko Yamaguchi, Japan
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POB049 **TRIM16 is an E3 ubiquitin ligase which directly binds and activates Caspases 2 and 3 leading to neuroblastoma cell apoptosis**
Belamy Cheung, Australia
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POB050 **HoxC9 activates the intrinsic pathway of apoptosis and is associated with spontaneous regression in neuroblastoma**
Hayriye Kocak, Germany
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POB051 **Modeling the G1-S Transition in Neuroblastoma**
Florian Lamprecht, Germany
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POB052 **NLRR2 inhibits tumor growth by activating ER stress signals and its down-regulation is associated with poor prognosis in neurobalstoma**
Md. Shamim Hossain, Japan
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POB053 **Possible role of Delta-like 1 homolog in the chemoresistant behavior of Neuroblastoma.**
Marjorie Flahaut, Switzerland
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Daniel Dreidax, Germany
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POB055 **Contribution Of Ataxia Telangiectasia Mutated (ATM) Loss Of Function To Neuroblastoma Progression.**
Stefano Mandriota, Switzerland
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POB056 **Mismatch repair protein expression and gene promoter methylation in paired neuroblastomas pre and post-chemotherapy**
Gail Halliday, United Kingdom
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POB057 **Role Of Carcinoembryonic Antigen-Related Cell Adhesion Molecule 1 (Ceacam1) In The Dna Damage Response Of Human Neuroblastoma Cells.**
Stefano Mandriota, Switzerland
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POB058 **FOXO3a Is A Critical Target of PI3K/AKT Pathway Activity In Neuroblastoma**
Evan Santo, Netherlands
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POB059 **Understanding the biological role of caspase-8 in neuroblastoma**
Devin Twitchell, United States
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POB060 **Frizzled receptor 6 (Fzd6) is a new marker for neuroblastoma stem cells.**
Sandra Cantilena, United Kingdom
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POB061 **BMI1 regulates neuroblastoma apoptotic cell death and differentiation**
Nobuhiro Akita, Japan
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POB062 **Reprogramming of human neuroblastoma cells using iPSC technology**
S.M. Rafiqul Islam, Japan
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POB063 **Identification of tumoral glial precursor cells in neuroblastoma**
Jaume Mora, Spain
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POB064 **Histone deacetylase inhibitors reduce the self renewal capacity of neuroblastoma tumor initiating cells**
Marie C. Schier, Germany
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POB065 **MYCN or ALKF1174L are sufficient to drive neuroblastoma development from neural crest progenitor cells**
Sven Lindner, Germany
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POB066 **From human embryonic stem cells to sympathetic neurons: A model for understanding neuroblastoma pathogenesis**
Jane Carr-Wilkinson, United Kingdom
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POB067 **Epigenetic Regulation of Differentially Expressed microRNAs in Neuroblastoma Cancer Stem Cell-like Population**
Saurabh Agarwal, United States
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POB068 **Interest of Orthotopic injections in NOD/SCID/Il2rg Null (NSG) mice to study cancer stem cells neuroblastoma**
Mona Beaunoyer, Canada
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POB069 **EFNA2 implication in stemness properties of neuroblastoma**
Hervé Sartelet, Canada
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POB070 **Isolation and verification of new neuroblastoma tumor-initiating cell lines**
Loen Hansford, Canada
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CLINICAL

POC01 **The Treatment of Children With Low And Intermedium Risk Neurobastoma**
Anatoly Kazantsev, Russian Federation
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POC02 **Phase I Trial of Polyethylene Glycol (PEG) Conjugated SN38 in Pediatric Patients (pts) with Recurrent or Refractory Neuroblastoma (NB) and Other Solid Tumors**
Rochelle Bagatell, United States
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POC03 **Double Scattered Proton Therapy (DSPT) versus Intensity Modulated X-Ray Therapy (IMRT) for Patients (pts) with High-Risk Neuroblastoma (HRNB)**
Christine Hill-Kayser, United States
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POC04 **MYCN-amplified neuroblastoma presenting with a unique histologic pattern due to focal protein expression: A case report**
Rie Suganuma, United States
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POC05 **Use of Multiplex Ligation-Dependent Probe Amplification to Evaluate Genetic Aberrations in Neuroblastoma – A Pilot Study in Singapore**
Shui Yen Soh, Singapore
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POC06 **Infants With Neuroblastoma Presenting With Symptomatic Epidural Compression**
Lucia Quaglietta, Italy
Abstract: Page 182

POC07 **A phase I/II study of 131I-Meta-Iodobenzylguanine (MIBG), hyperbaric oxygen (HBO) and Vitamin C in patients with recurrent neuroblastoma (NBL).**
Kathelijne Kraal, Netherlands
Abstract: Page 183

POC08 **Treatment with Long-term Topotecan Plus Cyclophosphamide in Children with Recurrent or Refractory Neuroblastoma: Hospital for Sick Children Experience**
Kaleem Ashraf, Canada
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POC09 **The expression of Retinoic Acid Related Genes in Neuroblastoma can predict the Patients' Prognosis.**
Keiji Tsuji, Japan
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POC10 **High Risk Neuroblastoma treated with the MSKCC guidelines at Hospital Sant Joan de Déu, Barcelona. Intensive Minimal residual Disease monitoring and Outcome.**
Jaume Mora, Spain
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POC11 **Development of an open-source, flexible framework for interinstitutional data sharing and collaboration**
Samuel Volchenboum, United States
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POC12a **Impact of post-induction Curie scores as prognostic marker in high risk neuroblastoma. A Children's Oncology Group report.**
Gregory Yanik, United States
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POC13 **Clinical features of neuroblastoma patients after discontinuation of mass screening**
Hiroshi Arai, Japan
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POC14 **Comparison of PCR and flow cytometry results of bone marrow involvement assessment**
Alexander Druy, Russian Federation
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POC15 **Biological analysis of the first nation-wide clinical trial for high-risk neuroblastoma by Japan Neuroblastoma Study Group (JNBSG)**
Kimikazu Matsumoto, Japan
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POC16 **ALARA: "As Low As Reasonably Achievable" radiation exposure to parents during molecular radiotherapy for neuroblastoma.**
Jennifer Gains, United Kingdom
Abstract: Page 185

POC17 **Evaluation of intensity modulated arc radiotherapy for dose escalation to tumour in high-risk neuroblastoma and reduction of high doses to uninvolved normal organs.**
Jennifer Gains, United Kingdom
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POC18 **Comparison of Circulating and Bone Marrow Neuroblastoma Cells with a Highly Sensitive Five-Gene TaqMan® Low Density Array Assay**
Araz Marachelian, United States
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POC19 **Comparison of a Sensitive Five-Gene Taqman® Low Density Array (TLDA) Assay for Tumor Cells in Bone Marrow and Blood with Histologic Bone Marrow Examination and Imaging for Disease Assessment in Patients with Recurrent/Refractory Neuroblastoma: A New Approaches to Neuroblastoma Therapy (NANT) Study**
Araz Marachelian, United States
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POC20 **Population Based incidence and improved survival of Patients with Recurrent Neuroblastoma in Ontario, Canada**
Paul Gibson, Canada
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POC21 **Infant Metastatic (4+ 4S) MYCN Non-amplified Neuroblastoma has a very high survival rate with moderate dose chemotherapy**
Mary Lou Schmidt, United States
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POC22 **A Trial Treatment Of Long-Term Maintenance Chemotherapy For Refractory Neuroblastoma : A Single Institution Experience In 7 Patients**
Shimozawa Katsuyoshi, Japan
Abstract: Page 187

POC23 **A novel high-risk neuroblastoma subset with abdominal primaries mimicking Wilms spreading to lungs but not bone marrow is defined by amplification of two distinct regions on chromosome 12**
Hanna Kryh, Sweden
Abstract: Page 187

POC24 **Ethnic differences in neuroblastoma indicated by the pattern of frequent ALK mutations in Vietnamese tumors; Eight novel tyrosine kinase domain mutations identified.**
Niloufar Javanmardi, Sweden
Abstract: Page 188

POC25 **Successful liver transplantation in an infant with stage 4S(M) neuroblastoma: Case report clinical and ethical issues**
Melanie Steele, Canada
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TRANSLATIONAL

POT001 **The Receptor Tyrosine Kinase AXL Contributes to Resistance of ALK-F1174L to TAE684 in Neuroblastoma**
Rani George, United States
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POT002 **The green tea compound Polyphenon E negatively modulates neuroblastoma-induced immuno-suppressive myeloid cells.**
Arturo Sala, United Kingdom
Abstract: Page 149

POT003 **Salmonella application is the most effective DNA vaccine delivery method for a survivin-based vaccination in neuroblastoma**
Stefan Fest, Germany
Abstract: Page 149

POT004 **Histone deacetylase 10 causes neuroblastoma cell survival by promoting Hsc70-mediated autophagic flux**
Ina Oehme, Germany
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POT005 **Nanoparticle (NP) Drug Delivery in Neuroblastoma (NB)**
Radhika Iyer, United States
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POT006 **Antitumoral effects of histone deacetylase 8 selective inhibitors**
Inga Wiegand, Germany
Abstract: Page 150

POT007 **Factors Involved in Resistance to Prolonged Antiangiogenic Therapy with Oral Metronomic Topotecan and Pazopanib in Neuroblastoma Mouse Model**
Sushil Kumar, Canada
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POT008 **Preclinical Evaluation of Antitumor Efficacy of the Hypoxia-Activated Prodrug TH-302 and Sunitinib in Neuroblastoma Mouse Models**
Sylvain Baruchel, Canada
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POT009 **In-vitro and in-vivo impaired proliferation and tumorigenicity of neuroblastoma cells after imetelstat treatment is dependent on telomere maintenance**
Libo Zhang, Canada
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POT010 **Identification of new MRP4 inhibitors from a library of FDA approved drugs**
Jamie I. Fletcher, Australia
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POT011 **HDAC11 controls mitotic cell cycle progression of neuroblastoma cells**
Theresa Maria Thole, Germany
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POT012 **FTY720 interferes with sphingosine-1-phosphate signaling in neuroblastoma, inhibiting tumor growth and enhancing the tumor-suppressive effect of topotecan in preclinical models.**
Mei-Hong Li, United States
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POT013 **Radiosensitization potential of bortezomib and SN38 in neuroblastoma**
Gregory Yanik, United States
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Zhijie Li, United States
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POT015 **Inhibition of MYCN by M606, a novel small molecule inhibitor identified through chemical library screening**
Murray Norris, Australia
Abstract: Page 152

POT016 **Targeting the ABCC4 transporter in neuroblastoma**
Michelle Henderson, Australia
Abstract: Page 153

POT017 **Increased endogenous trapping of topoisomerase I to escape from irinotecan cytotoxicity in resistant neuroblastoma tumors**
Gilles Vassal, France
Abstract: Page 153

POT018 **Hsp90 is a therapeutic target in TH-ALKF1174L/MYCN-neuroblastoma**
Elizabeth Tucker, United Kingdom
Abstract: Page 153

POT019 **Efficacy of mTORC1 inhibition in murine neuroblastoma**
Paul Wood, Australia
Abstract: Page 153

POT020 **Parvovirus H-1 Induces Oncolytic Effects In Human Neuroblastoma Cells In Vitro And In A Neuroblastoma Xenograft-Bearing Mouse Model**
Jeannine Lacroix, Germany
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Cecilia Dyberg, Sweden
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POT022 **Hypoxia and neuroblastoma: from bench to clinic**
Luigi Varesio, Italy
Abstract: Page 154

POT023 **Development, Characterization And Cytotoxic Activity Of strail-Targeted Liposomes Against Neuroblastoma**
Monica Loi, Italy
Abstract: Page 155

POT024 **Novel phage-display derived peptides for tumor- and vasculature-targeted therapies in neuroblastoma**
Fabio Pastorino, Italy
Abstract: Page 155

POT025 **Tumor-inhibiting and -promoting properties of drug-induced senescent neuroblastoma cells**
Sabine Taschner-Mandl, Austria
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POT026 **The HDAC6 inhibitor Tubastatin A synergizes with Bortezomib in Neuroblastoma**
Manila Hada, United States
Abstract: Page 155

POT027 **Targeting overexpression of XIAP in neuroblastoma by Smac mimetic LBW242 sensitizes for TNF-α independent apoptosis**
Holger Lode, Germany
Abstract: Page 156

POT028 **Biologically driven gene-set association analysis identifies new neuroblastoma susceptibility common DNA variations at region downstream of the NEFL gene**
Mario Capasso, Italy
Abstract: Page 156

POT029 **Exome and transcriptome sequencing of multiple tumors reveals a stable expressed somatic mutation profile in a patient with metastatic neuroblastoma**
Jun Wei, United States
Abstract: Page 156

POT030 **Genomic Evolution in Relapsed Neuroblastoma**
Shigeki Yagyu, Japan
Abstract: Page 156

POT031 **Next-generation sequencing: Integrated exotome analysis in human multiple neuroblastoma**
Eiso Hiyama, Japan
Abstract: Page 157

POT032 **Next-generation sequencing reveals differential expression of MYCN target genes in neuroblastoma (NB) and suggests the mTOR pathway as a promising therapy target in MYCN-amplified NBs**
Alexander Schramm, Germany
Abstract: Page 157

POT033 **Replication and cumulative genetic risk of GWAS-identified common variations for neuroblastoma in an Italian population**
Mario Capasso, Italy
Abstract: Page 157

POT034 **Identification of a new hereditary neuroblastoma predisposition locus at chromosome 2p25**
Andrew Wood, United States
Abstract: Page 158

POT035 **Genetic alterations are related to immune response in Neuroblastoma**
Marta Piqueras, Spain
Abstract: Page 158

POT036 **Therapeutic Targets For High-Risk Neuroblastoma By Functional Genomics**
Carla Grandori, United States
Abstract: Page 158

POT037 **Genetic variants associated with poor outcome in high-risk neuroblastoma are enriched with variants associated with in vitro cyclophosphamide resistance**
Navin Pinto, United States
Abstract: Page 158

POT038 **AADC gene expression, 18F-FDOPA uptake, and tumor differentiation in neuroblastic tumors**
Hsinyu Lee, Taiwan
Abstract: Page 159

POT039 **FDG:FDOPA uptake ratio by PET scans at diagnosis correlates with genomic type and treatment outcome of neuroblastoma**
Kai-Yuan Tzen, Taiwan
Abstract: Page 159

POT040 **Effects of different corticosteroids on neuroblastoma imaging and therapy**
Melanie Bayer, Germany
Abstract: Page 159

POT041 **Functional analysis of miRNA in drug resistant neuroblastoma cell lines**
Harry Harvey, Ireland
Abstract: Page 160

POT042 **Expression of 14q32.31 microRNA cluster is a new biomarker of relapse in favourable neuroblastoma**
Charles-Henry Gattolliat, France
Abstract: Page 160

POT043 **Mir-192 Directly Regulates Dicer1 In Neuroblastoma, Leading To A More Aggressive Disease**
Smadar Avigad, Israel
Abstract: Page 160

POT044 **In vivo modelling of early growth and spread of embryonic neural tumors.**
Seema Jamil, Sweden
Abstract: Page 160

POT045 **Tumor-associated macrophages promote neuroblastoma growth through upregulation of MYC and COX2 expression in a novel murine neuroblastoma model.**
Michael Hadjidaniel, United States
Abstract: Page 161

POT046 **Minimal residual disease monitoring in neuroblastoma patients by a set of real-time RT-PCR markers**
Aiko Tanaka, Japan
Abstract: Page 161

POT047 **Genetically Verified Tumor Cells In The Bone Marrow Of Patients With Localized Neuroblastoma – Frequency And Correlation With Known Risk Factors**
Peter Ambros, Austria
Abstract: Page 161

POT048 **Alternatively spliced NKp30 isoforms affect outcome in patients with metastatic neuroblastoma and minimal residual disease after induction chemotherapy**
Michaela Semeraro, France
Abstract: Page 162

POT049 **A three-gene expression signature model for risk stratification of patients with neuroblastoma**
Cinzia Lavarino, Spain
Abstract: Page 162

POT050 **CREB-binding protein regulates Ku70 acetylation in response to ionization radiation in neuroblastoma**
Roland Kwok, United States
Abstract: Page 162

POT051 **Mechanisms of CHD5 Inactivation in Neuroblastomas (NBs)**
Garrett Brodeur, United States
Abstract: Page 163

POT052 **The significance of Wip1 in neuroblastoma**
Jelena Milosevic, Sweden
Abstract: Page 163

POT053 **Human Neuroblastoma Cell lines with the Alternative Lengthening of Telomeres (ALT) Phenotype Manifest High Levels of Cytotoxic Drug Resistance**
Ahsan Farooqi, United States
Abstract: Page 163

POT054 **Tumor sphere specific CD133 regulation in neuroblastoma**
Hisanori Takenobu, Japan
Abstract: Page 164

POT055 **TrkA3 Isoform Expression Upregulates Stem Cell Markers and Correlates with Worse Outcome in Neuroblastomas (NBs)**
Anisha M. Simpson, United States
Abstract: Page 164

POT056 **Side population analysis and gene expression profiling identify Notch pathway genes in neuroblastoma**
Stephen Roberts, United States
Abstract: Page 164

POT057 **Phosphorylation status of Ascl1 regulates neuroblast self-renewal and differentiation**
Luke Wylie, United States
Abstract: Page 164

PROGRAMME

TUESDAY, JUNE 19, 2012
17:30 – 19:00
TUDOR 7

Parent's Symposium

Parent Committee: Tami Moscoe, Antonia Hudson, Anita Chow and Syd Birrell

5:30 - 5:35	Introductory Remarks (Dr. Sylvain Baruchel)
5:35 – 5:55	Session 1*
6:00 – 6:20	Session 2*
6:25 - 6:45	Session 3*
6:50 – 7:10	Closing Remarks (Networking, Fundraising and Awareness, Malcolm Burrows)
7:15 - 7:25	Final Remarks (James Fund)
7:25 – 9:00	Parents Reception

*Sessions:

During **each** of the three sessions, attendees can choose to participate in one of the following facilitated round-table discussions:

- New drugs and clinical trials (Dr. Julia Glade Bender, M.D., Medical Director of the Pediatric Cancer Foundation Developmental Therapeutics Program, Columbia University)
- ALK and personalized medicine (Dr. Yael Mosse, Pediatric Oncologist, Perelman School of Medicine, University of Pennsylvania)
- Immunotherapy (Dr. Alice L Yu, MD, PhD, Professor in Pediatrics , University of California in San Diego)
- Survivorship, late effects and aftercare (Dr. Lisa Diller, Chief Medical Officer, Dana Farber Children's Hospital Center)
- Relationships and social issues (Karen Fung, Social Worker, Haematology/Oncology Division, Hospital for Sick Children)

PROGRAMME

WEDNESDAY, JUNE 20, 2012
07:30 – 10:15
CONCERT HALL

Plenary Session 3: WHAT'S NEW IN "OMICS"
Session Chairs: Jan Molenaar and Isabelle Janoueix-Lerosey

07:30 - 08:30 **CONTINENTAL BREAKFAST (Canadian Ballroom)**

08:30 - 09:15 **PL10** **Will genomics provide more precise neuroblastoma therapies? Opportunities and challenges for the next decade.**
John Maris, United States
Abstract: Page 80

Dr. John Maris is currently 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 from 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 leads a team of over 50 faculty clinicians and scientists, and is responsible for the strategic direction of the pediatric cancer program at CHOP and Penn. Dr. Maris also serves as Director of the Pediatric Oncology Program in the Abramson Cancer Center at Penn. Dr. Maris has received several prestigious awards including election into the American Society of Clinical Investigation, the Oski award for outstanding pediatric oncologists, and the Berwick award at Penn for melding basic and clinical teaching. Dr. Maris was the fellowship program director at CHOP for a decade and played a major role in the training of the next generation of investigators in field of childhood cancer. He is the immediate past Chair of the Neuroblastoma Disease committee in the Children's Oncology Group (COG) and now serves on the COG Scientific Council. He is a current member of the Cancer Genetics Study Section at the NCI, and serves on many additional funding review committees and Scientific Advisory Boards.

Dr. Maris is internationally recognized as a leading expert in the field of pediatric oncology, especially in the disease neuroblastoma. His expertise is deep and broad, spanning basic genetics to clinical research and care. Dr. Maris has chosen to study this clinically important disease from all possible angles. His work on the genetic basis of human neuroblastoma has taken both traditional family-based linkage approaches and more recently whole genome association approaches. This work has resulted in his team discovering the major genetic causes of both hereditary and sporadic neuroblastoma. The genome-wide association study has amassed the largest collection of pediatric cancer cases ever assembled, and the ongoing study of 5000 neuroblastoma cases and 10,000 controls is a first in pediatric cancer, and has resulted in several major discoveries of predisposition genes, including the first example of a germline copy number variation being associated with cancer. Dr. Maris has shown an ability to translate these discoveries to the clinic, with several ongoing clinical trials based on work from his lab. For example, the discovery of activating mutations in the ALK oncogene as the cause of hereditary neuroblastoma led to the discovery of this gene being mutated somatically in sporadic neuroblastomas, followed closely by the preclinical work showing that ALK inhibition is potently cytotoxic to ALK mutated neuroblastomas. A clinical trial based on this work is ongoing, as is several other targeted therapeutic approaches. He is currently leading a large collaborative effort to sequence 200 human neuroblastoma genomes, and this will provide an unprecedented opportunity for translational science and impacting patient care.

09:15 - 09:30 **PL11** **Divergent ancestral genetic variation on chromosome 6p22accounts for racial disparities in survival in neuroblastoma.**
Navin Pinto, United States
Abstract: Page 81

09:30 - 09:45 **PL12** **Identification and functional relevance of two new neuroblastoma susceptibility loci at 6q16 within HACE1 and LIN28B**
Sharon Diskin, United States
Abstract: Page 81

09:45 - 10:00 **PL13** **Neural Crest-specific expression of Lin28b induces neuroblastoma in mice**
Johannes Schulte, Germany
Abstract: Page 81

10:00 - 10:15 **PL14** **A new 47-gene classifier for improved outcome prediction of non-high risk neuroblastoma patients – an international neuroblastoma consortium study**
André Oberthuer, Germany
Abstract: Page 82

10:15 - 10:45 **BREAK**

WEDNESDAY, JUNE 20, 2012
10:45 – 12:30
CONCERT HALL

Plenary Session 4: IMMUNOTHERAPY – Successes and Future Directions
Session Chairs: Paul Sondel and Frank Berthold

10:45 - 11:05 **PL15** **GD2-targeted immunotherapy of high risk neuroblastoma**
Alice Yu, United States
Abstract: Page 82

Dr. Yu has been pursuing translational medicine throughout her career. Her research focus lies in the immunotherapy of cancer and molecular biology of leukemia, neuroblastoma and breast cancer. As a pioneer of GD2-directed immunotherapy in neuroblastoma, Dr. Yu has taken a chimeric anti-GD2 from initial IND filing to phase I and II trials and chaired a pivotal COG phase III randomized study of the chimeric anti-GD2 in high-risk neuroblastoma. The latter demonstrated a 20% improvement in the 2 year event-free survival of this deadly cancer. This is considered a major advance in neuroblastoma for the last decade. Recently, she has spearheaded a randomized trial of globo H vaccine, another glycolipid antigen, for breast cancer in Taiwan and USA. Dr. Yu has served as a member of the COG Neuroblastoma Strategy Group, Acute Lymphoblastic Leukemia Biology Committee and T cell disease committee in USA.

11:05 - 11:25 **PL16** **Antibody-based GD2 targeted therapy for neuroblastoma (NB): past, present and beyond at Memorial Sloan-Kettering Cancer Center**
Nai-Kong V. Cheung, United States
Abstract: Page 82

11:25 - 11:40 **PL17** **Generation and characterization of a new anti-idiotype antibody ganglidiomab mimicking tumor-associated antigen disialoganglioside GD2 for active immunotherapy in neuroblastoma**
Nikolai Siebert, Germany
Abstract: Page 83

11:40 - 11:55 **PL18** **Targeting neuroblasts and neuroblast-supportive macrophages with dual-specific NKT cells**
Leonid Metelitsa, United States
Abstract: Page 83

11:55 - 12:10 **PL19** **Augmentation of NK and T cell infiltration into tumors by Intratumoral (IT) or Intravenous (IV) hu14.18-IL2 Immunocytokine (IC).**
Paul Sondel, United States
Abstract: Page 83

12:10 - 12:30 **Q&A and Future Directions – Session Chairs**

12:30 - 14:30 **LUNCH & POSTER VIEWING (Canadian Ballroom)**
Refer to page 51 for Posters

WEDNESDAY, JUNE 20, 2012
14:30 – 15:50
ONTARIO ROOM

Parallel Session 5: SIGNALING, APOPTOSIS, and TELOMERES
Session Chairs: Meredith Irwin and Carol Thiele

14:30 - 14:40	**OR31**	**Mitochondrial Bcl-2 family dynamics define therapy response and resistance in neuroblastoma** Kelly Goldsmith, United States *Abstract: Page 96*
14:40 - 14:50	**OR32**	**A synthetic-lethal siRNA screen identifies the spliceosome as a key Mcl1 regulator and therapeutic target in neuroblastoma** Theodore Laetsch, United States *Abstract: Page 96*
14:50 - 15:00	**OR33**	**CASZ1 suppresses neuroblastoma growth by recruiting epigenetic modifier NuRD and restoring Rb activity** Zhihui Liu, United States *Abstract: Page 96*
15:00 - 15:10	**OR34**	**RNA helicase A is essential for 1p36 gene KIF1Bβ tumor suppression in neuroblastomas.** Zhi Xiong Chen, Sweden *Abstract: Page 97*
15:10 - 15:20	**OR35**	**LSD1 histone demethylase expression interferes with stress response and DNA repair in a p53-dependent manner in human neuroblastoma cell lines** Alexander Schramm, Germany *Abstract: Page 97*
15:20 - 15:30	**OR36**	**Alternative Lengthening of Telomeres (ALT) in neuroblastoma tumors** Loretta Lau, Australia *Abstract: Page 97*
15:30 - 15:40	**OR37**	**Long Or Unchanged Telomeres Predict Recurrence In Neuroblastoma** Annalisa Pezzolo, Italy *Abstract: Page 98*
15:40 - 15:50	**OR38**	**Manipulating the Rac/Rho GTPase signalling in Neuroblastoma cells restores neuritogenesis and inhibits proliferation.** Ellen Westerhout, Netherlands *Abstract: Page 98*
15:50 - 16:20		**BREAK (Canadian Ballroom)**

WEDNESDAY, JUNE 20, 2012
14:30 – 15:50
CANADIAN BALLROOM

Parallel Session 6: CLINICAL AND BIOLOGICAL RISK FACTORS AND MRD
Session Chairs: Peter Ambros and Susan L. Cohn

14:30 - 14:40	**OR39**	**Quantification of bone marrow disease in high risk neuroblastoma patients by anti-GD2 immunocytochemistry – impact on survival. A SIOPEN High Risk Study.** Klaus Beiske, Norway *Abstract: Page 98*
14:40 - 14:50	**OR40**	**Clinical Relevance of Positive Marrow MRD following 2 Cycles of Anti-GD2 Immunotherapy** Irene Cheung, United States *Abstract: Page 99*
14:50 - 15:00	**OR41**	**Prospective Evaluation of MYCN Gene Amplification Status using Serum DNA of Neuroblastoma Patients** Tomoko Iehara, Japan *Abstract: Page 99*
15:00 - 15:10	**OR42**	**Combining gene set signatures and public databases identified a 20-gene predictor of survival in stage 4 neuroblastoma** Mario Capasso, Italy *Abstract: Page 99*
15:10 - 15:20	**OR43**	**Outcome Analysis of Non-high-risk Neuroblastoma Patients Enrolled on Children's Oncology Group Trials P9641 and A3961** Edward Attiyeh, United States *Abstract: Page 100*
15:20 - 15:30	**OR44**	**Highly Sensitive Quantitation of Neuroblastoma Cells in Bone Marrow with a Five-Gene TaqMan® Low Density Array Assay Identifies Patients at High-Risk for Disease Progression after Myeloablative Consolidation Therapy** Robert Seeger, United States *Abstract: Page 100*
15:30 - 15:40	**OR45**	**Factors that contribute to inferior survival of low-risk stage2B neuroblastoma patients: A Children's Oncology Group study.** Douglas Strother, Canada *Abstract: Page 100*
15:40 - 15:50	**OR46**	**Neuroblastoma in Older Children, Adolescents and Young Adults: A Report from the International Neuroblastoma Risk Group Project** Rebecca Deyell, Canada *Abstract: Page 101*
15:50 - 16:20		**BREAK (Canadian Ballroom)**

PROGRAMME

WEDNESDAY, JUNE 20, 2012
14:30 – 15:40
TUDOR 7/8

Parallel Session 7: NEW IDEAS
Session Chairs: Rani George and Jean Michon

14:30 - 14:37	**OR47**	**Post-chemotherapy Image Defined Risk Factors assessment is a useful tool to predict surgical risk.** Sabine Irtan, France *Abstract: Page 101*
14:37 - 14:44	**OR48**	**The prognostic value of the SIOPEN skeletal scoring method in high-risk stage 4 neuroblastoma by semi-quantitative I-123-mIBG scintigraphy.** Ruth Ladenstein, Austria *Abstract: Page 102*
14:44 - 14:51	**OR49**	**COUP-TF1, a transcription factor associated with the norepinephrine transporter, is essential for in vitro retinoic acid response in high-risk (HR) neuroblastoma** Haneen Abdella, United States *Abstract: Page 102*
14:51 - 14:58	**OR50**	**Massively parallel transcriptome sequencing in Neuroblastoma reveals specific mRNA splicing patterns controlled by RNA binding splicing factors** Javed Khan, United States *Abstract: Page 102*
14:58 - 15:05	**OR51**	**Whole-genome sequencing yields tumor-specific DNA rearrangements as targets for minimal residual disease detection in neuroblastoma** Esther M van Wezel, Netherlands *Abstract: Page 103*
15:05 - 15:12	**OR52**	**MYCN-DNA vaccination suppresses growth of MYCN overexpressing neurobastloma in a syngeneic mouse model** Alexander Stermann, Germany *Abstract: Page 103*
15:12 - 15:19	**OR53**	**Haploidentical stem cell transplantation and subsequent antiGD2 based immunotherapy for patients with relapsed metastatic neuroblastoma** Peter Lang, Germany *Abstract: Page 103*
15:19 - 15:26	**OR54**	**Aberrant activation of the RAS pathway confers resistance to targeted therapeutics in neuroblastoma** Sidong Huang, Netherlands *Abstract: Page 103*
15:26 - 15:33	**OR55**	**Non-polyalanine repeat expansion mutations of the PHOX2B gene dysregulate Sox10 expression and cause the neurocristopathy in the autonomic nervous system** Hideki Enomoto, Japan *Abstract: Page 104*
15:33 - 15:40	**OR56**	**Neuroblastoma stage 4S is a multifocal stem cell disease of defected neural crest precurser cells** Max van Noesel, Netherlands *Abstract: Page 104*
15:50 - 16:20		**BREAK (Canadian Ballroom)**

WEDNESDAY, JUNE 20, 2012
16:20 – 17:40
ONTARIO ROOM

Parallel Session 8: GENOMICS
Session Chairs: Angelika Eggert and Annie Huang

16:20 - 16:30	**OR57**	**Discovery of rare variants in TP53 associated with neuroblastoma** Sharon Diskin, United States *Abstract: Page 104*
16:30 - 16:40	**OR58**	**Whole exome sequencing of 59 neuroblastomas with different genomic subgroups, P1a or Ss, has revealed distinct pattern of mutations and pathways** Yuanyuan Li, Japan *Abstract: Page 105*
16:40 - 16:50	**OR59**	**Whole Genome Sequencing Revealed Recurrent ATRX Somatic Mutations Correlated with Age at Diagnosis and Telomere Length among Patients with Stage 4 Neuroblastoma** Nai-Kong Cheung, United States *Abstract: Page 105*
16:50 - 17:00	**OR60**	**Segmental Chromosome Aberratons In Localized Resectable Neuroblastoma Without MYCN Amplification Have A Strong Prognostic Impact In Patients Diagnosed Over The Age Of 18 Months But Not In Younger Patients** Inge Ambros, Austria *Abstract: Page 105*
17:00 - 17:10	**OR61**	**Characterization of the neuroblastoma transcriptome by RNA deep-sequencing: A study of the Sequencing Quality Control (SEQC) consortium** Matthias Fischer, Germany *Abstract: Page 106*
17:10 - 17:20	**OR62**	**Genome-wide massively parallel sequencing to characterize genomic rearrangements in neuroblastoma: from unbalanced translocations to chromothripsis** Isabelle Janoueix-Lerosey, France *Abstract: Page 106*
17:20 - 17:30	**OR63**	**Paired-end whole genome sequencing identifies chromothripsis in high stage neuroblastoma** Jan Koster, Netherlands *Abstract: Page 106*
17:30 - 17:40	**OR64**	**Genetic evolution of neuroblastoma is characterized by new chromosome breakpoints** Gudrun Schleiermacher, France *Abstract: Page 107*

WEDNESDAY, JUNE 20, 2012
16:20 – 17:40
CANADIAN BALLROOM

Parallel Session 9: EXPERIMENTAL THERAPIES (RX I)
Session Chairs: Rochelle Bagatell and Gilles Vassal

16:20 - 16:30	**OR65**	**Propranolol as a novel treatment for neuroblastoma** Jennifer Wolter, Canada *Abstract: Page 107*
16:30 - 16:40	**OR66**	**Anticancer Compounds that simultaneously suppress NFKB and activate p53 are highly effective at delaying tumor development and progression in the TH-MYCN mouse model of neuroblastoma** Michelle Haber, Australia *Abstract: Page 108*
16:40 - 16:50	**OR67**	**Volasertib (BI 6727), a second generation Polo-like kinase 1 (Plk1) inhibitor, is an active agent in preclinical neuroblastoma mouse models** Libo Zhang, Canada *Abstract: Page 108*
16:50 - 17:00	**OR68**	**4-HPR (fenretinide) sensitizes human neuroblastoma cells for ch14.18-mediated NK cell and complement killing** Holger Lode, Germany *Abstract: Page 108*
17:00 - 17:10	**OR69**	**In vitro and in vivo validation of ABT263 and YM155; new targeted compounds in apoptotic signaling** Jan Molenaar, Netherlands *Abstract: Page 108*
17:10 - 17:20	**OR70**	**Targeting the Hedgehog signal transduction pathway at the level of GLI inhibits neuroblastoma cell growth in vitro and in vivo** Malin Wickström, Sweden *Abstract: Page 109*
17:20 - 17:30	**OR71**	**A phase I study of bolus and metronomic cyclophosphamide with zoledronic acid and bevacizumab in children with recurrent or refractory neuroblastoma: a New Approaches to Neuroblastoma Therapy consortium trial** Julia Glade Bender, United States *Abstract: Page 109*
17:30 - 17:40	**OR72**	**Two step-Phase II study of imatinib mesylate in pediatric patients with unresponsive or relapsing metastatic neuroblastoma** Maria Valeria Corrias, Italy *Abstract: Page 109*

WEDNESDAY, JUNE 20, 2012
17:30 – 18:30
CANADIAN BALLROOM

Poster Viewing

BASIC

POB071 **Factors associated with recurrence and length of survival following relapse in patients with neuroblastoma: a pilot study**
Gail Halliday, United Kingdom
Abstract: Page 134

POB072 **Long-term vascular access at chemotherapy in children with oncology: optimisation, prevention and treatment of aftereffects**
Maxim Rykov, Russian Federation
Abstract: Page 134

POB074 **CDK4 inhibition increases chemosensitivity of MYCN-amplified neuroblastoma cells**
Sina Gogolin, Germany
Abstract: Page 135

POB075 **Radiosensitization of neuroblastoma cells after DNA-PK inhibition with NU7026**
M.E.M. Dolman, Netherlands
Abstract: Page 135

POB076 **The antifungal drug ciclopirox olamine as a potential therapeutic agent for the treatment of neuroblastoma**
Viktoryia Sidarovich, Italy
Abstract: Page 135

POB077 **A screening for natural products identifies isorhamnetin as a synergistic compound with 13-cis retinoic acid in neuroblastoma**
Pamela Gatto, Italy
Abstract: Page 136

POB078 **MR and Hemodynamic Response Imaging (HRI) for monitoring the efficacy of a novel anti-angiogenesis drug combination on a Neuroblastoma murine model**
Chani Komar, Israel
Abstract: Page 136

POB079 **Genetic and Epigenetic Determinants of Differentiation Potential**
Abraham Fong, United States
Abstract: Page 136

POB080 **The neuroblastoma and ganglion components of nodular ganglioneuroblastoma share the same genetic changes, distinct from Schwann cells**
Paola Angelini, Canada
Abstract: Page 136

POB081 **MLL is a strong candidate driver gene for high risk MYCN non amplified neuroblastomas: a multi-dimensional high-resolution genomic data mining approach**
Annelies Fieuw, Belgium
Abstract: Page 137

POB082 **Mapping the neuroblastoma epigenome: perspectives for improved prognostic biomarkers**
Maté Ongenaert, Belgium
Abstract: Page 137

POB083 **Promoter methylation analysis identifies prognostic methylation biomarkers in neuroblastoma**
Anneleen Decock, Belgium
Abstract: Page 137

POB084 **A map of genomic copy number alterations in neuroblastoma based on annotation-guided breakpoint detection**
Toby Hocking, France
Abstract: Page 137

POB085 **Translatome Profiling Of Neuroblastoma Cell Lines Reveals Extensive Translational Deregulation Of Histone Genes**
Alessandro Quattrone, Italy
Abstract: Page 138

POB086 **M2 macrophages express CD1d and are selectively targeted by NKT cells in neuroblastoma**
Leonid Metelitsa, United States
Abstract: Page 138

POB087 **The promoter methylation may lessen the increased pro-apoptotic impact of RASSF1A in triploid neuroblastoma found by mass screening**
Yasuhiko Kaneko, Japan
Abstract: Page 138

POB088 **Inactivation of hSgo1 shows synthetic phenotype to MYCN amplification.**
Yuko Murakami-Tonami, Japan
Abstract: Page 138

POB089 **PES1 is a MYCN-related driver gene of neuroblastoma and shows modified histone binding**
Masato Nakaguro, Japan
Abstract: Page 139

POB090 **Exploiting synthetic lethality for the Identification of therapeutic targets in MYCN-amplified neuroblastoma.**
Olesya Chayka, United Kingdom
Abstract: Page 139

POB091 **Mxi1 Inhibits N-Myc-Mediated Proliferation And Induces Neuroblastoma Cell Apoptosis**
Michael Armstrong, United States
Abstract: Page 139

POB092 **Neuroblastoma cell lines express embryonic neural crest stem cells genes**
Paola Angelini, United Kingdom
Abstract: Page 139

POB093 **NLRR3 negatively regulated by MYCN induces neuronal differentiation through proteolytic processing by ECEL1**
Jesmin Akter, Japan
Abstract: Page 140

POB094 **RUNX3, whose gene is mapped to chromosome 1p36, facilitates protein degradation of MYCN in neuroblastoma**
Tomoki Yokochi, Japan
Abstract: Page 140

POB095 **ARPP19 negatively regulates MYCN stability and enhances differentiation of neuroblastoma both in vivo and in vitro**
Md. Shamim Hossain, Japan
Abstract: Page 140

POB096 **NCYM, a MYCN antisense gene product, induces OCT4 promotes cell proliferation in neuroblastoma**
Yoshiki Kaneko, Japan
Abstract: Page 141

POB097 **The MYCN target gene AHCY drives methylation reactions and is thus involved in tumourigenesis**
Christina Schroeder, Germany
Abstract: Page 141

POB098 **Disrupting the N-Myc/Aurora-A complex as an approach to control N-Myc levels in childhood neuroblastoma**
Markus Brockmann, Germany
Abstract: Page 141

POB099 **The JARID1C histone demethylase is upregulated in aggressive neuroblastomas independent of MYCN amplification**
Alexander Schramm, Germany
Abstract: Page 142

POB100 **Exploitation of the chick embryonic microenvironment to reprogram MYCN-amplified neuroblastoma cells to a benign phenotype, lacking detectable MYCN expression**
Rachel Carter, United Kingdom
Abstract: Page 142

POB101 **CHD5 Promoter Regulation by MYCN in Human Neuroblastoma (NB)**
Mayumi Higashi, United States
Abstract: Page 142

POB102 **MYCN up regulation activates the folate metabolism and sensitizes cells for thymidylate synthase inhibitors**
Filip Pattyn, Belgium
Abstract: Page 143

POB103 **Mutation of the N-terminal T58 phosphorylation site of N-Myc stabilizes N-Myc protein expression and enhances its oncogenic potential**
Xiaodun Li, United Kingdom
Abstract: Page 143

POB104 **Integration of genomic and proteomic data identifies MYCN-regulated genes, proteins and interaction networks in neuroblastoma cells**
Samuel Volchenboum, United States
Abstract: Page 143

POB105 **Identification of synthetic lethal genes to MYCN-amplification**
Shubo Zhang, United States
Abstract: Page 143

POB106 **Distinct roles for the CXCL12 receptors, CXCR4 and CXCR7 in human neuroblastoma**
Julie Liberman, Switzerland
Abstract: Page 144

POB107 **High AXL promotes migration in non-MYCN amplified neuroblastoma cell lines**
Max van Noesel, Netherlands
Abstract: Page 144

POB108 **Prokineticins promotes neuroblastoma progression by maintaining a de novo population of c-KIT expressing cells**
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THURSDAY, JUNE 21, 2012
07:30 – 10:15
CONCERT HALL

Plenary Session 5: From Stem Cells to Spontaneous Regression
Session Chairs: Garrett Brodeur and Per Kogner

07:30 - 08:30 **CONTINENTAL BREAKFAST**

08:30 - 09:15 **PL20** **Nervous system tumor stem cells: the cancer stem cell hypothesis writ large**
Peter Dirks, Canada
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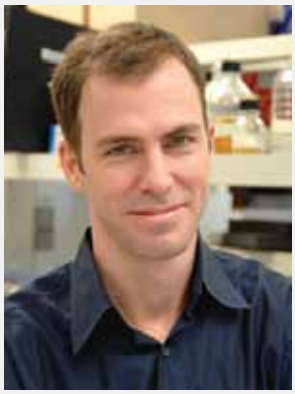

Peter Dirks is a neurosurgeon and senior scientist at the Hospital for Sick Children in the University of Toronto. His group was amongst the first to identify and characterize stem cell populations in human solid cancer. They are currently focused on elucidating a deeper understanding of the significance of cancer stem cells in human brain tumors, investigating their origins from normal brain cells, studying mechanisms governing their self renewal and proliferation, and developing novel therapeutics through cell based chemical biological and genetic screens.

09:15 - 09:30 **PL21** **Isolation and Characterization of a Novel Cancer Stem Cell-like Population in Neuroblastoma**
Jason Shohet, United States
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Stanley He, United States
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09:45 - 10:00 **PL23** **A Prospective Study of Expectant Observation as Primary Therapy for Neuroblastoma in Young Infants, a Children's Oncology Group Study**
Jed Nuchtern, United States
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10:00 - 10:15 **PL24** **Regression and differentiation in localized infant neuroblastoma**
Barbara Hero, Germany
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10:15 - 10:45 **BREAK**

THURSDAY, JUNE 21, 2012
10:45 – 12:00
CONCERT HALL

Plenary Session 6: Experimental Therapies Towards International Collaboration
Session Chairs: Sylvain Baruchel and Andrew Pearson

10:45 - 10:55 **PL25** **The Therapeutically Applicable Research to Generate Effective Treatments (TARGET) in Neuroblastoma Project**
John Maris, United States
Abstract: Page 85

10:55 - 11:15 **PL26** **New Agents for High-Risk Neuroblastoma: Combining, Refining, Redesigning, Selection and Discovery**
Julia Glade Bender, United States
Abstract: Page 85

Julia Glade Bender, M.D. is the Herbert Irving Assistant Professor of Clinical Pediatrics at Columbia University and the Medical Director of the Pediatric Cancer Foundation Developmental Therapeutics Program. She is recognized for her expertise in pediatric antiangiogenesis, and developed and chaired the first clinical trials of bevacizumab and pazopanib in children with relapsed solid tumors conducted though the Children's Oncology Group (COG). She is a special member of the New Approaches to Neuroblastoma Therapy Consortium and holds appointments on the COG Developmental Therapeutics and Neuroblastoma Steering Committees. She currently leads the COG Task Force on Maintenance Therapy for High Risk Neuroblastoma.

11:15 - 11:35 **PL27** **Accelerating Drug Development in Neuroblastoma – the SIOPEN ITCC Approach**
Andrew Pearson, United Kingdom
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11:35 - 11:50 **PL28** **Why are neuroblastoma patients ignored by the European Pediatric Medicine Regulation?**
Gilles Vassal, France
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11:50 - 12:00 **Q&A – Session Chairs**

12:00 - 13:00 **LUNCH (Canadian Ballroom)**

THURSDAY, JUNE 21, 2012
13:00 – 14:30
ONTARIO ROOM

Parallel Session 10: Predictive Markers & Clinical Trials
Session Chairs: Clare Twist and Thorsten Simon

13:00 - 13:10	**OR73**	**Curie and SIOPEN scoring in stage 4, high risk neuroblastoma. A report from the Metastatic Imaging Working Group of the International Neuroblastoma Response Criteria (INRC) Committee** Gregory Yanik, United States *Abstract: Page 110*
13:10 - 13:20	**OR74**	**The CURIE and the SIOPEN mIBG-scoring systems equally predict outcome in patients with stage 4 neuroblastoma: Results of the Cologne Inter-score Comparison Study** Boris Decarolis, Germany *Abstract: Page 110*
13:20 - 13:30	**OR75**	**Norepinephrine transporter (NET) protein, but not mRNA, expression is correlated with metaiodobenzylguanidine (MIBG) avidity in newly diagnosed neuroblastoma (NB) patients: A Report from the Children's Oncology Group (COG)** Vandana Batra, United States *Abstract: Page 110*
13:30 - 13:40	**OR76**	**Image-defined risk factors in localized thoracic neuroblastoma** Maike Reisberg, Germany *Abstract: Page 111*
13:40 - 13:50	**OR77**	**The role of imaging in detecting relapse in patients with Neuroblastoma. Can post-therapy surveillance programs be simplified?** Cormac Owens, Canada *Abstract: Page 111*
13:50 - 14:00	**OR78**	**Phase I Study of 131I-MIBG with Vincristine and Five Days of Irinotecan for Patients with Relapsed or Refractory Neuroblastoma** Steven DuBois, United States *Abstract: Page 111*
14:00 - 14:10	**OR79**	**Long-term outcome of MATIN, a schedule of high-administered activity Iodine 131 meta-iodobenzylguanidine and topotecan in neuroblastoma: A SIOPEN study** Mark Gaze, United Kingdom *Abstract: Page 111*
14:10 - 14:20	**OR80**	**Treatment of children over the age of one year with unresectable localized neuroblastoma without MYCN amplification: results of the Siopen Study** Alberto Garaventa, Italy *Abstract: Page 112*
14:20 - 14:30	**OR81**	**An epidemiological view on neuroblastoma trials over 30 years: Is there any progress?** Frank Berthold, Germany *Abstract: Page 112*

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THURSDAY, JUNE 21, 2012
13:00 – 14:30
CONCERT HALL

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Session Chairs: Patrick Reynolds and Mike Hogarty

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13:10 - 13:20	**OR83**	**Preclinical studies support therapeutic evaluation of targeting IL-6/JAK/STAT3 pathway in Neuroblastoma** Carol Thiele, United States *Abstract: Page 113*
13:20 - 13:30	**OR84a**	**Inhibiting Monocyte/Macrophage-Neuroblastoma Cell Interactions with Sorafenib Increases Tumor Cell Response to Cyclophosphamide and Topotecan** Yibing Xu, United States *Abstract: Page 113*
	OR84b	**Inhibiting Monocyte/Macrophage-Neuroblastoma Cell Interactions with Lenalidomide Increases Tumor Cell Response to Cyclophosphamide and Topotecan** Yibing Xu, United States *Abstract: Page 113*
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13:40 - 13:50	**OR86**	**Fenretinide and Vorinostat combination therapy for neuroblastoma** Belamy Cheung, Australia *Abstract: Page 114*
13:50 - 14:00	**OR87**	**Immunotherapy for Neuroblastoma by GD2-specific chimeric antigen receptor** John Anderson, United Kingdom *Abstract: Page 114*
14:00 - 14:10	**OR88**	**Antagonizing polyamine homeostasis prevents tumor initiation and lethal progression in complementary models of murine and human neuroblastoma** Michael Hogarty, United States *Abstract: Page 115*
14:10 - 14:20	**OR89**	**Phase I trial of a bivalent vaccine with escalating doses of the immunological adjuvant OPT-821, in combination with oral β-glucan for high-risk neuroblastoma (NB)** Brian Kushner, United States *Abstract: Page 115*
14:20 - 14:30	**OR90**	**Phase I Study of Anti-GD2 Humanized 3F8 (hu3F8) Monoclonal Antibody (MoAb) in Patients with Relapsed or Refractory Neuroblastoma (NB).** Ellen Basu, United States *Abstract: Page 115*

PROGRAMME

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CONCERT HALL

Closing Remarks

Towards ANR 2014!
Dr. Sylvain Baruchel, ANR 2012 Conference Chair

ABSTRACT BOOK

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PRECISION MEDICINE: A NEWLY REVISED INRC (3) WS11

WS11

Consensus Statement on the Revised International Neuroblastoma Response Criteria

Julie R. Park[1], Rochelle Bagatell[2], Frank Berthold[3], Ariane Boubaker[4], Penelope Brock[5], Susan Burchill[6], Susan L. Cohn[7], Wendy B. London[8,9], John M. Maris[2], Kieran McHugh[5], Jed G. Nuchtern[10], Andrew Pearson[11], Nita L.Siebel[12], Gudrun Schleiermacher[13], Katherine K. Matthay[14], Dominique Valteau-Couanet[15]

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Background: The International Neuroblastoma (NB) Response Criteria (INRC) were last updated in 1993 and have significant limitations in accurately defining response at metastatic sites (bone and bone marrow). The INRC provide limited guidance for incorporation of standard imaging modalities (123I-MIBG imaging), and no guidance for incorporation of FDG-PET or evolving techniques for quantification of marrow disease. Current response criteria for recurrent malignancies are based on cross-sectional imaging of measurable disease. Because recurrence of high risk NB primarily occurs at metastatic sites that cannot be measured by cross-sectional imaging, our ability to assess the activity of novel therapeutic agents is impaired. Also, a lack of consensus regarding the definition of treatment failure further limits international clinical trial collaboration.

Methods: An NCI-sponsored international meeting was held to develop consensus guidelines for assessment of response in patients with newly diagnosed and recurrent NB. Data obtained from COG, NANT, SIOP-EN and GPOH contemporary clinical trials were analyzed. A limited institution retrospective trial will assess the association between primary tumor response (measured in 1, 2 or 3-dimensions) and change in image-defined risk factors and completeness of surgical resection. Molecular data will be analyzed to define a group of patients at risk for early treatment failure.

Results: Individual response components of a revised INRC will include primary tumor dimensions, metastatic disease assessment using 123I-MIBG imaging and bone marrow morphologic assessment. 123I-MIBG and PET scans replace bone scan for assessment of metastatic bone disease. Overall response categories will be defined as complete response, partial response, minor response, stable disease and progressive disease.

Conclusions: Standardization in risk group stratification and response assessment are paramount to enabling international clinical trial comparisons and collaborative trial development. We are now poised to develop a revised INRC and an internationally accepted approach to characterize patients at highest risk for early treatment failure.

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TARGETED RADIOPHARMACEUTICAL THERAPY OF NEUROBLASTOMA WS21 – WS28

WS21

Targeting the norepinephrine transporter for imaging and therapy of high risk neuroblastoma

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Neuroblastoma, the most common extra- cranial solid tumor in children, is derived from neural crest cells. 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. Metaiodobenzylguanidine (MIBG) is an aralkylguanidine norepinephrine analogue originally developed to visualize tissue of sympathetic neuronal origin, which has now become an essential tool for neuroblastoma staging and response. MIBG actively enters cells by the human norepinephrine transporter (hNET) expressed in 90% of neuroblastomas, prompting the use of radiolabeled MIBG for targeted radiotherapy in these tumors. Early phase trials have confirmed the activity of 131I-MIBG in relapsed neuroblastoma, with response rates of about 30%, but the technical aspects of administration of large amounts of radioactivity in young children and the limited access have hindered incorporation into treatment of newly diagnosed patients. Clinical studies of 131I-MIBG have identified myelosuppression as the main dose-limiting toxicity, necessitating stem cell reinfusion at higher doses. Recent studies focusing on the use of 131I-MIBG in combination with chemotherapy or myeloablative regimens have shown activity and tolerability in refractory disease. Initial studies of no-carrier added MIBG suggest the possibility of a higher therapeutic ratio. The early promise of MIBG for imaging and therapy have led to new challenges and questions, which will be addressed in this symposium: more precise dosing with better dosimetry measurements, new radiopharmaceuticals with different radioisotopes for better cellular penetration or different ligands for neuroblastomas lacking MIBG avidity, radiosensitizers to improve the efficacy, and improving patient compliance and safety and increased access. Future challenges must address the best therapeutic use of MIBG for newly diagnosed patients, and the best way to combine this treatment with radiosensitizers and other targeted therapies, such as immunotherapy or small molecule inhibitors.

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WS22

Pretherapy dosimetry with I-124 MIBG PET/CT

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Background: For 131I-metaiodobenzylguanidine (MIBG) therapy of neuroblastoma, individualized radiation dosimetry can be achieved by pretherapy imaging using 124I-MIBG. I-124 has a half-life of 4.2 days and emits positrons so that positron emission tomography (PET) can be employed for imaging. PET is a superior imaging modality in terms of quantitative accuracy over planar scintigraphy or single photon emission computed tomography (SPECT). In addition, the half-life of I-124 allows 4-5 days of imaging time window so that the organ and tumor dosimetry data can be easily converted to those of I-131.

Methods: In order to establish the correlation of the human norepinephrine transporter (hNET) expression and 124I-MIBG uptake, we performed in vitro cell uptake study using the NB1691 human neuroblastoma cell line that expresses a high level of hNET. In addition, 124I-MIBG in vivo PET/CT was performed on murine models xenografted using hNET-overexpressed NB1691 and unmodified NB1691 cells. 124I-MIBG was administered intravenously, and microPET/CT was performed at 5 time points over 95 hours. In vivo 124I-MIBG biokinetics data were obtained in major organs and tumors from reconstructed PET/CT images for radiation dosimetry.

Results: In vitro cell uptake study showed that there is increased uptake of 124I-MIBG in NB1691 cells in comparison to uptake in control cells. However, in vivo imaging using the animal model showed no prominent uptake of 124I-MIBG in unmodified NB1691 tumors; however, the 124I-MIBG uptake in hNET-overexpressed NB1691 tumors was highly visualized. Organ dosimetry estimated the mean effective dose of 0.31 mSv/MBq for 124I-MIBG when the animal data were extrapolated to human equivalents. Tumor radiation dose calculation was also possible assuming the simplest sphere tumor model.

Conclusions: 124I-MIBG PET/CT imaging techniques showed utility in estimating organ and tumor radiation dose for both 124I-MIBG and 131I-MIBG assuming the same biodistribution for both. The tumor dosimetry for nonspherical geometries and considerations of surrounding activities will need further investigation. From the success of the preclinical imaging studies, we are currently initiating the 124I-MIBG imaging trial in patients who will receive the 131I-MIBG therapy.

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WS23
Pre-clinical testing of radiosensitizers with MIBG
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Background: We previously reported that combining [131I]MIBG with the topoisomerase I inhibitor topotecan induced long-term DNA damage and supra-additive toxicity to noradrenaline transporter (NAT)-expressing cells and xenografts. This combination treatment is undergoing clinical evaluation. To further enhance the efficacy of [131I]MIBG + topotecan, the present study investigated interaction with PJ34 (an inhibitor of Poly(ADP-ribose) polymerase [PARP-1]) and disulfiram (an anti-alcoholic drug and possible radiosensitizer by virtue of the induction of oxidative stress and inhibition of proteasome activity) in vitro and in vivo.

Methods: Combinations of PJ34 with [131I]MIBG + topotecan and of disulfiram with [131I]MIBG were assessed, by combination index analysis, for synergistic kill of clonogens from SK-N-BE(2c) (neuroblastoma) and UVW/NAT (NAT gene-transfected glioma) cells. Efficacy in vivo was measured by growth delay of tumour xenografts. We also assessed DNA damage by γ-H2AX assay, cell cycle progression by FACS analysis and PARP-1 activity in treated cells.

Results: In vitro, all schedules of combinations of PJ34 with [131I]MIBG + topotecan induced supra-additive toxicity to and increased DNA damage in SK-N-BE(2c) cells but only simultaneous administration of PJ34, [131I]MIBG and topotecan induced enhanced kill of UVW/NAT clonogens. PJ34 with [131I] MIBG + topotecan induced G2 arrest in all cell lines, regardless of the schedule of delivery. Simultaneous administration of PJ34 with [131I]MIBG + topotecan to athymic mice significantly delayed the growth of SK-N-BE(2c) and UVW/NAT xenografts compared with [131I]MIBG + topotecan treatment in the absence of PJ34. Copper was necessary for the radiosensitizing activity of disulfiram which enhanced the efficacy not only of external beam radiation but also of [131I] MIBG.

Conclusion: The anti-tumor efficacy of [131I]MIBG + topotecan combination treatment was increased by PARP-1 inhibition in vitro and in vivo. This indicates the potential benefit of three-way combination therapy. Furthermore, disulfiram may have therapeutic potential in combination with [131I]MIBG and copper supplementation.

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WS24
Clinical Trials of 131I-metaiodobenzylguanidine (MIBG) with Concomitant Radiation Sensitizers for the Treatment of Neuroblastoma
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MIBG is a targeted radiopharmaceutical that is among the most active agents for patients with relapsed or refractory neuroblastoma. Radiation sensitizers are drugs that augment the anticancer activity of radiotherapy. An optimal radiation sensitizer for use in combination with MIBG would have anti-neuroblastoma activity as well as non-overlapping toxicity with MIBG. One group has evaluated pretreatment with cisplatin-based chemotherapy followed by MIBG and demonstrated that this approach is feasible both in patients with relapse and in patients with newly diagnosed neuroblastoma. Based on strong preclinical data showing additive activity, one group evaluated MIBG in combination with concomitant topotecan and demonstrated that this approach was feasible. The New Approaches to Neuroblastoma Therapy (NANT) consortium then conducted a phase 1 trial of vincristine and irinotecan with concomitant MIBG, using irinotecan 20 mg/m2/dose on a 5 day/week x 2 weeks schedule. The maximum tolerated dose of MIBG with this combination was 18 mCi/kg, though 25% of patients had grade 3 diarrhea. UCSF has since completed a pilot study evaluating MIBG in combination with vincristine and irinotecan 50 mg/m2/ dose on a 5 day/week x 1 week schedule. This combination was also tolerable up to MIBG doses of 18 mCi/kg, with lower rates of diarrhea. Preclinical studies have demonstrated that vorinostat, a histone deacetylase inhibitor, both sensitizes neuroblastoma cells to radiation and upregulates norepinephrine transporter expression by neuroblastoma cells. Based on these data, the NANT consortium is conducting an ongoing phase 1 study of MIBG in combination with concomitant vorinostat. Other potential radiation sensitizers that may be considered for future combination studies include PARP inhibitors, aurora A kinase inhibitors, and PI3 kinase inhibitors. Ultimately, randomized studies will be needed to determine whether the addition of radiation sensitizers improves response rates compared to
single-agent MIBG.

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WS25
Lu-177-DOTATATE for targeted therapy of neuroblastoma
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Background: Several radionuclides, including 177-Lutetium and 90-Yttrium, chelated with DOTA to various somatostatin analogues, including octreotide and octreotate, have been used for targeted therapy of metastatic adult neuroendocrine cancers expressing somatostatin receptors. Such peptide receptor radionuclide therapy has been shown to be a safe and effective treatment for this patient group, and is now regarded as standard practice. Neuroblastoma cells sometimes express somatostatin receptors. The aim of this study was to extend the use of Lu-177-DOTATATE (LuDO) to patients with neuroblastoma shown on diagnostic imaging with 68-Gallium DOTATATE PET/ CT to express somatostatin receptors.

Methods: Patients with relapsed or refractory neuroblastoma were considered eligible for LuDO therapy if uptake on the diagnostic scan in tumour equal to or higher than that of the liver. Patients typically received three administrations of 7.3GBq at two month intervals.

Results: Of the 6 children treated, 5 had stable disease by the response evaluation criteria in solid tumors (RECIST). Of these 5 children, 2 had an initial metabolic response and reduction in the size of their lesions, and 1 patient had a persistent partial metabolic response and reduction in size of the lesions on CT, although the disease was stable by RECIST. One had progressive disease. Three children had grade 3 and 1 child had grade 4 thrombocytopenia. No significant renal toxicity has been seen.

Conclusions: LuDO treatment of children with neuroblastoma is feasible, and partial metabolic responses may result. The principal toxicity is myelosuppression, principally thrombocytopenia, which may be dose limiting. Further formal evaluation in a Phase II trial is planned.

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WS26

Development of α-particle emitting meta-[211At]-astatobenzylguandidine for neuroblastoma therapy.

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The majority of patients with high-risk neuroblastoma are not cured with current multimodality treatment, and survivors often have significant long-term morbidity (1). Targeted therapies for cancer rely on exploiting molecular aberrations unique to the tumor cell compared to host tissues. This is especially critical in the developing child. The norepinephrine transporter (NET) is differentially overexpressed on the cell surface of most human neuroblastomas and provides a highly specific mechanism for directing uptake of NET-ligands into the tumor. Metaiodobenzylguanidine, radiolabeled with the β-emitter Iodine-131 (131I), is one such compound, and we have extensive experience with this agent as a targeted radiotherapeutic in relapsed or refractory neuroblastoma (2-5). However, 131I-MIBG is most effective against bulky tumor masses due to the radiobiologic properties of β-energy emission, and does not significantly impact isolated tumor cells or microscopic clusters. This likely explains the clinical observation that responses in the bone marrow compartment can be incomplete and transient. Zalutsky and colleagues have previously developed Astatine-211 (211At), a short-lived (7.2 hour) α-emitting halogen with considerably more potent and focused cytotoxicity, as a potential targeted radiotherapeutic in neuroblastoma (6). Practical issues have limited further preclinical and clinical development, but here we seek to address these issues and address the unmet need of developing novel effective targeted radiotherapeutic strategies for neuroblastoma, with future applications to related cancers such as malignant pheochromocytoma/paraganglioma. Our long-term goal is to improve outcomes for children and adults with neuroblastoma using targeted radiotherapy. The immediate objective is to develop -particle emitting approaches using highly characterized preclinical models and rational combinatorial strategies. Our central hypothesis is that targeted radiotherapy with the α-particle emitting NET-ligand meta-[211At]-astatobenzylguanidine (211At-MABG) will be safe and effective against the NET-expressing cancer neuroblastoma. At ANR 2012 we will report on recent progress in 1) developing reproducible in vitro and in vivo models to study 211At-MABG; 2) recent progress in synthesizing high specific activity 211At-MABG at the University of Pennsylvania; and 3) discuss our immediate and long term plans towards an early phase clinical trial of 211At-MABG.

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WS27

Comparison among 18F-FDOPA PET, 18F-FDG PET, and 123I-MIBG scan in neuroblastic tumors

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Background: Neuroblastic tumors (NTs) can theoretically be imaged by 18F-fluoro-dihydroxyphenylalanine (18F-FDOPA) positron emission tomography (PET), a recently emerged diagnostic tool for neuroendocrine tumors. In this study, we compared the accuracy among 18F-FDOPA PET, 123I-MIBG scan, and 18F-FDG PET in NTs.

Methods: From 2008 to 2011, patients with tissue-proven NTs receiving 18F-FDOPA PET at initial diagnosis and/or during follow-ups were included in this analysis. Using histopathology as the gold standard, the sensitivity and specificity of 18F-FDOPA PET were compared to those of 123I-metaiodobenzylguanidine (123I-MIBG) scintigraphy and 18F-fluorodeoxyglucose (18F-FDG) PET by McNemar test.

Results: Fifty tumors from 34 patients, including 42 NTs and 8 lesions without viable tumor cells, were eligible for analysis. The patients (20 boys and 14 girls) had a median age of 2.8 (range, 0.2–8.6) years, with advanced stage (INSS 3 or 4) in 26/34 (76%) and MYCN amplification in 7/32 (22%). 18F-FDOPA PET clearly visualized the primary NTs and probable metastatic lesions in sites such as lymph nodes, bone/bone marrow, and cranium. Using histopathology as the gold standard, 18F-FDOPA PET showed a sensitivity of 97.6% (87.4%–99.9%) and a specificity of 87.5% (47.3%–99.7%). In tumors with concomitant studies, 18F-FDOPA PET demonstrated a higher sensitivity than 123I-MIBG scan (100% vs. 75%, n=18, P=0.0455) or 18F-FDG PET (97% vs. 87%, n=46, P=0.0455), while the specificity was similar between 18F-FDOPA PET and 123I-MIBG scan (P=0.16), or between 18F-FDOPA PET and 123I-MIBG scan (P=0.32). Of note, 4 of 17 NTs (24%) were MIBG-negative but were all successfully detected by 18F-FDOPA PET.

Conclusions: In comparison to classical 123I-MIBG and 18F-FDG scans, 18F-FDOPA PET showed a superior sensitivity in detecting and tracking NTs, and it is especially useful in visualizing MIBG-negative NTs. 18F-FDOPA PET may become a powerful imaging tool for the functional assessment of NTs in the future.

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WS28

Development of a MIBG treatment facility, nuts and bolts

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Iodine-131-labeled Meta-Iodobenzylguanidine (131I-MIBG) is a highly effective and relatively non-toxic agent that is well-established for the treatment of refractory neuroblastoma and is a promising agent in development for inclusion into frontline high-risk neuroblastoma therapy. Treatment with 131I-MIBG presents challenges that are unique compared to those encountered when using standard chemotherapeutic agents, and the establishment of a therapeutic MIBG program requires a great deal of planning, availability of hospital resources and a multidisciplinary approach. Factors important to consider when developing an MIBG treatment facility include: 1. Commitment of a core group of individuals representing oncology (including physician staff, nursing staff and child life specialists), nuclear medicine (including physician staff, medical physicists and technologists) and radiation safety, 2. Identification of an appropriate physical facility for treatment and design of shielding to minimize the radiation exposure of caregivers and individuals in adjacent spaces, 3. Identification of a radiopharmacy for isotope supply and consideration of the logistics of ordering and receiving the agent, 4. Development of standard operating procedures to allow for good patient care that is done in compliance with state and federal standards for occupational and community radiation exposure, 5. Design of treatment protocol(s) to allow for broad availability of MIBG with appropriate institutional regulatory approval and 6. Creation of educational tools for patients and caregivers as well as medical staff describing general concepts of radiation and radiation safety. Once the MIBG program is established and patients are receiving treatment, regular meetings of the core group should be scheduled to review programmatic and specific patient issues from a multidisciplinary perspective and to ensure that staff education is ongoing and addressing issues raised during patient treatments.

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CELLULAR AND ANIMAL MODELS OF NEUROBLASTOMA: SEARCHING FOR THE MOST PREDICTIVE PRE-CLINICAL MODELS WS31 – WS36

WS31

Establishing validated neuroblastoma cell lines and xenografts from post-mortem blood samples

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Background: Neuroblastoma (NB) cell lines and direct xenografts provide important laboratory models. The COG Cell Line & Xenograft (XG) Repository (www.COGcell.org) establishes, characterizes, and distributes cell lines and XGs from childhood cancers. We have obtained samples at disease progression (PD), including post-mortem (PM) peripheral blood (PB) with circulating tumor cells (CTCs), to initiate NB cell lines in vitro and direct XGs which should manifest therapeutic resistance.

Methods: Tumor was minced and PB or bone marrow (BM) mononuclear cells were cultured in IMDM + 20% FBS + 4mM L-Glutamine + insulin, transferrin, selenous acid at 37°C in 20% O2/ 5% CO2. A subset of samples was also cultured in bone marrow level hypoxia (5% O2,) and/or in Neurobasal-A Medium (NAM). Three samples were also injected directly into NOD/SCID mice. Lines were validated by detecting tyrosine hydroxylase (TH) by RT-PCR, short tandem repeat (STR) analysis (compared to patient sample), and lack of Epstein-Barr Virus (EBV) genome by PCR.

Results: The COG Repository has received 23 PM samples and established 15 continuous cell lines (1/6 tumor, 10/12 PB, 3/4 BM, 1/1 pleural fluid) and 3 direct xenografts (all from PB). The take rate for PM cell lines was 65% while PB samples were highest at 83%. Cell lines were established for all seven samples cultured in 5% O2 and 4 of 6 in NAM. Direct XG establishment was 100%. All cell lines and XGs were validated to original patient using STR analysis, expressed TH and were EBV-free. From non-PM samples, 158 lines (98 from pre-therapy samples and 60 from PD) and 3 XG's (all from PD) have been established.

Conclusions: PM samples enable establishing cell lines and XGs and these models may more closely reflect the tumor biology of subjects enrolled in early-phase trials. It is vital to indicate the benefits of PM samples for research to physicians, patients, and parents. The cell lines and XGs established from PM samples will provide an ongoing resource for cancer genomic, biological, and preclinical therapeutic studies of therapy-refractory NB.

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WS32

Establishment of a condition for tumor sphere formation from primary neuroblastoma tissues

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Background: One of the technical problems of neuroblastoma (NB) studies is the failure to establish long-term surviving tumor spheres from primary tumor tissues, although tumor sphere formation from bone marrow metastasis in NB patients has been successful (Hansford et al., Cancer Res. 67, 2007). This limits the studies to fully understand the biology of NB.

Methods/Results: We recently established an allograft model by serially allografting subcutaneous tumors initiated from primary tumors of MYCN transgenic mice (Huang et al., Cancer Res. 71, 2011). Here, we could obtain long-term surviving tumor spheres from allografted NB, but not primary NB, using the Hansford method. We asked the reason of this failure for primary NB. We found several characteristics of primary NB which differed from allografted NB. Thus, subcutaneously grafted tumors from primary NB grew much less than those from allogafted NB. Some genes were strikingly differently expressed, e.g., TH and Phox2a were high, and Pax6 was low in primary NB. Interestingly, primary NB also highly expressed some embryonic stem cell markers including oct4, musashi, klf4 and c-myc. Based on these observations, we thought that a suitable culture condition for primary NB could be invested. After testing several conditions, a condition finally enabled us to maintain long-term surviving tumor spheres from primary NB. There was no exhaustion even after 30 times passages. Importantly, the sphere cells exhibited expression profiles similar to those of primary NB, and the profiles did not change after passages. Furthermore, these sphere cells could form tumor in vivo, and had a potent property of differentiation to neurons.

Conclusions: The success of establishment of tumor-spheres from primary NB will allow studies to compare primary NB and metastatic NB, e.g., tumor initiating cells and molecular networks critical for tumorigenesis and metastasis, and consequently will contribute to identification of molecular targets for therapy.

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WS33

Functional Profiling of Neuroblastoma Tumor-Initiating Cells

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Introduction: Intratumoral heterogeneity is thought to result from a functional cellular hierarchy, including a minor sub-population of so-called cancer stem cells or tumor-initiating cells (TICs). This specific population which harbors normal stem cell features is alone responsible for initiation and maintenance of the tumor. Although the identification and targeting of TICs to eradicate the disease represent an essential clinical and biological challenge, this population has not yet been identified in NB.

Methods: Microarray time course analysis of serial neurospheres passages from metastatic NB cells was used as an original strategy to isolate and characterize NB TICs. Functional evaluation of TICs subsets was determined by in vivo orthotopic cell implantations in nude mice.

Results: A predictive association was established between one of the most pertinent stem cell property, self-renewal, as assessed by serial neurosphere formation, and clinical aggressiveness in primary tumors. Moreover, cell subsets gradually selected during serial neurosphere culture harbored increased in vivo tumorigenicity, specifically highlighted in a natural (orthotopic) microenvironment. A micro-array time-course analysis of serial neurospheres passages of NB cells allowed us to specifically "profile" the NB stem cell-like phenotype, and to identify several relevant genes (ALDH1A2, CD133, MDR1, GPR177, EDNRB, ABCA1, NOTCH3, and ROBO1) as neurospheres/self-renewal markers. Based on combined neurosphere markers expression, distinct tumorigenic cell sub-populations were identified, also shown to pre-exist in primary NB. However, CD133/MDR1 markers cell sorting of the tumor failed to recapitulate the functional TIC phenotype, highlighting the complexity of the TIC model in NB.

Conclusion: Our data supports the NB stem-like cells as a dynamic and heterogeneous cell population strongly dependent on microenvironmental signals and add novel candidate genes as potential therapeutic targets in the control of high-risk NB.

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WS34

Genomic profiling of ALKF1174L and TH-MYCN transgenic neuroblastoma mouse models provides insights into the dynamic process of tumor formation

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Introduction: Establishing mouse neuroblastoma (NB) models that faithfully recapitulate human disease is of utmost importance in order to understand the complex biology of the disease in more detail and to offer modalities for pre-clinical in vivo testing of new therapeutic compounds.

Aims/methods: A new transgenic NB mouse model, driven by the ALKF1174L oncogene was established and compared with and crossbred to the TH-MYCN mice. These model systems were characterized using genome and m(i)RNA-ome profiling to assess how well they recapitulate human NB.

Results: ALKF1174L driven tumors occur with 40% penetrance and appear after 130 days or later. Genomic profiles of four tumors were remarkable in that they represented the diverse spectrum of aberrations observed in human NB, as exemplified by an endogenous MYCN amplification and a segmental chr11-gain covering a syntenic region almost entirely encompassing human chr17. The TH-MYCN mouse with accelerated tumor formation showed silent profiles or few genomic imbalances. Interestingly, the number of genomic aberrations was correlated with the time of tumor appearance. Of further interest, the most frequently occurring imbalance in all mouse tumors is chr3-gain. Since chr3 contains no syntenic regions recurrently implicated in human NB, the significance of this intriguing finding remains to be established. In relation to transcriptome analysis, we analysed two expression signatures that were established on human NB tumors. High MYC(N) miRNA activity score was observed in the TH-MYCN tumors whereas high ALK mRNA activity score was measured in ALK tumors. Inversely, a mRNA and miRNA signature established on the mice data, did show to correlate with survival in human NB tumors.

Conclusions: Our data shed new light onto the dynamics of genomic alterations in TH-MYCN and ALKF1174L driven NB formation. Furthermore, we show that transcriptional perturbations mimic those observed in human NB thus validating these mouse models for cross-species integrated genomics.

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WS35

A new MYCN-driven neuroblastoma mouse model using Cre-driven conditional expression of MYCN

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Background: In the first neuroblastoma (NB) mouse model, MYCN overexpression driven by a TH core promoter caused NB development. Although representing an excellent and broadly used tool, some limitations exist: 1. pronucleus injection with transgene integration into a less well-defined locus potentially yields less robust MYCN expression, 2. tumors predominantly originate from abdominal ganglion structures, 3. bioluminescent tumor imaging is impossible and 4. tumor incidence is 70% in the 129x1/SVJ but low in the C57Bl6 background, reducing the potential for combination with other cancer-relevant alleles. We aimed to overcome these limitations with a new mouse model using Cre-conditional MYCN expression in the neural crest.

Methods: The CAG-LSL-MYCN-IRES-Luciferase vector (LSL-MYCN) was introduced into the ROSA26 locus, and mice were crossbred with DBH-iCre mice to target MYCN expression to the neural crest. Tumors were detected by bioluminescent imaging, characterized by histology, immunohistochemistry, PCR and western blotting.

Results: Abdominal tumors developed with almost 100% penetrance at 50-100 days of age in mice heterozygous for LSL-MYCN;DBH-iCre in a mixed C57Bl6/129 strain background. Tumors were detectable by bioluminescent imaging, and arose predominantly from the adrenals, but occasionally also from superior cervical or celiac ganglia or from paravertebral structures in the thorax. They consisted of small round blue cells and expressed the NB tumor markers, TH, DBH and Phox2b. Several tumors were shown to originate from adrenal structures by histological analysis and by following tumor growth with high frequency ultrasound. The macroscopic tumor appearance, primary tumor sites, tumor histology and marker gene expression confirmed these tumors as NB, and western blotting confirmed strong MYCN expression.

Conclusions: Here we present a new NB mouse model with conditional expression of MYCN from a defined genomic locus in the neural crest. This model overcomes several limitations, and has the potential to improve investigations technically difficult for the current model.

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WS36

Caspase-8 deficiency enhances neuroblastoma metastasis in vivo - a new mouse model for metastatic neuroblastoma

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Background: Half of all neuroblastoma patients are diagnosed with extensive metastasis, yet little is known about the process, in part, due to the lack of animal models. The most widely employed transgenic model, the TH-MYCN mouse exhibits limited metastasis. Here we describe the first genetic immunocompetent mouse model for metastatic neuroblastoma with enhanced frequency of secondary tumors in the bone marrow, the most common site for dissemination of the disease in human patients.

Methods: In order to recapitulate the two most frequent alterations in metastatic neuroblastoma, amplification of MYCN and loss of caspase-8 expression, mice with neural crest specific deletion of caspase-8 (Casp8fl3-4 x TH-Cre) were crossed with the neuroblastoma prone TH-MYCN mouse. Tumors were identified by ultra-sound imaging, genomic PCR assay, histology and immunohistochemistry. Molecular and cellular changes in the tumor cells due to deficiency of caspase-8 were analyzed by expression microarrays, sphere-forming capacity and by orthotopic and tail vein injections and sequential passages in immunocompetent mice.

Results: While over expression of MYCN by itself rarely caused bone marrow metastasis (5% incidence, 1/21mice) combining MYCN overexpression and caspase-8 deletion significantly increased bone marrow metastasis (37% incidence, 10/27 mice, p=0.014). In contrast, loss of caspase-8 expression did not alter the site, incidence, or latency of the primary tumor. Interestingly, the loss of caspase-8 expression in only a portion of the primary tumor cells was sufficient to enhance metastasis and complete loss of caspase-8 expression in all the cells of the primary tumors did not increase metastasis incidence.

Conclusions: The immunocompetent mouse model described is valuable for studying neuroblastoma metastasis and treatment strategies and indicates that caspase-8 functions as a metastatic tumor suppressor gene in neuroblastoma. The results suggest a mechanism of collaboration between subsets of cells in the primary tumor with and without caspase-8 expression that enhances the metastatic process.

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NURSING SYMPOSIUM NS0 – NS5

NS0

I-131 Metaiodobenzylguanidine therapy for Neuroblastoma

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Neuroblastoma is diagnosed in approximately 700 children each year within the United States. Roughly 50% of these patients have high risk disease and of those cases we cure approximately 35%. Consequently we are faced with nearly 250 patients who will ultimately suffer a relapse of their neuroblastoma. In the year 2012 we do not have a known cure for neuroblastoma that recurs following the standard therapy which includes induction chemotherapy, high dose consolidation chemotherapy, stem cell transplant, local radiation therapy, followed by immunotherapy. I-131 Metaiodobenzylguanidine (MIBG) has been proven in clinical trials to be the most active agent that we have for recurrent/refractory neuroblastoma. A phase 2 trial demonstrated a 36% response rate in children with recurrent/refractory neuroblastoma. We at The Children's Hospital of Philadelphia have a well established program where we have been regularly providing MIBG therapy to patients with Neuroblastoma for decades. Providing this radioactive therapy can be challenging due to the specialized environment and dedicated team needed to do so. With proper preparation and education this therapy can be safely provided to patients within the hospital setting.

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NS1

The role and function of a case manager for children with neuroblastoma and their families

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Purpose: The main purpose of this study was to examine and evaluate the effectiveness of a case manager charged with the care of children with neuroblastoma and their families in a medical center in Taipei two years after the position was first created.

Methods: A retrospective design was adopted. Personal interview and chart review were used to collect data. Qualitative data were analyzed by content analysis.

Results: Since the position of case manager was created, a total of 29 children diagnosed with neuroblastoma are under good management and keep in contact with the health care team; this compares with 98 neuroblastoma children on the follow-up list over the previous 25 years. Therefore, overall, the role of the case manager in taking care of the neuroblastoma children and their families can be considered successful. The major functions of case manager can be itemized as follows: (1) arranging clinical visits when children were admitted for treatment/check-up, a total of 737 visits were made with an average time of 62.4 minutes; (2) conducting follow-up phone calls, a total of 265 calls were made with an average time of 10.86 minutes; (3) making check-up arrangements, giving families flexibility and priority; (4) listening to caregivers' venting of their emotions; (5) offering consultation; (6) providing an easy access service 24 hours a day; and (7) pro-actively building of a network through forming a parent support group and interacting on a blog.

Discussion: It is recommended that this position should be transferred into the hospital human resource system to allow more comprehensive care to be provided to children with cancer and their families.

Conclusion and Implications: The model of case manager for neuroblastoma children and their families may be applied to provide better care to children with other cancers and improve the quality of medical services.

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NS2

Administering Chimeric Antibody,what it means for Bedside Nurses

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Background: The Children's Oncology Group study ANBL0032 is a phase III trial investigating chimeric antibody therapy to treat patients with high risk neuroblastoma. As a result of the numerous hospitalizations and expected toxicities associated with the initiation of the chimeric antibody therapy, this protocol requires highly complex inpatient nursing care.

Methods: Due to the intricacies of this protocol, extensive staff education and admission planning was required. Knowledge translation and transfer methodology was utilized for staff education, patient/family education, purchase of equipment, creation of core group of expert RN's, bed planning, and relationship building with multidisciplinary team members.

Results: Through our experience in caring for over five patients amounting to more than 25 admissions, our institution has gained success by effectively collaborating and forming relationships with multidisciplinary team members. Due to the particulars of this protocol, 58 RN's underwent extensive staff education through structured education sessions.

Conclusion: Our nurses have been able to obtain the knowledge to successfully care for these patients and with this knowledge they have been able to support, educate, and empower families through this stage of their journey. We were able to identify and outline the administrative implications associated with the delivery of chimeric antibody therapy, side effects and supportive considerations, and to discuss bedside nursing implications and practicalities in relation to the implementation of this protocol.

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NS3

Study of Carrier Use and Administration of LXS Fenretinide Powder in Patients with Recurrent/Refractory Neuroblastoma within the New Approaches to Neuroblastoma Therapy (NANT) Consortium Study: NANT 2004-04

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Background: The majority of patients diagnosed with high-risk neuroblastoma are less than 4 years old. Children with refractory and recurrent high-risk neuroblastoma have an extremely poor prognosis. Fenretinide is a cytotoxic retinoid with preclinical/clinical data suggesting activity against neuroblastoma. Initially administered in capsular form, fenretinide is now available in an oral powder formulation (4HPR/LXS) developed to ease administration to young children and increase drug levels.

Methods: A phase 1 study of 4HPR/LXS was conducted within the NANT Consortium. 57 patients have enrolled; 32 in the initial dose escalation (Slim Fast required as carrier) and 25 in the fixed dose pharmacokinetic cohort (liberalized carrier). Drug administration diaries were utilized to monitor compliance during nursing assessments.

Results: A maximum tolerated dose was not attained during the dose escalation (352mg/m2-2210mg/m2), rather volume of drug powder was limiting, and plasma levels reached plateau. A dose of 1500mg/m2 was chosen for the expansion cohort with the schedule change from divided twice to three times daily to reduce the powder volume required per dose. Eight patients withdrew due to inability to effectively administer the drug in Course 1(range 4 - 25 years; median 12). Of the 19 patients who successfully took at least 2 courses (range 3-18 years; median 9), common food carriers included: non fat chocolate drinks/puddings (6), peanut butter (6), apple sauce (4), sunny delight fruit beverage (4), and extemporaneous fruit "smoothies" (2). 90% of patients used multiple carriers. One patient took drug via G-tube.

Conclusion: Patients who were successful utilized multiple individualized carrier-regimens during multiple courses. All patients unable to take 4HPR/LXS powder were identified in the first course of treatment. Age was not the predominate reason for ineffective delivery. There is an increased need for collaboration between nursing and patient/families to facilitate administration of novel oral agents and optimize compliance.

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NS4
The Road to a Canadian 131 I-MIBG Program
Denise Mills

It is known that relapsed neuroblastoma is extremely difficult to treat and patients with high-risk neuroblastoma have a poor prognosis. Treatment with Iodine-131-labeled meta-iodobenzylguanidine (131I-MIBG) has shown to be safe and efficacious in patients with progressive, refractory or relapsed high-risk neuroblastoma. Access to 131I-MIBG therapy for Ontario children is limited by the lack of a pediatric radiopharmaceutical facility. While access is available at several children's hospitals in the United States and a centre in Quebec, travel, accommodation and unplanned hospitalization expenses make this option unrealistic for most Ontario patients. A five year forecast to determine clinical demand for Ontario and out of province patients was completed and demonstrated the need for 131I-MIBG therapy in Ontario. A formal business and functional program plan was developed and funding from a philanthropic source was secured for the capital expenses. A dedicated team from oncology, nuclear medicine, occupational health and safety and several consultant services have been working on all aspects of the project. Objectives of the project include securing approval of the Canadian Nuclear Safety Commission, room design, and operational planning. When final approval from the Ontario Ministry of Health and Long Term Care is granted the next phase of the project construction of the 131I-MIBG suite, will begin. The success of the program will be dependent on the education and training of the multidisciplinary team that will be caring for and supporting the patients and families who will be receiving 131I-MIBG therapy.

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NS5
Late effects in survivors of childhood neuroblastoma
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Until recently, survival of high-risk neuroblastoma was poor. Consequently, there are few long-term survivors who were treated with intensive therapies such as hematopoietic stem cell transplantation or antibody therapy. However, even survivors of low and intermediate risk neuroblastoma are at risk for late effects as a consequence of their tumor surgery, or the chemotherapy and radiation used to treat their cancer. Further, the opsoclonus-myoclonus-ataxia syndrome has been associated with long-term decrements in neurocognitive function. This talk will explore the late effects observed to date in survivors of neuroblastoma, and will draw on literature regarding survivorship in other childhood cancer diagnoses to present the late effects that we anticipate observing as more children become long-term survivors of high-risk neuroblastoma. It will focus on sequelae of specific therapies such as platinum agents, anthracyclines and radiation therapy, and discuss the impact of a serious cancer diagnosis on functional and psychosocial outcomes. Finally, it will cover issues around the care of long-term survivors of neuroblastoma, including the need for guideline driven follow-up care, and successful transition from pediatric to adult follow-up as these children age.

PL00
Personalized Treatment for Children with Neuroblastoma: An Old Paradigm with New Tools
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Risk-based strategies have been used to treat children with neuroblastoma for decades. Although the International Neuroblastoma Risk Group (INRG) classification system was established to facilitate risk-based treatment strategies, the current system was based on the genetic markers that were available prior to 2002. The development of technologies capable of analyzing the whole genome has led to an explosion of new prognostic genetic discoveries including chromosomal aberrations and "omic" profiles. Recent studies have also led to a better understanding of the key molecular events that drive tumorigenesis and the identification of promising new "druggable" targets that have led to truly personalized approaches to treatment. Efforts to define additional key mutational events which are ongoing are likely to direct us to additional targets for personalized medicine. It will also be critical to advance our knowledge of the host genome, as germline genetic variation can influence drug toxicity and response. To facilitate neuroblastoma research, data on over 11,500 patients collected by the INRG Task Force have been made available to investigators from around the world. While seminal studies have already been conducted with these data, it is not currently possible to link the INRG data to other databases that contain biobank, genomic, or other molecular information. To ensure that investigators are able to perform more complex genetic research studies, we are developing a web-based, interactive INRG database (iINRGdb). This database will have expanded patient data fields for more detailed demographic, treatment, and outcome information, and tools to facilitate data requests and data sharing. Most importantly, technology to link the current INRG data set to tissue-bank ("biobank") and genomic databases is being developed. The creation of the iINRGdb will provide physicians, scientists, and other members of the INRG community with access to multiple datasets pertaining to childhood neuroblastoma, including biological data, phenotypic measures, and clinical outcomes, enabling studies that will move us closer to developing "personalized" approaches for all children with neuroblastoma.
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PL01
Future Anti-Cancer Therapeutic Targets: Putting the Carts Before the Horses?
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Tumour progression is driven by genetic aberrations that affect multiple oncogenes and tumour suppressor genes (TSGs). For the last three decades, the "Oncogene Revolution" prompted investigators to concentrate on the development of agents against oncogenes, with the goal of blocking cell growth and metastasis. It has now become clear that the cancer cell genome is too varied and the number of oncogenes too numerous for this strategy to work effectively for most tumours. Consequently, it may be time to consider targeting genes that are involved in sustaining the cancer cell phenotype. Two such classes of genes are those involved in cancer cell metabolic adaptation and maintenance of aneuploidy.

In this presentation, I present evidence supporting our contention that we should be able to pursue the development of agents targeting molecules involved in tumour metabolism and aneuploidy. Our laboratory has already identified DJ-1 (PARK7) and CPT1c as important regulators of these pathways. Another promising candidate is isocitrate dehydrogenase, mutations of which have been discovered in human brain cancers as well as certain leukemias and lymphomas. I will also discuss recent data from our and other laboratories suggesting that tumours which are genomically unstable deregulate specific genes to retain their chromosomal integrity during mitosis. The tumour cell signaling pathways altered by these genetic aberrations are intimately involved in mediating metabolic adaptation and maintaining aneuploidy. We propose that the affected genes represent a promising new class of anti-cancer targets.
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PL02
A New Model of Neuroblastoma in Zebrafish to Evaluate Candidate Genes involved in Neuroblastoma Pathogenesis
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PL03a
ALKF1174L is a driving oncogene of neuroblastoma in transgenic mice
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Background: Activating anaplastic lymphoma kinase (ALK) mutations occur in most familiar and in 10% of sporadic neuroblastomas (NB), but the role of mutated ALK in tumorigenesis remains elusive. We here demonstrate that targeted expression of the most frequent and aggressive variant, ALKF1174L, is tumorigenic in mice.

Methods: Transgenic mice were generated by pronucleus injection of the CAGCS-LSL-ALKF174L-IRES-Luciferase vector. Founders were crossbred with DBH-iCre mice to target expression of ALK to the neural crest and with TH-NMYC mice to explore synergism between ALKF1174L and MYCN. Tumors were characterized using histology, immunohistochemistry, electron microscopy, PCR, western blotting, aCGH and mRNA microarrays. Transgenic tumor-bearing mice and nude mice carrying human NB xenografts were treated with either crizotinib or NVP-TAE-684 orally.

Results: Of the mice transgenic for LSL-ALKF1174L and DBH-iCre, 5 of 12 developed tumors between 130 and 351 days of age. Tumors resembled human NB in morphology, metastasis pattern, gene expression and subcellular structures. Array CGH reveled that this ALK-driven NB mouse model recapitulated the genetic spectrum of the human disease. Chromosomal aberrations were syntenic to those in human NB, including 17q gain and MYCN amplification. Targeted ALKF1174L and MYCN co-expression by crossbreeding LSL-ALKF1174L and TH-NMYC mice revealed a strong synergism in inducing NB with minimal secondary hits. Treatment of ALKF1174L transgenic mice with the NVP-TAE-684 ALK inhibitor induced complete tumor regression. NVP-TAE-684 treatment of SY5Y-derived xenografts afforded similar encouraging results in contrast to crizotinib, which failed to successfully treat NB xenografts harboring ALKF1174L.

Conclusions: We conclude that an activating mutation within the ALK kinase domain is sufficient to drive NB, and selected ALK inhibitors show promise to treat NB harboring ALK mutations. Since this model faithfully recapitulates all major genetic subtypes, it offers unique opportunities for NB research and will be of particular importance to explore ALK-targeted therapy of NB.

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PL03b

The ALKF1174L mutation potentiates the oncogenic activity of MYCN in a mouse model of neuroblastoma.

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Background: Mutations in the kinase domain of ALK occur in 8-12% of sporadic neuroblastoma (NB) cases. ALKF1174L is the most common somatic mutation, encodes a potent, constitutively active kinase and is frequently associated with MYCN amplification in a subset of NB patients with poor outcome. Taken together, these observations suggest an important role for mutant ALK in the genesis of NB and a likely role in the acquisition of an aggressive phenotype in association with MYCN amplification.

Methods: To model a potential interaction between ALKF1174L and MYCN we generated a transgenic mouse model in which overexpression of ALKF1174L is targeted to the neural crest under control of the tyrosine-hydroxylase (TH) promotor. TH-ALKF1174L mice were interbred with TH-MYCN animals producing tumor-penetrant compound hemizygotes (TH-ALKF1174L/MYCN) that were characterized using standard histopathologic and microarray-based techniques.

Results: TH-ALKF1174L (hemi- or homozygote) mice exhibited no overt phenotype but TH-ALKF1174L/MYCN developed aggressive NB tumours (40 day latency, 100% penetrance) in excess of what is typically observed with TH-MYCN hemi- or homozygotes. Histology was comparable to that of human tumours positive for ALKF1174L and MYCN amplification. Transcriptional profiling showed upregulation of Pi3k/mTor and Mapk signaling, inhibition of MYCN-induced pro-apoptotic targets, enrichment of murine Mycn transcripts and hallmarks consistent with MYCN oncoprotein stabilization

Conclusions: We present a novel murine transgenic model of NB that expresses ALKF1174L and MYCN. The model defines major in vivo readouts of ALKF1174L signaling and demonstrates mechanistic interactions between ALKF1174L and MYCN in the genesis of aggressive NB. ALKF1174L directly potentiates MYCN by: 1) activating signaling pathways that enhance MYCN-driven oncogenesis, 2) upregulating MYCN expression, and 3) inhibiting MYCN-driven apoptosis. This model should provide an ideal platform for future studies to dissect in vivo functions of ALK, the molecular basis underlying ALK-MYCN interactions in NB tumorigenesis and to screen novel ALK-targeted agents for neuroblastoma.

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PL04

Efficacy of crizotinib in children with relapsed/refractory ALK-driven tumors including neuroblastoma: A Children's Oncology Group Phase 1 Consortium Study

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Background: Genetic aberrations in the ALK gene are found in neuroblastoma and other tumors. Crizotinib, a small molecule inhibitor of ALK, is active in ALK-translocated non-small cell lung cancers (NSCLC). We performed a phase 1 dose-escalation and pharmacokinetic (PK) trial of crizotinib in patients with refractory neuroblastoma and other ALK-driven tumors.

Methods: Crizotinib was administered bid without interruption in cycles of 28 days. Six dose levels (100, 130, 165, 215, 280, 365 mg/m2/dose) have been fully evaluated (A1). Patients with confirmed ALK fusion proteins, mutations or amplification (A2) could enroll at one dose level lower than part A1 and those with NB could enroll on a separate stratum (A3). PK studies were performed on day 1 and at steady state (SS). ALK genomic status in NB tumor tissue was evaluated, when available.

Results: 77 patients were enrolled, 62 fully evaluable for toxicity [median (range) age 9.9 yrs. (1.1–21.3 In A1, 2/7 patients developed DLT (grade 3 dizziness, grade 5 intra-tumoral hemorrhage) at 215 mg/m2, and 2/6 patients developed DLT (grade 4 liver enzyme elevation, grade 4 neutropenia) at 365 mg/m2. Mean (±SD) Cave (=AUC0-12h/12h) of crizotinib at SS was 466±114 ng/mL at 215 mg/m2/dose (n=5), 443±121 ng/mL at 280 mg/m2/dose (n=8), and 720±230 ng/mL at 365mg/m2/dose (n=4). In the 27 patients enrolled with neuroblastoma, 8 had an ALK mutation identified in a molecular diagnostic laboratory (5 R1275, two germline; 2 F1174L; 1 F1245), consistent with the expected distribution of mutations. Patients were treated at 100mg/m2/dose (n=1), 130mg/m2/dose (n=1), 165mg/m2/dose (n=2), 215mg/m2/dose (n=2), 280mg/m2/dose (n=1), and at 365mg/m2/dose (n=1). 5 patients had early disease progression, all treated at lower doses with the exception of 1 patient who had isolated CNS disease. Crizotinib has seen been shown to penetrate poorly though the blood-brain barrier. 1 adolescent with a somatic F1174 mutation remains on study with stable disease (8 cycles). The 2 patients with germline mutations both achieved complete responses and remain on therapy (15 and 6 cycles) at different dose levels. Of the 19 NB patients enrolled with unknown ALK status, 1 has had a CR and remains on study after 24 cycles, and 6 have stable disease (for 29, 15, 10, 9, 8, and 7 cycles respectively).

Conclusions: Inhibition of ALK in pediatric patients with neuroblastoma resulted in objective anti-tumor activity or stable disease and occurs with minimal toxicity.

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PL05

NCYM, a novel MYCN cis-antisense gene product, stabilizes MYCN and contributes to aggressiveness of human neuroblastoma

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Background: Neuroblastoma (NB) with MYCN amplification shows aggressive behavior and possesses highly metastatic potential. Here, we show that NCYM, a cis-antisense gene of MYCN, encodes a nuclear protein which is co-expressed with MYCN in human NBs. The NCYM protein stabilizes MYCN to promote metastasis of NB caused in the NCYM/ MYCN double transgenic mice.

Methods: The expression levels of NCYM and MYCN mRNA were measured by quantitative real-time PCR. The standard in vitro procedures were used for molecular analyses of NCYM. The roles of NCYM in vivo were examined by generating the NCYM/ MYCN double transgenic (tg) mice.

Results: The NCYM gene was 100% co-amplified with MYCN in primary NBs. NCYM mRNA expression was significantly correlated with amplification ($p<0.001$) and expression ($p<0.001$) of MYCN as well as poor clinical outcome ($p<0.001$) in 106 NBs. The NCYM-specific antibody we generated showed translation of NCYM to be a 12 kDa protein which is evolutionally conserved only in human and chimpanzee. The knockdown of NCYM down-regulated expression of MYCN protein and induced massive apoptosis in human CHP134 NB cells. NCYM interacted with both MYCN and GSK3beta, a kinase regulating the stability of MYCN protein, and inhibited GSK3beta-mediated phosphorylation and degradation of MYCN. In the adrenal tissues of NCYM tg mice, NCYM protein interacted with GSK3beta and stabilized MYCN protein, suggesting that GSK3beta inhibition by NCYM stabilizes MYCN protein both in vitro and in vivo. In contrast to MYCN tg mice, the NCYM/ MYCN double tg mice frequently developed both abdominal and thoracic NBs with marked distant metastases in lymph nodes, lung, intracranium and ovary that is rather similar to human NB with MYCN amplification.

Conclusions: The NCYM enhances the aggressiveness of human NB through the stabilization of MYCN. Targeting NCYM could be one of the promising future strategies to treat the high-risk NBs.

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PL06

Whole Genome Sequencing of Neuroblastoma identifies frequent Chromothripsis and Neuritogenesis Gene Defects.

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Background: The pathogenesis of neuroblastoma has for a long time been enigmatic, as only few gene defects were identified in this

tumour. Frequently detected gene alterations are limited to MYCN amplification (20%) and ALK activations (7%).

Methods: Here we present a whole genome sequence analysis of 87 neuroblastoma of all stages.

Results: Neuroblastoma showed few amino acid changing gene mutations, with low stage tumors even significantly less than high stage tumors. Recurrent mutations were found in ALK and TIAM1. In contrast, analysis of structural defects identified in 18% of high stage neuroblastoma a local shredding of chromosomes, known as chromothripsis6. These tumors are associated with a poor outcome. Chromothripsis could disrupt an entire chromosome, with a preference for chromosome 5, or more localized regions where it mediated loss of heterozygosity or gene amplification. The paired-end sequence technology identified a second type of frequent defects: deletions and duplications of small regions, often not larger than a few exons. Also such defects were more frequent in high stage tumors. They recurrently affected genes functioning in neuronal growth cone biology. Defects in ODZ2, ODZ3 and ODZ4 together affected 18% of stage 4 tumors. ODZ family genes regulate neuritogenesis, as observed in C. elegans, Drosophila and mice and can induce neurite extensions in neuroblastoma cells. Also PTPRD and CSMD1, which are both involved in neuronal growth cone stabilization, were frequently affected. In addition, ATRX, TIAM1 and a series of regulators of the Rac/Rho pathway were mutated, further implicating defects in neuritogenesis in neuroblastoma. Most tumors with defects in these genes were high stage neuroblastoma without MYCN amplification.

Conclusion: The genomic landscape of neuroblastoma reveals two novel molecular defects, chromotripsis and neuritogenesis gene alterations, which frequently occur in high risk tumors.

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PL07

Prognostic Subgroups of High-Risk (HR) Neuroblastoma (NB) Patients Are Identified by Analysis of Peripheral Blood Stem Cells (PBSC) with a Highly Sensitive TaqMan® Low Density Array (TLDA) Assay for Five Neuroblastoma-Associated Genes: Children's Oncolog

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Background: The COG-A3973 trial for HRNB demonstrated no difference in 5-year EFS/OS for patients randomized to receive purged (40%±3%/50%±3%) versus non-purged (36%±3%/51%±3%) autologous PBSC after myeloablative chemotherapy. We assessed PBSC with a 5-gene TLDA assay to determine frequency of positive tests; correlation of positivity with EFS/OS; and effect of purging.

Methods: Newly diagnosed HR NB patients (n=486) were randomized at study entry to have PBSC, which were collected after two chemotherapy cycles, purged with five antibodies/immunomagnetic beads or not purged. All received the same induction, myeloablative consolidation, and isotretinoin post-consolidation therapy; 78 also received post-consolidation ch14.18 antibody. PBSC from the first day of collection and pre- and post-purging were evaluated using a five-gene TLDA assay (CHGA/DCX/DDC/PHOX2B/TH). Results are reported as positive/negative for TLDA signal and as the geometric mean expression (cycle threshold) of the detection genes (DGS=detection gene score; lower DGS=higher mRNA).

Results: 463 of 486 patients had PBSC collected, and 372 were transplanted. Immunocytology detected tumor cells in 4/460 day 1 PBSC and in none after purging. Among 245 patients with day 1 samples available for TLDA analysis, 122 (50%) were positive: 68/129 (53%) from purged and 54/116 (47%) from non-purged arms. Five-year EFS correlated with the DGS: 20%±7% for DGS<25th percentile; 33%±5% for DGS>25th percentile; 51%±5% for non-detectible (P=0.0002)(positive DGS range=32.3-39.9). Five-year OS paralleled EFS (30%±8%, 45%±5%, and 62%±4%, respectively; P=0.0007). The 245 patients whose PBSC were evaluated with TLDA were representative of all 486 patients. TLDA analysis of 52 before/after purging pairs of PBSC showed that pre-purge positive signals became negative (52%), weaker (28%), or slightly stronger (20%).

Conclusions: Five-gene TLDA analysis of PBSC provides a prognostic biomarker that likely reflects the early response to induction chemotherapy. Failure of purging to improve outcome may be due to incomplete purging and/or residual tumor in patients.

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PL08

Final Results from the HR-NBL1/SIOPEN Trial favour Busulphan-Melphalan as Superior Myeloablative Therapy (MAT) for High Risk Neuroblastoma.

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Aims: The HR-NBL1 trial of the European SIOP Neuroblastoma Group randomised 2 MAT regimens with the primary aim to demonstrate superiority based on event free survival (EFS).

Patients and Methods: At randomisation closure, 1577 high risk neuroblastoma patients (944 males) had been included since 2002. Response eligibility criteria prior to randomisation after Rapid COJEC Induction (J Clin Oncol, 2010) ± 2 courses of TVD (Cancer, 2003) included complete bone marrow remission and ≤ 3, but improved, mIBG positive spots. The MAT regimens were BuMel (oral busulfan till 2006, 4x150mg/m2, or after 2006 intravenous use according to body weight and melphalan 140mg/m²/day) and CEM (carboplatin ctn. infusion (4xAUC 4.1mg/ml.min/day), etoposide ctn. infusion (4x338mg/m²day or 4x200mg/m²/day*), melphalan (3x70mg/m²/day or 3x60mg/m²/day*. *reduced if GFR<100ml/min/1.73m²)). A minimum of 3x10E6 CD34/kgBW PBSC were requested. Local control included surgery and radiotherapy of 21 Gy. A total of 598 patients were randomised (296 BuMel, 302 CEM). The median age at randomisation was 3 years (1-17.2) with a median follow up of 4.4 years.

Results: A significant difference in EFS in favour of BuMel (3-years EFS 49% vs. 35%) was observed as well as for overall survival (3-years OS 60% vs. 48%, p=0.003). This difference was mainly related to the relapse and progression incidence, which was significantly (p<0.001) lower with BuMel (48% vs. 61%). The severe toxicity rate up to day 100 (ICU and toxic deaths) was below 10%, but was significantly higher for CEM (p=0.012). The acute toxic death rate was 3% for BuMel and 5% for CEM (NS). The acute MAT toxicity profile favours the BuMel regimen in spite of a total VOD incidence of 17% (grade 3: 3%) Bumel 24% (grade 3: 4%) vs. CEM 10% (Grade3: 1%).

Conclusions: BuMel was demonstrated to be superior to CEM and hence is recommended as standard treatment.

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PL09

HSCT improves outcome for high risk neuroblastoma: What's next?

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PL10

Will genomics provide more precise neuroblastoma therapies? Opportunities and challenges for the next decade.

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PL11

Divergent ancestral genetic variation on chromosome 6p22 accounts for racial disparities in survival in neuroblastoma.

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Background: More high-risk disease and a worse outcome are observed in children with neuroblastoma who self-report as black versus white. We sought to determine whether genetic variation would explain this racial disparity.

Methods: After quality control, we analyzed 511,836 germline genetic variants in 2,709 children with neuroblastoma enrolled on ANBL00B1 from 2001 to 2009. Genetic variation was summarized by conducting principal components analysis. Principal component 1 (PC1) separated patients with African ancestry from all others. PC1 was used as a continuous variable for ordinal regression with risk group and a Cox proportional hazard model of EFS. To identify genetic mechanisms for the observed disparities, we developed a method using genome-wide variation data applied to high-risk versus non-high risk samples. We identified a comprehensive list of loci with significant divergence between ancestral populations. Each locus was tested for association with high-risk phenotype using logistic regression with the proportion of African ancestry as covariate. Top SNPs were added to multivariate models of EFS to determine if any top associations could abrogate observed disparities.

Results: PC1 was associated with both risk (P = 0.007) and EFS (P = 0.037). 72 population-divergent SNPs were nominally associated with high-risk disease ($p < 0.001$). The risk allele for one of the top SNPs: rs9295536 ($P = 9.2 \times 10^{-8}$) was more common in the African ancestral population, was associated with high-risk phenotype and poor outcome in all patients, and validated in a Caucasian-only subanalysis. In multivariate testing, this SNP abrogated the PC1 association with EFS (P = 0.18).

Conclusions: A SNP with high divergence between ancestral populations on chromosome 6p22 accounts for the observed racial disparity in survival and is also a common genetic variant associated with survival in patients derived from either European or African ancestry (PBonferroni <0.05). Studies to elucidate the function of this SNP are underway.

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PL12

Identification and functional relevance of two new neuroblastoma susceptibility loci at 6q16 within HACE1 and LIN28B

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Background: Our neuroblastoma (NB) genome-wide association study (GWAS) has identified several validated susceptibility alleles, but much of the heritability to this disease remains undefined.

Methods: We compared germline single nucleotide polymorphism (SNP) genotypes of 2,101 NB cases and 4,202 controls of European ancestry genetically matched by multi-dimensional scaling. Significant discovery results were replicated in an Italian cohort of 350 cases and 780 controls and an African American cohort of 365 cases and 2,491 controls. Correlative and mechanistic studies were performed in NB cell lines and tumor tissues.

Results: In addition to confirming previously reported associations (NEJM 2008, Nat Genet 2009, Nature 2010, PLoS Genet 2011), two new loci were identified at 6q16 implicating major cancer genes. The first signal was within the HECT domain and ankyrin repeat containing, E3 ubiquitin protein ligase 1 gene (HACE1; rs4336470, $P = 3.5 \times 10^{-13}$, OR 1.27, 95% CI: 1.19–1.36), and the second within lin-28 homolog B (LIN28B; rs17065417, $P = 8.4 \times 10^{-9}$; OR 1.38, 95% CI: 1.23-1.54). NB cell lines homozygous for the rs17065417 risk allele showed increased LIN28B mRNA and protein expression and down regulation of let-7 miRNAs. Significant growth inhibition was observed upon depletion of LIN28B in NB cells homozygous for the rs17065417 risk allele; cell lines with one copy of the protective allele showed no change in growth upon LIN28B knockdown. Primary tumors with advanced stage showed significantly lower HACE1 and higher LIN28B expression, consistent with tumor suppressive and oncogenic roles respectively. Decreased HACE1 expression was associated with poor survival, even within the high-risk subset. However, we observed that lower LIN28B expression was associated with increased risk of relapse in high-risk patients. Pathway analysis suggests that tumors with high LIN28B are more proliferative, but show a deficit in a wide array of markers associated with innate and adaptive immunity.

Conclusion: Common variation at 6q16 is associated with NB and implicates HACE1 and LIN28B as susceptibility genes. Similar to other genes identified in our GWAS, the germline variants identified here likely play an important role not only in tumor initiation but also disease progression via cis-effects on major cancer genes.

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PL13

Neural Crest-specific expression of Lin28b induces neuroblastoma in mice

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Background: Overexpression of Lin28b has been reported in neuroblastomas (NB) and other malignancies. Lin28b is known to repress expression of let-7 miRNAs, which target MYCN, but Lin28b tumor-initiating capacity and oncogenicity has not yet been investigated in vivo. We overexpressed Lin28b in the murine neural crest to determine if Lin28b can drive neuroblastomagenesis.

Methods: Lin28b was conditionally expressed by knock-in of the CAG-LSL-Lin28b-IRES-Luciferse vector (LSL-Lin28b) into the ROSA26 locus. Mice were crossbred with DBH-iCre mice to target expression to the neural crest. Arising tumors were characterized using histology, immunohistochemistry, PCR and western blotting, and maintained via serial transplantation.

Results: Abdominal tumors developed in 4 of 16 DBHiCre;LSL-Lin28b transgenic mice at 36-56 days of age. Autopsy revealed uni- or bilateral adrenal tumors in all mice, reflecting the most frequent localization of human NB. Thoracic tumors and tumors originating from the superior cervical or celiac ganglia were also observed. Tumors consisted of small round blue cells and expressed the NB markers, DBH, TH and Phox2b. The macroscopic tumor appearance, primary tumor sites, tumor histology and marker gene expression confirmed these tumors as NB. Successful serial transplantation in immunocompromized mice supported that the primary tumors from this model system were fully transformed malignant tumors. Both the Lin28b and MYCN proteins were strongly expressed in all tumors and members of the let-7 miRNA family were significantly downregulated.

Conclusions: We demonstrate that overexpressing Lin28b in the neural crest can drive NB tumor formation in mice, supporting LIN28B as an important oncogene for NB and potentially for other malignancies. Our results suggest that, similar to human NB, MYCN is at least in part induced via downregulation of let-7 miRNAs by Lin28b overexpression in mice. Therapeutic approaches aimed at inhibiting Lin28b or let-7 family re-expression may be useful therapeutic approaches to circumvent MYCN addiction in NB.

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PL14

A new 47-gene classifier for improved outcome prediction of non-high risk neuroblastoma patients – an international neuroblastoma consortium study

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Background: Despite promising results of gene-expression based outcome predictors for neuroblastoma, the question which patients might eventually benefit from such genomic classifiers remains unanswered to date. In this study, we aimed to generate a clinically applicable neuroblastoma gene-expression classifier and to identify patients who will profit from risk estimation using this predictor.

Methods: Single-color 44K microarray gene-expression profiles were generated from 650 internationally collected primary neuroblastoma tumors. Using a training set of 75 patients with maximally contrasting courses (patients who succumbed to disease vs. patients with ≥1000 days event-free survival (EFS) without cytotoxic treatment) a novel 47-gene classifier was built using a support vector machine (SVM) algorithm. External classifier validation was performed by predicting an independent test set of 575 samples and comparing the prediction results with patients' outcome.

Results: The 47-gene SVM classifier achieved an overall external classification accuracy of 96% (sensitivity 95%, specificity 97%) and significantly separated patients with contrasting clinical courses (5-year EFS favorable 0.83±0.02 vs. unfavorable 0.35±0.03; 5-year OS 0.98±0.01 vs. 0.55±0.04, both p<0.001). Furthermore, the predictor reliably distinguished patients with contrasting outcome in subgroups with clinical low-, intermediate-, and high-risk of death from disease (low-risk (LR) 5-year OS 0.99±0.01 vs. 0.81±0.09; Intermediate Risk (IR): 1.0 vs. 0.71±0.09; High-Risk (HR) 0.87±0.07 vs. 0.45±0.04; all p<0.001). Additionally, the classifier accurately identified all patients with MYCN non-amplified stage 2 and 3 disease >548 days of age who succumbed to disease as unfavourable (EFS 0.95±0.05 vs. 0.08±0.08; OS 1.0 vs. 0.44±0.12, both p<0.001).

Conclusions: The 47-gene SVM predictor can contribute to improved treatment stratification of both LR and IR risk patients. A strategy to implement this classifier into the German neuroblastoma trial as a stratifying marker with therapy reduction for favorably classified and treatment intensification for unfavorably classified patients is currently being developed.

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PL15

GD2-targeted immunotherapy of high risk neuroblastoma

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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. Clinical trials of 3 anti-GD2 mAbs have documented their toxicity profile and anti-tumor activities. A pivotal phase III randomized trial in high risk neuroblastoma has recently demonstrated a significantly improved outcome with 20% reduction in relapses when ch14.18 + cytokines + 13cis-retinoid acid were administered after stem cell transplant. This is the first clinical trial to show a substantive increase in survival in well over a decade for this dreadful disease. The 20% improvement in prevention of relapse for children with neuroblastoma receiving immunotherapy makes this therapy the new standard of care. This is also the first successful cancer immunotherapy to target a glycolipid antigen.Three 2nd generation GD2-targeted agents have been developed with an aim to ameliorate toxicities and/or increase efficacy. The first agent is mAb1A7, an anti-idiotypic antibody against anti-GD2, serving as a surrogate GD2 antigen for active immunotherapy of neuroblastoma. A pilot trial of mAb1A7 showed induction of endogenous anti-GD2 antibodies with little toxicities. The 2nd agent, hu14.18-IL2 is a fusion protein of humanized anti-GD2 and IL-2. The maximum tolerated dose (MTD) was12 mg/m2/day, ~50% of that for ch14.18, with similar toxicities as those reported with IL-2 + anti-GD2 mAbs. Antitumor activity was noted in both phase I and II studies in neuroblastoma evaluable only by MIBG and/or bone marrow histology, but no responses for patients with measurable disease. Hu14.18K332A is a humanized ch14.18 with a mutation to alanine at lysine 322 that limits its ability to fix complement and thereby reduces the pain associated with ch14.18, while retaining ADCC capabilities. MTD in a phase I study was 70 mg/m^2/d x 4, which exceeded 2 fold of that for ch14.18. The efficacy of these agents awaits further clinical investigations.

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PL16

Antibody-based GD2 targeted therapy for neuroblastoma (NB): past, present and beyond at Memorial Sloan-Kettering Cancer Center

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From the first phase I study of murine anti-GD2 monoclonal antibody (MAb) 3F8 in 1987 to the most recent randomized proof of clinical benefit with the combination of ch14.18 with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-2 (IL-2) in 2010, efficacy of antibody-based GD2-targeted therapy of NB is now generally accepted. Yet, there are unresolved issues - pain side effects associated with anti-GD2 therapy and the absolute requirement of cytokine being the most pressing.

We undertook a retrospective analysis of 169 high-risk stage 4 patients enrolled in consecutive 3F8 protocols. All patients underwent 5-drug induction, second look surgery, with addition of 2-4 rounds of second line therapy if there was refractory disease. Patients were enrolled onto 3F8 immunotherapy once CR/VGPR was achieved, and primary site consolidated with local radiation. We made the following observations: (1) GM-CSF (sc better than iv) activated CD11b on myeloid cells was highly correlated with PFS, (2) 3F8 ± GM-CSF was effective in eradicating marrow minimal residual disease (MRD), (3) failure to achieve early MRD remission was the strongest adverse predictor of PFS and OS, (4) missing ligand(s) for KIR receptors on natural killer cells (NK) impacted PFS and OS, (5) human anti-mouse antibody (HAMA) response was a strong positive predictor of OS, (6) relapse was primarily at isolated sites, and (7) isolated CNS relapse was salvageable by combining surgical resection, CSI + focal radiation, intrathecal^{131}I-MAb 8H9 therapy and low dose chemo-immunotherapy. Concurrent with 3F8 ± GM-CSF studies, clinical trials using 3F8 + oral barley glucan (to activate CD11b), 3F8 + oral yeast glucan, sc anti-3F8 anti-idiotypic MAb A1G4, iv 131I-3F8 radioimmunotherapy, and sc GD2/GD3 conjugate vaccines have been completed, while 3F8 + adoptive NK cell therapy and 3F8 dose-escalation + GM-CSF are ongoing. Humanized 3F8 (hu3F8) is currently in phase I clinical trial, balancing antibody-dependent cell-mediated cytotoxicity (ADCC) versus complement-mediated cytotoxicity (CMC), immunogenicity versus idiotype network, pharmacokinetics versus pain side effects. Using crystal structure and *in silico* modeling, binding of hu3F8 to GD2 can be further improved by selective amino acid mutations. It is feasible to produce next generation hu3F8 with increased affinity for antigen and for Fc-receptor. Bifunctional constructs (e.g. engaging T cells or multistep targeting) have also successfully passed the proof of concept stage in mouse experiments. From blueprint to production cell-lines, and from laboratory scale to GMP production, we are testing the model for translating novel antibody-based biologics to the clinic for the treatment of NB in a timely fashion using more accessible and affordable technology within the non-profit budget constraints.

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PL17

Generation and characterization of a new anti-idiotype antibody ganglidiomab mimicking tumor-associated antigen disialoganglioside GD2 for active immunotherapy in neuroblastoma

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Background: Disialoganglioside GD2 is a highly expressed but poorly immunogenic antigen in neuroblastoma (NB). In order to induce an active immune response against GD2, an anti-idiotype antibody (AITAB), which mimics GD2 may overcome this problem.

Materials: A new AITAB called ganglidiomab was developed by immunizing balb/c mice with murine anti-GD2 mAb 14G2a followed by generation of hybridomas. Binding to anti-GD2 antibodies 14G2a, ch14.18 and hu14.18 as well as to NK-92 cells expressing scFv(ch14.18)-zeta receptor (NK-92tr) was analyzed by a standard ELISA and flow cytometry. GD2 specific lysis of NB cells by NK-92tr cells was evaluated by a 51Cr release assay. Binding affinity to anti-GD2 antibodies 14G2a, ch14.18 and hu14.18 was determined by Biacore analysis. RNA isolation from hybridoma cells, RT-PCR, cloning and sequencing of DNA fragments encoding for the variable regions of the heavy (VH) and light chain (VL) of ganglidiomab were done using standard molecular biology techniques. In vivo, anti-GD2 immune response was demonstrated in A/J mice after vaccination with ganglidiomab-Al(OH)3.

Results: We demonstrate binding of ganglidiomab to 14G2a, ch14.18 and hu14.18 as well as to NK-92tr cells. Importantly, ganglidiomab competitively inhibited both binding of these anti-GD2 antibodies and NK-92tr cells to the nominal antigen GD2 and GD2 specific lysis of NB cells by NK-92tr cells clearly indicating anti-idiotypic characteristics. Moreover, the dissociation constants of ganglidiomab from anti-GD2 antibodies ch14.18, 14G2a and hu14.18 were shown to be similar compared to another AITAB 1A7 using "steady state" analysis. The sequences of frameworks (FRs) and complementarity determining regions (CDRs) of ganglidiomab were identified by homology search in the Kabat database. Finally, an anti-GD2 immune response could be induced in vivo.

Conclusion: We generated and characterized a new anti-idiotype antibody ganglidiomab which mimics GD2 providing an important baseline for development of DNA and protein vaccines against NB.

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PL18

Targeting neuroblasts and neuroblast-supportive macrophages with dual-specific NKT cells

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Background: The infiltration of primary tumors with V 24-invariant Natural Killer T cells (NKTs) is associated with good outcome neuroblastoma. The mechanistic studies revealed that instead of attacking tumor cells directly, NKTs target CD1d-positive tumor-associated macrophages (TAMs). However, effective immune control of tumor may also require direct and specific attack against the tumor cells.

Methods: We genetically modified expanded NKTs using a retroviral vector encoding a chimeric antigen receptor (CAR) that targets GD2 ganglioside which is highly expressed by neuroblastoma cells. The functional activity of the native TCR and CAR.GD2 in the gene-modified NKTs was tested using CD1d+ TAMs and GD2+ neuroblastoma cells, respectively. Next, we examined various co-stimulatory endodomains encoded in cis with the CAR.GD2 (CD28, CD137, OX40 and their combinations) to enable optimal CAR-mediated signaling for NKT cell cytotoxicity, cytokine production, proliferation, and survival.

Results: We found that all examined CAR.GD2 constructs render NKTs highly cytotoxic against GD2-positive neuroblastoma cells while retaining their native CD1d-restricted cytotoxicity. However, only the "co-stimulatory" CAR.GD2 NKTs that express CD28, OX40, and/or CD137 underwent rapid proliferation upon specific stimulation that enabled clinical scale expansion of the gene-modified NKTs. While adoptive transfer of the parental NKTs only transiently suppressed growth of metastatic neuroblastoma in humanized NOD/SCID/IL-2γ(null) mice, NKTs expressing CAR.GD2 with CD28 or CD137 endodomains had potent and long-lasting anti-metastatic activity. Furthermore, we discovered a striking and previously unanticipated Th2 (IL-4 and IL-10) and Th1 (IFNγ and GM-CSF) polarization of NKTs expressing CAR.GD2/CD28 and CAR.GD2/CD137, respectively.

Conclusions: NKTs engineered to express CAR.GD2/CD137 have potent anti-tumor activity via targeting both neuroblasts and neuroblast-supportive TAMs as well as via production of IFN and GM-CSF that activate multiple types of anti-tumor effector cells. These results establish the potential of NKTs to serve as a novel platform for anti-tumor CAR therapy in neuroblastoma and other types of cancer.

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PL19

Augmentation of NK and T cell infiltration into tumors by Intratumoral (IT) or Intravenous (IV) hu14.18-IL2 Immunocytokine (IC).

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Background: Hu14.18-IL2 (APN301, Apeiron Biologics) is an immunocytokine (IC) consisting of IL2 linked to each IgG heavy chain of the anti-GD2 hu14.18 mAb. Phase-II trials of IV hu14.18-IL2 IC in neuroblastoma (NBL) and melanoma (by COG and UWCCC) are underway. Activity was demonstrated in our initial COG Phase-II trial. Furthermore, cell depletion studies in mice, and KIR/KIR-L genotyping in our COG Phase-II study, showed that NK cells play an important role in the anti-tumor effects observed in NBL. Our prior studies in NBL-bearing mice showed enhanced anti-tumor activity with intratumoral IC treatment (IT-IC). We report here on recent studies in NBL-bearing mice that address the local and distant immunological mechanisms for IC-mediated anti-NBL activity.

Methods: A/J mice bearing measurable-established subcutaneous NXS2 NBL were treated with IT-IC, IV-IC or PBS. We analyzed tumor growth, survival, tumor histology and phenotype of tumor infiltrating lymphocytes (TILs).

Results: IV-IC induced anti-tumor effects against small tumors. Survival and inhibition of tumor growth were superior for mice receiving IT-IC. Depletion studies showed that both T cells and NK cells were involved in the potent antitumor effects of IT-IC. This is consistent with significant increases in NK cell and T cell infiltration in tumors [detected by both immunohistochemistry (IHC) and by flow cytometry (FC)] in mice receiving IT-IC. Within the IT-IC group, the smallest pre-treatment tumors showed the most tumor shrinkage. In all treatment groups, the percentage of NK or CD8 T cells in the tumor correlates inversely with tumor size. IT-IC and IV-IC treatment induced increased NKG2D effector receptors on intratumoral NK cells and CD8 T cells, but not on splenic NK cells; supporting the localized tumor targeting by IC treatment.

Conclusions: IV-IC and IT-IC treatments induce tumor-specific changes in TILs. IT-IC is more effective than IV, and merits clinical testing.

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PL20

Nervous system tumor stem cells: the cancer stem cell hypothesis writ large

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PL21

Isolation and Characterization of a Novel Cancer Stem Cell-like Population in Neuroblastoma

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Introduction: Neuroblastoma (NB) is an aggressive childhood malignancy derived from neural crest precursors. Previous studies have suggested that ex vivo-derived populations enriched for tumorigenicity (Tumor Initiating Cells) may represent a cancer stem cell-like population, which drives tumorigenesis and drug-resistant disease relapse. We report the identification and purification of a neuroblastoma subpopulation, isolated directly from all patient biopsy material, xenografts, and cell lines without ex vivo manipulation, which demonstrates lineage specific tumorigenesis in vivo and has a molecular and cellular phenotype highly similar to induced pluripotent stem cells (iPSCs).

Methods: Fluorescence-activated cell sorting (FACS) and flow cytometry were used to quantify the expression of CD114 on cell lines, xenografts, and patient biopsy samples at diagnosis and at tumor resection after chemotherapy. In vivo orthotopic tumorigenesis experiments were performed in NOD-SCID mice with fluorescent-tagged subpopulations, isolated from GFP and RFP transduced cell lines. Flow cytometry-based BRDU cell cycle analysis, small-RNA sequencing and gene expression profiling were used to further evaluate CD114+ and CD114- subpopulations extensively from three neuroblastoma cell lines.

Results: CD114+ cells are remarkably distinct from CD114- populations. They demonstrate a 'stem cell-like' cell cycle (prolonged S-Phase and short G1/G0 phase), a microRNA signature consistent to that found in iPSC populations, and express genes down-regulated upon neuronal differentiation. Additionally, in vivo, tumors generated from CD114+ orthotopic xenografts (100% CD114+ cells) recapitulate the original subpopulation (<1% CD114+ cells). As seen in our clinical biopsy data, this population is enriched upon in vivo exposure to chemotherapy.

Conclusion: We have successfully identified a novel subpopulation of neuroblastoma, highly reminiscent of a true cancer stem cell population. This population is phenotypically similar to iPSCs and is found in every primary biopsy specimen evaluated to data. Further evaluation of epigenetic and molecular features may permit the development of stem cell-targeted therapeutics for neuroblastoma.

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PL22

Elevated Myc levels in CASZ1 Haploinsufficient Murine Embryonic Stem Cells Prevents Neurogenesis

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Background: Tumor suppressor CASZ1(1p36.22) is often lost in poor prognosis neuroblastomas (NB) with an undifferentiated histopathology. Differentiation of NB cells upon CASZ1 restoration indicates CASZ1 loss may contribute to the undifferentiated phenotype during NB tumorigenesis. How CASZ1 functions during normal mammalian development is unknown. Using the murine embryonic stem cell (mESC) in vitro embryogenesis model, we investigated the effects of CASZ1 haploinsufficiency and CASZ1 reconstitution on differentiation programs.

Methods: Differentiation in mESCs, wild-type (WT) and Casz1 gene-trap (CAS+/-mESCs), was evaluated using mESC neurogenesis and adipogenesis assays. For rescue experiments, CAS+/-mESCs were transfected with a bacterial artificial chromosome containing the full human CASZ1 genomic locus (ESC-BAC-CASZ1). Gene expression was evaluated by qPCR and western blotting.

Results: Analysis of CAS+/-mESCs revealed elevated N-MYC protein (>5.5 fold) and higher c-Myc mRNA (~5.5 fold) compared to WT mESCs. Unlike WT mESCs, during neural differentiation CAS+/-mESCs exhibit a 5.4 fold increase in Gata2 mRNA (mesodermal lineage) and a 3.4 fold decrease in Pax6 mRNA (neuroectodermal lineage). CAS+/-mESCs maintain adipogenesis potential, but neurogenesis potential is lost as evidenced by a failure to extend ß-III tubulin positive neuritic processes. However in the ESC-BAC-CASZ1 there is normalization of CASZ1 mRNA (1-2 fold) and protein (0.6-1.2 fold) levels, a 3.8 fold decrease in N-MYC protein levels, and a 2 fold decrease in c-Myc mRNA relative to CAS+/-mESCs. Moreover, during neural differentiation Gata2 and Pax6 expression normalize to 0.5 and 2 times WT mESCs, respectively. ESC-BAC-CASZ1also demonstrated full neurogenesis potential with restoration of ß-III tubulin expressing neurites.

Conclusions: The data shows CASZ1 haploinsufficent mESCs are unable to implement a neural differentiation program. We propose the CASZ1 associated elevations in Myc expression, possibly through polycomb dysregulation, perpetuate repression of neuroectodermal lineage markers, such as Pax6, while directing cells into aberrant lineages, as evidenced by increased, mesodermal lineage marker, Gata2 expression.

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PL23

A Prospective Study of Expectant Observation as Primary Therapy for Neuroblastoma in Young Infants, a Children's Oncology Group Study

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Background: Neuroblastoma is the most common malignant tumor in infants, and in young infants, 90% are located in the adrenal gland. Although surgical resection is standard therapy, multiple observations suggest that expectant observation could be a safe alternative. The objective of this study was to demonstrate that expectant observation of young infants with adrenal masses would result in excellent overall and event-free survival (OS and EFS).

Methods: A prospective study of infants less than 6 months of age with small adrenal masses and no evidence of spread beyond the primary tumor was performed at all participating Children's Oncology Group institutions. Parents could choose observation or immediate surgical resection. Serial abdominal sonograms and urinary VMA and HVA measurements were performed over a ninety-week interval. Infants experiencing a 50% increase in the volume of the mass or urine catecholamine values, or an increase in the HVA/VMA ratio >2 were referred for surgical resection.

Results: 88 patients were enrolled, 84 elected observation and 4 chose immediate surgery. 16 observation patients ultimately had surgery; 9 had INSS stage 1 neuroblastoma, 2 had higher stage neuroblastoma (2B and 4S), 2 had low grade adrenocortical neoplasm, 2 had adrenal hemorrhage and one had extralobar sequestration. The two patients with adrenocortical tumors were resected for >50% increase in tumor volume. The 3-year EFS for a neuroblastoma event was 97.7±2.3%. The overall survival was 100% with median follow-up of 2.9 years. 81% of patients on the observation arm were spared resection.

Conclusions: Expectant observation of infants with small adrenal masses led to excellent EFS and OS while avoiding surgical intervention in a large majority of the patients.

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PL24

Regression and differentiation in localized infant neuroblastoma

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Background: Neuroblastoma in infancy often shows spontaneous regression, which, however, not always will lead to complete remission. This study aimed to explore the course of disease and histology beyond infancy in patients observed in wait-and-see strategies.

Methods: Data of infants with localised (INSS stage 1, 2 or 3), histological proven neuroblastoma without MYCN amplification diagnosed between 1995 and 2010 were included in the analysis. Chemotherapy was scheduled in case of symptoms or progression. Otherwise the tumor was either resected or observed.

Results: Of 540 infants, 362 were treated at diagnosis (chemotherapy n=100, 18.5%, complete or nearly complete resection: n=262, 48.5%), while 178 started observation strategies (33%). During the first year of life, 45 of 178 patients with observational strategies started chemotherapy (25.3%), 45 patients underwent resection (25.3%), and 35 tumors regressed with no or only minimal residuals left (CR, VGPR, 19.7%). At the end of the first year of life, major tumor residuals (PR, NR) were found in 53 patients (29.7%). Of these, 15 patients achieved CR or VGPR by spontaneous regression in the second year of life, in 21 patients the tumor residual was observed beyond the second year of life, two patients were treated with chemotherapy because of local progression, and in 15 patients the tumor residual was resected (13 complete, 2 incomplete) 5 to 75 months after diagnosis. Signs of differentiation were seen in the tumors resected beyond the second year of life in 10 out of 13 patients from whom initial histological samples were available, leading to ganglioneuroblastoma intermixed in three patients and to ganglioneuroma in one patient.

Conclusion: Localized infant neuroblastoma may not only undergo spontaneous regression, even beyond infancy, but also differentiate spontaneously into ganglioneuroma or ganglioneuroblastoma.

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PL25

The Therapeutically Applicable Research to Generate Effective Treatments (TARGET) in Neuroblastoma Project

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Background: Identification of oncogenic drivers of high-risk neuroblastoma (NB) should provide tractable targets for new therapies.

Methods: A total of 496 children age 1.5-5 years at diagnosis with INSS Stage 4 NB were identified for the NB TARGET project. Illumina SNP array (N=388), Affymetrix HuEx array (N=252) and next generation sequencing data (N=45 RNAseq, N=20 whole genomes and N=249 whole exomes) were generated from primary tumor specimens and matched normal DNAs, and data were integrated to define the mutational landscape of neuroblastoma.

Results: SNP array data confirmed complex chromosomal rearrangements with a striking paucity of recurrent homozygous deletions and focal amplification except for MYCN and ALK. Exon-level expression analysis showed enhanced complexity of putative splicing events in MYCN driven tumors, and identified robust signatures of disease outcome. Next generation sequencing data showed a low rate of somatic mutation (median of 17 somatic mutations per protein coding genome), confirmed ALK as the most commonly mutated gene, and uncovered chromatin remodeling and canonical MAPK signalling alterations as pathways recurrently targeted by somatic mutational events.

Conclusion: High-risk neuroblastoma is a disease driven by copy number alterations that influence broad alterations in mRNA expression. It is very likely that heritable genetic features and epigenetic alterations, in concert with copy number changes, have a major impact on high-risk NB phenotype. These data create challenges for personalized medical approaches based on easily quantifiable alterations in DNA sequence.

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PL26

New Agents for High-Risk Neuroblastoma: Combining, Refining, Redesigning, Selection and Discovery

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High-risk neuroblastoma remains a significant clinical problem as more than half of affected children will eventually succumb to disease that is either primary refractory or subsequently relapsed. Treatment for newly diagnosed patients includes multiple cycles of intensive induction cytotoxic therapy, followed by consolidation with high dose chemotherapy, stem cell rescue and local radiation. In North America, maintenance differentiation and immunotherapy with isotretinoin, GD2- targeted antibody and cytokines has become the standard of care. This approach has reached the limits of acceptability toxicity. Introduction of new agents into this paradigm remains challenging. Efforts have focused on combining novel therapeutics agents with each other and chemotherapy, refining and redesigning currently utilized immunotherapeutics and retinoids and the ongoing discovery of minimally toxic therapies. First steps are likely to include screening for activating ALK tyrosine kinase mutations and early, selective introduction of small molecule inhibitors such as crizotinib into combination chemotherapy. Other molecularly targeted agents including inhibitors of angiogenesis, other growth pathways (e.g. mTOR, AKT, IGF, Trk), novel cytotoxics (e.g. aurora kinase inhibitor MLN8237), and epigenetic modifiers (e.g. histone deacetylase inhibitor vorinostat) will need to show both feasibility and efficacy in combinatorial and selection design phase 2 trials in relapsed patients. Novel anti-GD2 antibodies such as the immunocytokine hu14.18-IL-2 or the genetically modified hu14.18K322A will be of interest if they can be shown to have enhanced activity or an improved side effect profile over the standard ch14.18. Similar added benefit will be expected of the synthetic retinoid fenretinide, or molecular therapies combined with isotretinoin. Rapid study evolution will require the definition of novel efficacy endpoints. Innovative therapeutics like oncolytic viruses, marine-derived anti-cancer compounds, new agents interfering with microtubule formation and miRNA mediated therapies are ongoing avenues for discovery.

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PL27

Accelerating Drug Development in Neuroblastoma – the SIOPEN ITCC Approach

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PL28

Why are neuroblastoma patients ignored by the European Pediatric Medicine Regulation?

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On January 26th, 2007, the Pediatric Medicine regulation was launched in Europe to provide better medicines for children. This regulation is based on rewards, incentives and obligations for Pharmaceutical Companies to study their drugs in children, when appropriate. The regulation was expected to facilitate access to anticancer drugs which are in development in adults and to increase significantly the number of those drugs in clinical development for children and adolescents in Europe. As a result in the paediatric oncology community there was great anticipation and hope for children suffering with cancer.

After 5 years of the regulation being in place, changes occurred in the field of new drug development in pediatric oncology, in Europe, and in the US and worldwide as well. All Pharmaceutical Companies now consider Pediatric Oncology and submit pediatric investigation plans (PIPs) to the European Medicine Agency before filing their anticancer drug in adults or claim a waiver. More than 41 out of 500 PIPs have been approved for 35 anticancer compounds. However, there is no significant increase in the number of drugs in pediatric phase I in Europe and the large majority of patients with a life-threatening advanced disease are still denied access to innovative therapies. Unfeasible or barely feasible PIPs are approved (development of 2 b-raf inhibitors in patients aged 12 to 18 with advanced V600E B-RAF metastatic melanoma) while major and urgent needs are ignored. Neuroblastoma is a case in point. There is no PIP of any drug in neuroblastoma among the 41 oncology PIPs while there are 3 PIPs in children with chronic myeloid leukemia and 4 PIPs in children with non-Hoghkin lymphoma. However, several drugs with an approved PIP were of interest for neuroblastoma, such as IGF1R inhibitor and anti-angiogenic compounds. Even crizotinib, the Alk inhibitor, which is approved in the US and nearly approved in Europe for the treatment of EML4-ALK non small cell lung cancer received a class waiver on May 2010, because there is no lung cancer in children.

Thus, the needs of neuroblastoma patients are ignored by the European Pediatric Medicine regulation for two reasons: i) the implementation of the regulation is entirely driven by the adult indication ii) Pharmaceutical Companies consider drug development for children with cancer (and other diseases as well) as a regulatory obligation to comply with rather than a R&D strategy to address pediatric needs. It is important to keep in mind that this does not impact only Europe. There is an urgent need to improve the implementation of this regulation and to increase the role of academic groups in defining needs, priorities and development strategies.

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PARALLEL SESSIONS

ALK

OR01 – OR07

OR01

ALK-based therapeutic stratification of patients with neuroblastoma

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Background: Genetic studies have firmly established the anaplastic lymphoma kinase (ALK) oncogene as a tractable molecular target in neuroblastoma. This provides the basis for implementation of ALK genomic status screening as a therapeutic stratification tool.

Methods: Primary tumor DNA from 1596 patients representative of the natural spectrum of neuroblastoma was analyzed for ALK genomic status. Detailed clinical correlative analyses were performed. We investigated 18 mutants in cell culture-based systems and assessed biochemical activity using a radioactive peptide-based assay to determine activating potential, ability to drive tumor formation, and response to pharmacologic inhibition with crizotinib.

Results: ALK is mutated in 8% of cases with mutations distributed across the phenotypic spectrum. Amino acid substitutions at locations found to be altered in earlier studies accounted for 86% of mutations (R1275, F1174, F1245 in decreasing frequency). Seventeen low-frequency mutation sites were discovered (10 not previously identified). Overall, the presence of an ALK mutation was predictive of worse event-free survival (EFS) (p=0.001 by log-rank; n=1584). Within the high-risk patient group, 10% harbor an ALK mutation and 4% high-level amplification. In a multivariable Cox model of EFS (n=1210 with complete data), ALK mutation was independently significantly prognostic, with 1.4(±0.39) times greater risk for an event than those without an ALK mutation (p=0.02), even after simultaneous adjustment for INSS stage 4 (hazard ratio[HR]=2.9±0.6), MYCN amplification (HR=1.7±0.4), 11q aberration (HR=1.8±0.4), age ≥18 months (HR=1.6±0.3), and diploidy (HR=1.2±0.2). The three most common mutations are highly active. Enhanced enzyme activity that is independent of receptor autophosphorylation appears to be a surrogate marker for transforming ability of mutations and Km,ATP is a surrogate for sensitivity to kinase inhibition with ATP competitors such as crizotinib, with predicted relative resistance in F1174 and F1245 variants.

Conclusions: Integration of genomic, biochemical, structural and functional assessment of ALK mutations predicts responsiveness to ALK inhibition therapy.

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OR02

The growth factor Midkine plays a critical role in the tumorigenesis of neuroblastoma via Notch2 signaling

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Background: Midkine (MK) is a growth factor highly expressed in various cancers including neuroblastoma (NB). We previously reported that plasma MK level was the reliable marker for poor prognosis, but its involvement in the tumorigenesis of NB has been obscure.

Methods & Results: Using MYCN Tg mice as a model for NB, we compared the gene expressions in normal superior mesenteric ganglion (SMG), precancerous SMG and tumor tissues. MYCN Tg mice at 2-week showed a precancerous histology in SMG, in which some part of SMG was occupied with undifferentiated neuroblasts. MK was highly expressed in those cells. By crossing MYCN Tg mice with MK-/- mice, MYCN Tg / MK-/- mice were obtained. Those mice exhibited a suppressed tumor incidence compared with MYCN Tg / MK+/+ mice. Next, we explored the downstream of MK to elucidate its mechanism of action. In MYCN Tg / MK-/- mice, tumor cells showed the attenuation of Notch2 signaling. The nuclear translocation of Notch2 intracellular domain was diminished. In addition, mRNA of some Notch target genes were reduced by the knockdown of MK and were restored by the treatment with recombinant MK in NB39 cells. To further investigate the downstream of MK, we screened the genes whose expressions were decreased in MK-/- tumor by DNA microarray analysis. Twelve such candidate genes exhibited a significant relationship with poor prognosis in clinical data from R2 (5 genes: $p<0.05$, 4 genes: $p<0.01$, 3 genes: $p<0.001$). Furthermore their expressions were also responsive to both the knockdown and the treatment of MK in NB39 cells.

Conclusions: The results indicate that MK is a critical factor involved in the tumorigenesis of NB. We also identified several downstream candidates whose expressions could be directly regulated by MK probably via Notch2. They would provide a novel molecular target for NB therapy.

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OR03

MDK promotes survival of neuroblastoma cells by signaling through ALK and other receptors

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Background: Midkine (MDK) is overexpressed in several malignancies including neuroblastoma. It is unclear whether MDK participates in neuroblastoma oncogenesis and MDK signaling pathways in neuroblastic cells are only partially understood. One of the receptors of MDK is the Anaplastic Lymphoma Kinase (ALK), a receptor tyrosine kinase expressed in neuroblastic tissues and recently reported as a key player in neuroblastoma oncogenesis. We explored whether MDK can control neuroblastoma cell proliferation and survival and analyzed the role of ALK and other known MDK receptors in MDK signal transduction in neuroblastoma.

Methods: siRNAs were used to knockdown MDK or its receptors in vitro in the neuroblastoma cell lines IGR-NB8, IMR-32 (both ALK wild-type), and LAN-1 (ALK F1174L). Cytotoxicity of each siRNA was evaluated by MTS assay. In vivo, MDK overexpression was achieved through DNA electrotransfer in skeletal muscle in a mouse model. Tumor engraftment and growth under MDK influence was evaluated on WT ALK IGR-NB8-R neuroblastoma xenografts.

Results: MDK silencing significantly reduced neuroblastoma cell viability between 30% and 65% in all three cell lines tested. Cytotoxicity of MDK knockdown was associated with high MDK expression but independent of ALK status or expression. MDK overexpression in vivo accelerated and enhanced tumor engraftment in IGR-NB8-R xenografts. MDK silencing resulted in reduced ALK activation and protein expression in both wild-type and mutated ALK neuroblastoma cells. Moreover, silencing of at least one receptor of MDK, including ALK resulted in significant cytotoxicity in all three cell lines. This underlines that MDK activates both ALK-dependent and independent survival pathways in neuroblastoma.

Conclusions: MDK is a driving factor for multiple cell survival pathways in neuroblastoma cells and therefore represents a relevant therapeutic target in neuroblastoma.

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OR04

Anaplastic Lymphoma Kinase (Alk) Expression Is An Independent Prognostic Factor In Neuroblastoma Patients And Correlates Well With Alk Inhibitor Response In Vitro.

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Background: ALK mutations occure in 3-11% of the neuroblastoma (NBL) patients and are considered oncogenic. We determined the role of ALK protein levels on survival of NBL patient tumors. Secondly, we tested in vitro responsiveness to ALK inhibitors in wild-type and mutated NBL cell lines.

Methods: Immunohistochemistry (IHC) for total ALK protein was performed on Formalin-Fixed, Paraffin-Embedded material of 71 NBL, 12 ganglioneuroblastoma (GNBL) and 20 ganglioneuroma (GN) patients. The percentage of ALK positive cells was startified in 4 Categories; Category 1 (n=40): < 20% ALK-positive cells, category 2 (n=25): 20-50% ALK positive cells, category 3 (n=25): 50-75% ALK positive cells, and category 4 (n=13): 75-100% ALK positive cells.

Results: The four categories correlate significantly with overall survival in NBL, GNBL and GN (p=0.002, Logrank test). ALK mutations were identified by sequencing in 2/63 NBL (3.2%) and 2/11 NBL (18.2%) patients. Importantly, in NBL and GNBL patients ALK protein expression remains a significant factor in a multivariate model (Cox Proportional Hazard model) including the COG risk stratification ((p<0.0001), which encompasses stage and NMYC status (see table).

In NBL cell lines, we studied the role of ALK levels in the response to ALK inhibitor TAE684 in vitro . TAE684 response correlates well with ALK protein levels (r=-0.813, p=0.001). ALK mutated cell lines show higher ALK levels and a better TAE684 response (LC50 values 14.9 fold lower, p=0.004) than WT and amplified cell lines.

In conclusion, 1. ALK protein expression is an independent predictor of overall survival in NBL patients and can be easily tested by IHC. 2. TAE684 response in vitro is not mutation dependent, but dependent on expression level.

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OR05

Mechanisms of acquired resistance in crizotinib-treated neuroblastoma

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Background: Mutation and amplification of anaplastic lymphoma kinase (ALK), a receptor tyrosine kinase (RTK) expressed on the majority of neuroblastoma tumors, leads to increased autophosphorylation and activation, and has been associated with decreased event-free survival. Crizotinib, an orally available small-molecule inhibitor of ALK, is currently in phase 1 trials in pediatrics, and although pharmacological inhibition of kinases in other cancers has significantly improved survival, resistance inevitably develops.

Methods: To mimic resistance development, we treated the ALK-expressing human neuroblastoma-derived cell line NB1643 with gradually increasing doses of crizotinib in vitro over a period of 3 months, verified resistance to inhibitor treatment, and studied resulting genetic, transcriptional, and protein changes in 3 subclonal populations generated by serial dilution of resistant cells under drug pressure.

Results: Western blotting was used to confirm inhibition of phospho-ALK and rule out drug efflux as a resistance mechanism. Sanger sequencing of the ALK TKD revealed no additional crizotinib-induced mutations and no copy number variations were detected by SNP Array using the Illumina 1M chip. We next used phospho-array technology to evaluate changes in the activation state of a panel of RTKs as well as downstream signaling molecules. In comparison to untreated cells, crizotinib-resistant cells had up-regulated activated epidermal growth factor receptor(EGFR) and ERK, as well as other downstream signaling molecules. Native EGFR up-regulation was confirmed by transcriptional analysis of single cells and no mutations were detected by Sanger sequencing of the EGFR TKD. Drug combination studies showed that dual inhibition of ALK and EGFR or ERK enhanced growth inhibition.

Conclusions: Taken together, these data suggest that ALK inhibition in neuroblastoma leads to up-regulation of additional pathways and implies the need to develop therapeutic strategies to target these additional pathways when ALK inhibition is used clinically.

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OR06

Combined inhibition of ALKF1174L and downstream signaling leads to tumor regression and prolongation of survival in transgenic mice with ALKF1174L/MYCN tumors.

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Background: The ALKF1174L mutation is associated with intrinsic and acquired resistance to crizotinib in neuroblastoma (NB) and ALK-rearranged cancers, respectively, and co-segregates with MYCN amplification in NB.

Methods: To identify therapeutic strategies that circumvent crizotinib resistance in NB, we utilized a transgenic mouse model expressing ALKF1174L and MYCN (described in abstract by Berry et al.) for the studies described below. Transgenic mice with palpable tumors were treated with 100 mg/kg crizotinib or 20 mg/kg Torin2 as single agents, or, 100 mg/kg crizotinib and 20 mg/kg Torin2 in combination for 7 days. Trials were monitored by MRI.

Results: Crizotinib treatment of ALKF1174L/MYCN mice did not affect tumor growth or volume. Analysis of the PI3K/AKT/mTOR and MAPK pathways in these tumors revealed that treatment with crizotinib led to a minimal decrease in pAkt but no discernible effects on mTOR or MAPK signaling. We therefore determined if these tumors could be rendered sensitive to crizotinib by concurrent blockade of mTOR using Torin2, an ATP-competitive inhibitor of mTOR. When given alone, Torin2 ablated MYCN-expressing tumors with marked destabilization of the oncoprotein. However, Torin2 was ineffective as a single agent in ALKF1174L/MYCN tumors even with clear evidence of PI3K/mTOR blockade. By contrast, combined use of crizotinib with Torin2 suppressed tumor growth and prolonged survival compared to the single agent-treated groups, and correlated with inhibition of pALK, PI3K/AKT/mTOR and MAPK signaling and induction of apoptosis.

Conclusions: Thus, our TH-ALKF1174L/MYCN model exhibits persistent activation of PI3K/mTOR and MAPK signaling and recapitulates the crizotinib resistance of human NB. We show that combined treatment with a downstream pathway inhibitor overcomes the resistance of these tumors to crizotinib. This model should provide an ideal platform for future studies to screen novel targeted agents for their ability to inhibit ALK oncogenicity in neuroblastoma.

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OR07

Association of ALKF1174L expression with altered vasculature in transgenic murine models of neuroblastoma

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Introduction: The F1174L mutation in the tyrosine kinase domain of anaplastic lymphoma kinase (ALK) co-associates with amplification of MYCN in a group of neuroblastoma (NB) patients with very poor outcome. In NB tumours, aggressive biology is linked to a hypervascular phenotype, making anti-angiogenic therapeutic strategies of clinical interest. ALK is known to modulate several genes involved in tumour vasculogenesis. To investigate whether expression of ALK influences vascular morphology of MYCN-driven tumours, we compared histopathology and non-invasive magnetic resonance imaging (MRI) parameters of NB GEMMs that express MYCN alone (TH-MYCN) or MYCN with ALKF1174L (TH-ALKF1174L/TH-MYCN).

Methods: We compared gross appearance, H&E, endomucin and smooth muscle actin staining, tumour uptake of Hoechst 33342 (perfusion) and Evans Blue (permeability) using fluorescence microscopy, and vascular corrosion casting. Expression arrays focusing on modulation of angiogenic targets was performed. A multi-parametric approach was used to quantify functional vascular MRI biomarkers, including fractional blood volume (fBV, %); transverse relaxation rate R2* (s-1), a proxy for paramagnetic [deoxyhaemoglobin]; vessel size index (VSI, μm); vascular permeability/perfusion (KTRANS, min-1).

Results: Major differences in tumour-associated vasculature were revealed. H&E staining of the TH-MYCN tumours revealed the presence of blood lakes, which were absent from TH-ALKF1174L/TH-MYCN tumours. A macroscopic reduction in vasculature was apparent in vascular casts of ALKF1174L-mutated tumours. Tumours from TH-ALKF1174L/TH-MYCN mice had a significantly lower fBV (10.4±2% versus 21.9±3%, $p=0.01$, $n\geq7$), and a significantly slower R2* rate (74±7s-1 versus 109±9 s-1, $p<0.01$, $n\geq8$) compared with TH-MYCN mice. There was no significant difference in KTRANS, VSI, Hoechst 33342 or Evans Blue uptake.

Conclusion: This study demonstrates that expression of ALKF1174L in the context of MYCN significantly alters the vasculature of NB tumours. These changes may have important implications for the delivery of therapeutic agents and may in part explain the enhanced penetrance and progression of these murine tumours in vivo.

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PARALLEL SESSIONS

miRNA
OR08 – OR14

OR08

MYCN directly regulates long non-coding RNA expression in neuroblastoma

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Background: With the discovery of non-coding RNA genes, the complexity of the transcriptome and its regulation has increased dramatically. Apart from the well-studied small non-coding microRNAs (miRNAs), several thousands of long non-coding RNAs (lncRNAs) have recently been discovered. Like miRNAs, lncRNAs appear to predominantly function as regulators of gene expression and are believed to be integral components of various signaling networks.

Methods: To determine whether lncRNAs are involved in the MYC/MYCN transcriptional network we designed a custom high-throughput RT-qPCR lncRNA profiling platform, capable of measuring 1718 human lncRNAs, and evaluated lncRNA expression upon MYCN activation in the SHEPMYCN-ER model system. Differential lncRNAs were further validated in primary neuroblastoma tumors and other tumor entities with increased MYC activity. MYCN and MYC binding to lncRNA promoters was assessed using ChIP-sequencing and lncRNA functions were studied through RNAi-mediated lncRNA knockdown.

Results: LncRNA expression profiling of a SHEPMYCN-ER time-series revealed 12 up regulated and 22 down regulated lncRNAs upon MYCN activation. For a subset of these lncRNAs, we confirmed differential expression in 366 primary neuroblastoma tumor samples and 32 neuroblastoma cell lines with and without MYCN amplification. Differential lncRNA expression was further validated in MYC-amplified cell lines from different tumor entities (neuroblastoma, prostate cancer, colon cancer) treated with the MYC inhibitor JQ1. ChIP-sequencing for both MYC and MYCN in neuroblastoma and other tumor entities revealed strong MYC and MYCN binding in the lncRNA promoters, accompanied by a H3K4me3 – H3K36me3 signature indicative for active transcription and elongation. RNAi-mediated knockdown of a MYC/MYCN activated lncRNA drastically reduced viability in different MYCN-amplified neuroblastoma cell lines.

Conclusions: Our findings demonstrate that lncRNAs are implicated in the MYC/MYCN transcriptional network in neuroblastoma and other tumor entities with activated MYC signaling. LncRNA perturbation experiments suggest that MYC/MYCN-activated lncRNAs have oncogenic properties and could serve a novel targets for therapy.

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OR09

miR-542-3p exerts tumor suppressive functions in neuroblastoma by downregulating survivin

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Background: MicroRNAs (miRNAs) are small, non-coding RNAs deregulated in neuroblastoma (NB) and other cancers. We previously reported a signature of 42 miRNAs to be highly predictive for NB outcome. This signature included miR-542-3p, which was downregulated in tumors from patients with adverse outcome. Here we analyzed the influence of miR-542-3p expression on major aspects of NB tumor biology.

Methods: Pre-existing datasets (real-time miRNA expression, next-generation RNA sequencing and mRNA expression microarrays) from primary NBs were re-analyzed to assess expression of miR-542-3p, miR-542-5p and survivin. MiR-542-3p or -5p were re-expressed or survivin was downregulated (siRNA) in the SHEP, IMR-32 and SK-N-BE NB cell lines, and resulting cell viability, proliferation and apoptosis were assessed. Reporter assays were performed in SHEP and HEK293 cells to determine if miR-542-3p directly targets survivin. Combined overexpression of survivin and miR-542-3p was conducted in SHEP cells. NB xenografts in nude mice were intravenously treated with miR-542-3p nanoparticles.

Results: MiR-542-3p and miR-542-5p were expressed in equimolar ratios in primary NBs, and expression inversely correlated with poor prognosis. Re-expression of miR-542-3p in NB cell lines reduced cell viability and induced apoptosis and cell cycle arrest. In contrast, this effect was only minor after re-expressing miR-542-5p. Survivin was downregulated upon miR-542-3p re-expression in NB cell lines, and was inversely correlated with miR-542-3p expression in primary NBs. Reporter assays confirmed that miR-542-3p directly targeted survivin. Survivin knockdown via siRNA phenocopied the effect of miR-542-3p re-expression, and combined overexpression of survivin and miR-542-3p partially rescued the miR-542-3p re-expression phenotype. Targeting NB xenografts in mice with miR-542-3p nanoparticles repressed expression of survivin and induced apoptosis.

Conclusions: MiR-542-3p exerts tumor suppressive functions in NB, at least in part, by targeting survivin. Initial evidence from miR-542-3p re-expression in vivo points to a promising potential of nanoparticle-targeted delivery of selected miRNAs as a novel therapy approach.

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OR10

Enrichment analysis for MYCN pathway genes in focal genomic gains and losses identifies new components of the MYCN-miRNA regulatory network in neuroblastoma

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Background: Particular patterns of large chromosomal deletions, gains and amplifications in neuroblastoma have been described in detail and delineate three major genomic subgroups. However, comprehensive analyses of focal aberrations have thus far received much less attention. The finding of a 5 kb gain containing exclusively the MYCN activated miR-17-92 cluster in a neuroblastoma cell line lead us to hypothesize that clinically relevant focal genomic gains and losses may be implicated in neuroblastoma and that such DNA copy number changes may specifically target MYCN regulated genes.

Methods: High resolution DNA copy number data of 190 neuroblastoma tumor samples and 33 neuroblastoma cell lines were analyzed for focal DNA copy number aberrations. Accompanying mRNA and miRNA data, available for most of these samples, were used for additional data mining purposes.

Results: We detected significant enrichment for up regulated MYCN target genes in focally gained and amplified regions, suggesting that DNA copy number variants can further reinforce particular MYCN downstream effects in tumor cells. In the deleted regions, enrichment for predicted target genes of MYCN up regulated miRNAs was observed. Using an integrated data mining approach and subsequent experimental validation, RGS5, homozygously deleted in one neuroblastoma cell line, was identified as a target of MYCN up regulated miRNAs. RGS5 encodes a regulator of G protein signaling implicated in vascular normalization and has not previously been reported in neuroblastoma oncogenesis.

Conclusions: With this unique approach, we show for the first time that focal genomic gains in neuroblastoma are enriched for direct MYCN target genes, while focal genomic deletions are enriched for genes down regulated through MYCN regulated miRNAs. Using an integrated genomic approach we confirmed the latter observation and expanded the MYCN-miRNA controlled regulatory network. Given the emerging role of RGS5 in tumor angiogenesis, this gene may represent an important target for anti-angiogenic therapy.

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OR11

MYCN and HDAC2 cooperate to repress miR-183 signaling in neuroblastoma

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Background: MYCN is a master regulator controlling many processes necessary for tumor cell survival. Inhibition of HDAC activity suppresses malignant properties of MYCN-amplified neuroblastoma cells. Here, we unravel a micro-RNA network, at least, partially causing these tumor-suppressive effects.

Methods: Expression changes from pan-HDACi treatment were analyzed by miRNA profiling. Transfection of neuroblastoma cell models and mouse xenografts were used to assess the role of candidate miRNAs in neuroblastoma biology. Regulatory mechanisms were investigated by (re-)ChIP.

Results: Of the 1000 miRNAs, pan-HDACi treatment most strongly induced miR-183 expression. Up-regulation reached 80-fold in a time-course using qRT-PCR analysis, validating microarray results. Similar effects were observed in vitro in 4 neuroblastoma cell lines and a primary tumor sphere culture as well as in neuroblastoma xenografts. Enforced miR-183 expression induced apoptosis and inhibited proliferation and anchorage-independent colony formation of MYCN-amplified cells in vitro and strongly reduced xenograft growth in mice. Clinical relevance is supported, since strong miR-183 expression in primary neuroblastomas significantly correlated with favorable event-free survival in patients. Experiments to identify the HDAC(s) involved in miR-183 transcriptional regulation showed that HDAC2 depletion induced miR-183. Conversely, HDAC2 overexpression reduced endogenous miR-183 and counteracted the induction caused by HDAC2 depletion. ChIP revealed that HDAC2 was recruited to the miR-183 promoter, and that HDAC2 depletion enhanced miR-183 promoter-associated histone H4 pan-acetylation, suggesting epigenetic changes preceded transcriptional activation. Because H3K27 trimethylation at the miR-183 promoter was higher and endogenous miR-183 expression was up to 15-fold lower in MYCN-amplified cells, we tested whether MYCN recruits HDAC2 to the miR-183 promoter, and detected MYCN and HDAC2 in common complexes at this site.

Conclusions: These data reveal the tumor suppressive properties of miR-183 in neuroblastoma that are jointly repressed by MYCN and HDAC2, and suggest a possible novel way to bypass MYCN in treating patients with MYCN-amplified tumors.

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OR12

MiR-137 is epigenetically silenced in MYCN amplified neuroblastomas and targets the polycomb repressive complex 2 (PRC2) component EZH2

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Introduction: Aberrant epigenetic modifications are important events in the process of tumor formation. Recent studies have shown that epigenetic silencing of tumor suppressor miRNA's can directly add to tumor formation.

Aims and Methods: Given the established role of miRNA's in neuroblastoma (NB), we hypothesized that epigenetic silencing of miRNA's would contribute to NB. To investigate this hypothesis, we performed an integrated analysis of miRNA profiles from 14 murine neuroblastomas (TH-MYCN and ALK-driven) and 200 human NB, and MBD-sequencing data marking genome-wide DNA methylation at high resolution in 8 human NB cell lines and 45 primary tumors.

Results: Among 523 profiled murine miRNA's, 21 miRNA's were differentially expressed in MYCN-driven versus ALK-driven tumors. For miR-137, we confirmed significant lower expression levels in MYCN-driven human tumors and low expression levels were associated with more aggressive phenotype ($p<0.001$). Interestingly, as observed in other cancer entities, its transcription start site (TSS) was more frequently hypermerhylated in more aggressive human NB tumors and MYCN amplified (MNA) NB cell lines, corresponding with reduced expression of miR-137. This DNA hypermethylation coincided with loss of the activating histon mark H3K4me3 at the level of the predicted TSS in MNA NB cell lines as opposed to non-MNA NB cell lines. Through integration of m(i)RNA expression data and survival data, targets of miR-137 were subsequently prioritized. EZH2, a known target of miR-137 and a core component of the Polycomb Repressor Complex 2, displayed increased expression in more aggressive tumors, matching its recent reported status of oncogene in NB. A similar expression pattern was apparent for AKT2, a major downstream effector of the PI3K pathway, one of the most potent pro-survival pathways in cancer.

Conclusion: Our results indicate that miR-137 is methylated in MNA NB and that this miRNA may act as a tumorsuppressor through down regulation of EZH2 and AKT2.

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OR13

Establishment of a miRNA/mRNA regulatory network through integrated analysis of neuroblast and neuroblastoma expression profiles

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Background: MicroRNAs (miRNAs) play an essential regulatory role in many normal cellular functions and development as well as diseases, including cancer. For neuroblastoma, as for other embryonic tumours, it has been assumed that oncogenesis results from the disruption of normal developmental processes. The sympatho-adrenal progenitor cells located within the adrenal gland and sympathetic ganglia are considered as the normal counterpart for the neuroblastoma cells.

Methods: To investigate whether miRNAs, implicated in the development of the sympathetic nervous system, are also involved in neuroblastoma development, we compared miRNA and mRNA expression profiles of 101 primary untreated neuroblastoma tumours to those of human neuroblasts, dissected from foetal adrenal glands.

Results: A total of 60 differentially expressed miRNAs were identified and included into an integrative genomic analysis using mRNA expression profiles in order to build an miRNA/mRNA transcriptional network controlling genes implicated in neuronal development and neuroblastoma. MiR-204 emerged as a strong putative network component and was shown to directly target PHOX2B, a gene known to act as master regulator of sympathetic nervous system development and implicated in familial neuroblastoma. The network was further expanded using target enrichment analysis, gene ontology data, literature data, miRNA target prediction databases and miRNA-mRNA correlation analyses. One of the components of this network was SOX11, which was shown to be directly targeted by miR-204 and miR-542-3p and to be indirectly regulated by miR-133b, all of which are expressed at higher levels in the neuroblasts as compared to the tumours. A reduction in colony formation capacity was observed upon knockdown of SOX11 underscoring the oncogenic potential of this gene. Conclusions: Our data allowed to describe a putative miRNA/mRNA regulatory network implicated in neuroblastoma. The identified network components may provide a further basis for development of miRNA targeted therapies.

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OR14

Outcome prediction of neuroblastoma patients using microRNA gene expression profiling in both fresh frozen and archived tumor samples

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Introduction: Current risk classification criteria for neuroblastoma patients result in suboptimal classification, contributing to less effective cancer therapy. By developing a miRNA prognostic signature we aim at achieving higher risk stratification accuracy and thus better neuroblastoma survival rates.

Methods: 430 mature human microRNAs were profiled in 51 fresh frozen tumor samples upon which a set of 25 prognostic miRNAs was identified. The signature was tested in 179 fresh frozen tumor samples and validated in an independent set of 304 fresh frozen samples and 75 formalin-fixed paraffin-embedded (FFPE) samples.

Results: The 25-gene miRNA signature could accurately predict progression-free survival (PFS) and overall survival (OS) ($p<0.0001$) in the test cohort, independently from currently used risk predictors. Patients with increased risk for shorter PFS and OS could also be identified within the high-risk subgroups from the test cohort and the validation cohort. Remarkably, the signature could also predict OS and PFS in the FFPE sample set ($p<0.01$).

Conclusions: In this study we present the largest neuroblastoma miRNA expression study so far, including more than 500 neuroblastoma samples originating from fresh frozen primary tumor biopsies and 75 FFPE samples. We established and validated a robust miRNA classifier, able to identify patients with higher risk for adverse outcome within the current high-risk group. Given the successful classification using FFPE material, we are currently collecting large FFPE sample cohorts to allow prognostic classification within current treatment groups. Given the low survival rates and low response to treatment, special attention will be dedicated to identification of ultra high-risk patients in the current high-risk, allowing more accurate patient assignment to new upcoming treatment strategies.

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PARALLEL SESSIONS

MYC-N

OR15 – OR22

OR15

Anaplastic Lymphoma Kinase (ALK) regulates initiation of transcription of MYCN in neuroblastoma cells.

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Neuroblastoma is a neural crest derived embryonal tumour of the postganglionic sympathetic nervous system and a disease with several different chromosomal gains and losses, which include MYCN amplified neuroblastoma on chromosome 2), deletions of parts of the chromosomes 1p and 11q, gain of parts of 17q and triploidy. Recently, activating mutations of the ALK (Anaplastic Lymphoma Kinase) RTK (Receptor Tyrosine Kinase) gene have been described in neuroblastoma. A meta-analysis of neuroblastoma cases revealed that ALK mutations (49 of 709 cases) in relation to genomic subtype were most frequently observed in MYCN amplified tumours (8.9 %),

correlating with a poor clinical outcome (1). MYCN proteins target proliferation and apoptotic pathways, and play an important role in the progression of neuroblastoma. Here we show that both wild type and gain-of-function mutants in ALK are able to stimulate transcription at the MYCN promoter and initiate mRNA transcription of the MYCN gene in both neuronal and neuroblastoma cell lines. Further, this stimulation of MYCN gene transcription and de novo MYCN protein expression is abrogated by specific ALK inhibitors, such as crizotinib (PF-2341066), NVP-TAE684, and by siRNA to ALK resulting in a decrease in proliferation rate. Finally, co-transfection of ALK gain-of-function mutations together with MYCN leads to an increase in transformation potential. Taken together, our results indicate that ALK signaling regulates initiation of transcription of the MYCN gene providing a possible explanation for the poor clinical outcome observed when MYCN is amplified together with activated ALK.

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OR16

Direct effects of Bmi1 on p53 protein stability inactivates oncoprotein stress responses in neuroblastoma precursor cells at tumor initiation

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Background: The Polycomb trans-repressor protein, Bmi1, is an oncogene and co-factor for Myc-induced malignancy. We have previously shown that Bmi-1 was necessary for neuroblastoma tumor initiation in TH-MYCN transgenic mice, and, that p53 signal responses were repressed in rest cells prior to malignant transformation. Neuroblastoma, can arise from postnatally persistent embryonal rest cells and is uniquely characterised by the absence of p53 mutations.

Methods: We used human neuroblastoma cell lines, murine primary ganglia cells cultured in vitro from TH-MYCN transgenic mice, adenoviral vectors, co-immunoprecipation, and ubiquitination assays, to examine the hypothesis that Bmi-1 and other Polycomb complex proteins, Ring 1A and 1B, reduced p53 protein stability as a cofactor for rest formation.

Results: Here we show that Bmi1 expression exhibited a stepwise increase during the progression from rest to tumor in tissues from TH-MYCN mice. Bmi1 knockdown in perinatal TH-MYCN ganglia restored both death sensitivity to NGF withdrawal, and p53 expression. In human neuroblastoma cells, the p53 protein half-life increased following Bmi1 knock-down, whereas, exogenous Bmi1 overexpression reduced p53 protein half-life. SHEP neuroblastoma cells carry a homozygous p14ARF deletion, demonstrated an increased p53 protein stability following Bmi1 knock-down. Thus, the Bmi1 effect on p53 protein stability was independent of p14ARF transcriptional regulation. Endogenous p53 was co-immunoprecipitated with Flag-tagged Bmi1. Ring-1A and 1B proteins directly bound to endogenous or overexpressed p53. Overexpressed Bmi1 increased p53 binding to the Ring proteins. We observed increased p53 polyubiquitination in both NBL-S and p14ARF-deleted SHEP neuroblastoma cells overexpressing Bmi1. Conversely, Bmi1 knock-down markedly reduced p53 ubiquitination. Both Ring1A and 1B induced p53 mono- and di-ubiquitination, and Bmi1 markedly increased the efficacy of this reaction.

Conclusion: Bmi1 can directly bind and ubiquitinate p53 in neuroblastoma cells, as a mechanism of p53 signal repression during the initial steps of MycN-driven neuroblastoma tumorigenesis.

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OR17

Targeting the MYCN Signaling Pathway by Inhibiting the Histone Demethylase JMJD2B

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Background: DNA binding of the MYCN transcription factor, frequently amplified in high-risk neuroblastoma, depends on the presence of both consensus binding sites and epigenetic modifications that expose these sites to MYCN protein. We sought to determine whether JmjC domain-containing histone demethylases, which alter chromatin configuration and DNA transcription, influence MYCN signaling.

Methods: The human-derived neuroblastoma cell lines SKNBE2, NB1691 and SKNAS were used for in vitro studies. Transient transfection or lentiviral vector-mediated transduction was used to overexpress or silence JMJD2B or MYCN. Co-immunoprecipitation, microarray analyses, and ChIP-PCR were used to analyze molecular changes in these modified cells.

Results: Co-immunoprecipitation studies demonstrated that the histone demethylase JMJD2B physically interacts with MYCN and appeared to be required for expression of most MYCN-responsive genes. Microarray analysis of genetically manipulated cells further confirmed significant overlap (>60%) of JMJD2B and MYCN downstream targets. Consistent with the role of JMJD2B in modulating MYCN activity, loss of JMJD2B significantly inhibited tumor cell proliferation. A bioinformatics approach was then used to identify compounds whose gene expression profiles inversely matched that of high-risk neuroblastoma, as they might suppress oncogenic driving events. One compound, ciclopirox (CPX), which potently suppressed neuroblastoma cell proliferation and induced tumor cell differentiation, also induced a dramatic increase in transcription-silencing histone methyl marks, including H3K9me3/me2. Further analysis revealed that CPX inhibited the enzymatic activity of JMJD2B while gene expression profile analysis demonstrated that there was significant overlap among genes that were downregulated by CPX and loss of JMJD2B expression. In addition, CPX was found to downregulate expression of MYCN targets, suggesting that CPX blocks the MYCN pathway by inhibiting histone demethylases, including JMJD2B.

Conclusion: We have identified JMJD2B as an important, novel epigenetic modulator of MYC pathway. Targeting this pathway using compounds such as CPX might be a promising new strategy for the treatment of neuroblastoma.

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OR18

The class III histone deacetylase SIRT2 stabilizes N-Myc oncoprotein by transcriptional repression of the E3 ubiquitin-protein ligase NEDD4

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Background: The class III histone deacetylase SIRT2 has recently been shown to promote tumor cell proliferation and survival. N-Myc oncoprotein is well-known to modulate gene transcription. However, much less is known about N-Myc protein stabilization and degradation. The E3 ubiquitin-protein ligase NEDD4 induces protein degradation by targeting substrate proteins for ubiquitination and degradation.

Methods: Gene and protein expression was analysed by real-time RT-PCR and immunoblot, protein stability was examined by pulse chase assays, and transcriptional target genes of SIRT2 was identified by Affymetrix gene array studies. The binding of SIRT2 protein to NEDD4 gene promoter was tested by chromatin immunoprecipitation assays, and the binding of NEDD4 protein to N-Myc protein was examined by protein co-immunoprecipitation assays. The direct effect of NEDD4 on N-Myc protein ubiquitination and degradation was investigated by in vitro and in vivo ubiquitination assays.

Results: SIRT2 expression was up-regulated by N-Myc in neuroblastoma cells. Conversely, SIRT2 enhanced N-Myc protein stability and promoted cancer cell proliferation. Affymetrix gene array studies revealed that the gene most significantly repressed by SIRT2 was the E3 ubiquitin-protein ligase NEDD4. Consistent with this finding, SIRT2 repressed NEDD4 gene expression by directly binding to the NEDD4 gene core promoter. Importantly, NEDD4 directly bound to N-Myc protein and targeted N-Myc protein for ubiquitination and degradation. Small molecule SIRT2-selective inhibitors reactivated NEDD4 gene expression, reduced N-Myc protein expression, and suppressed neuroblastoma cell proliferation. In human neuroblastoma tissues, high levels of SIRT2 and low levels of NEDD4 expression correlated with poor patient prognosis.

Conclusions: Our data reveal a novel pathway critical for N-Myc protein stability, and provide important evidences for potential application of SIRT2 inhibitors for the prevention and therapy of N-Myc-induced neuroblastoma.

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OR19

Suppression of neuroblastoma tumorigenesis using ENU mutagenesis in the TH-MYCN mouse model of neuroblastoma

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Background: N-ethyl-N-nitrosourea (ENU) mutagenesis is a powerful tool for elucidating new genes/pathways that are important to a particular disease of interest. In the TH-MYCN model of neuroblastoma, 100% of mice homozygous for the MYCN transgene develop tumors by 7.5 weeks of age. The aim of this study was to use ENU to cause heritable mutations that result in delay or abrogation of tumor development in this model, in order to identify genes involved in neuroblastoma tumorigenesis and potential targets for therapy.

Methods: Male homozygous mice, treated with cyclophosphamide to temporarily ablate their tumors, were subsequently injected with ENU prior to mating with female homozygotes. Progeny were screened for a delay in tumor development by palpation. To determine the mutation(s) responsible for delayed tumor development, exome sequencing and traditional backcrossing were used.

Results: Of 1716 viable offspring screened, 50 mice displayed a delay in tumor onset of >7.5 weeks. Of these mice, 5 lines showed heritability. In one line, the founder mouse developed a tumor at 49 weeks of age, an unprecedented delay compared to untreated homozygous mice. On excision the tumor was found to be highly avascular compared to the haemorrhagic tumors normally observed. This phenotype is inherited as a dominant Mendelian trait, with 50% of 22 offspring of this founder developing neuroblastoma by 6 weeks and the remainder displaying suppression of tumor development - either failing to develop tumors at all (>52 weeks), or in two cases developing avascular tumors (18-25 weeks). Mapping and sequencing have localised the mutation to the proximal end of chromosome 4.

Conclusions: ENU mutagenesis screening is a powerful in vivo approach for identifying genes associated with neuroblastoma tumor development. By employing a phenotype-driven methodology that assumes no bias towards any gene or pathway, it has the potential to provide novel targets for neuroblastoma therapy.

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OR20

Identification and partial characterization of novel Aurora kinase inhibitors to target MYCN destabilization

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Background: Amplification of the transcription factor MYCN occurs in ~25% of neuroblastoma and marks high-risk disease. MYCN thus represents a prominent therapeutic target in neuroblastoma, however the development of small molecule inhibition of transcription factors remains challenging. The stability of MYCN protein in neuroblastoma is regulated by upstream signaling through the PI3K/mTOR pathway, and by a kinase-independent scaffolding function of Aurora kinase A. Is it possible to generate kinase inhibitors of Aurora A that also disrupt kinase-independent scaffolding functions? Type II inhibitors bind to their target kinases through movement of the activation helix and alpha-C loops, leading to changes in secondary structure, and potentially disrupting kinase-independent scaffolding functions. We hypothesize that type II inhibitors of Aurora kinase A will therefore block Aurora kinase while also disrupting scaffolding functions, destabilizing interactions between MYCN and Aurora A, and driving degradation of MYCN.

Methods: An array of standard MYCN amplified and non amplified cell lines were acquired including SK-N-BE2, NB1691, Kelly, and SH-EP cells. Primary cell lines were generated from TH-MYCN mice as well as fresh, patient tumors from surgical procedures done at UCSF. Lentiviral and bacterial constructs were generated to express MYCN and Aurora A kinase (Addgene) and mutants were generated by PCR-based site-directed mutagenesis (Stratagene). Western blots and in vitro Aurora A kinase assay were performed as previously described.

Results: Using MYCN protein level as a readout, we screened a panel of novel type II molecules built around a diaminopyrimidine scaffold (a basis for known dual Aurora A/B inhibitors). Among 32 initial molecules, we identified 3 candidates that blocked Aurora Kinase A, and that dramatically decreased MYCN protein in neuroblastoma cells. To validate a post-transcriptional mechanism of MYCN destabilization, we performed secondary assays against MYCN proteins mutant at T58 and S62, phosphorylation sites critical to proteolytic degradation. Among our 3 candidate inhibitors, a single compound lost activity against T58 and S62 mutants. In vitro kinase assays indicate that this and derivative candidate compounds potently inhibited Aurora A kinase at nanomolar concentrations. Using published crystal structures of Aurora A and structure activity relationships among our candidates, we are currently screening a second generation of compounds to identify additional candidate molecules, and provide further insight into mechanism.

Conclusions: These results indicate a novel class of Aurora A kinase inhibitors targeted specifically to disrupt Aurora's interaction and stabilization of MYCN and represent a new strategy for targeting high-risk neuroblastoma.

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OR21

Paucity of early tumor-driving gene mutations in MYCN amplified neuroblastoma

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Background: We recently performed whole genome sequencing of a large series of neuroblastoma (Molenaar et al., Nature, in press). Only few recurrent amino-acid changing mutations were detected. Instead, each tumor carried a series of particular mutations, of which a contribution to neuroblastoma pathogenesis was difficult to establish. We therefore asked whether we could distinguish early tumor driving mutations from late tumor maintaining aberrations and passenger events.

Methods: We used primary tumor samples from four MYCN amplified tumors and 4 cell lines derived from these tumors and in two cases also bone marrow matastasis derived cell lines. We performed paired-end whole genome sequencing of all samples. Data was integrated with mRNA expression data and analyzed using the R2 bioinformatic platform.

Results: We hypothesized that primary tumors, metastases and cell lines derived of them share tumor-driving mutations, but might differ in late or passenger-type gene defects. Validation of the technology was performed using two technical duplicates. This allowed identification of criteria to optimize specificity and sensitivity. In the tumors and cell lines, we sequence-validated all 73 amino-acid changing somatic mutations detected in the tumors and cell lines of the four patients. Comparison of tumors and corresponding cell lines classified most mutations as late or passenger-type. There remained 10, 8, 1 and 0 early amino-acid changing mutations in the neuroblastomas of the four patients.

Conclusion: These findings indicate that most amino-acid changing mutations detected in neuroblastoma are late or passenger mutations. Primary neuroblastomas are already heterogeneous with respect to their DNA defects.. The paucity of early tumor-driving mutations detected in this systematic sequencing study of neuroblastoma urges to reconsider alternative oncogenic mechanisms like haploinsufficiency and dosage effects. A predominance of such mechanisms over gene-mutations as tumor-driving events would strongly bear on the development of targeted therapies and personalized treatment in neuroblastoma.

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OR22

NLRR1, a direct target of MYCN, regulates cell growth both in vitro and in vivo and can be a therapeutic target against high-risk neuroblastoma

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Background: Neuronal leucine-rich repeat protein (NLRR) 1 is a member of NLRR family and highly expressed in unfavorable NB, whereas NLRR3 expression is high in favorable subsets. We previously reported that NLRR1 is a direct target of MYCN and its up-regulation induces cell proliferation by sensitizing the downstream signaling of several tyrosine kinase receptors. We here hypothesized that NLRR1 may play a physiological role in tumorigenesis and development and that it could be a potential therapeutic target.

Methods: To test our hypothesis, we have employed xenograft formation assay using NLRR1 stably expressing cells. NLRR1 knockout mice were generated by conventional targeting strategy. A set of novel hybridomas against NLRR1 were subjected to screening by growth inhibition assay.

Results: As overexpressed NLRR1 elevated cell proliferation in NB cells, NLRR1 overexpression enhanced tumor growth in mice with worse survival than control. To examine its physiological function in cell proliferation, we generated NLRR1-deficient mice and found that the deletion of Nlrr1 gene resulted in approximately 18% less body weight at weaning than control littermates. The growth defect was confirmed in MEFs showing less proliferation rate and phosphorylated ERK in NLRR1-deficient MEFs compared to wild-type. The reduced phospho-ERK was also observed in primary cultured neurons from NLRR1-deficient embryos, suggesting that NLRR1-deficient cells have less efficient signal transmission upon growth factors treatment, thereby impairing cell growth. Furthermore, the candidate monoclonal antibodies we generated against NLRR1 extracellular domain were examined by cell growth assay, showing that NLRR1 antibody suppressed cell growth in NB cells and potentiated EGFR inhibitor-mediated growth suppression.

Conclusions: These data suggest that NLRR1 acts as a key regulator of cell proliferation in NB and embryonic development. Growth inhibitory effect of NLRR1 antibody observed in this study points to a potential therapy of NB by targeting NLRR1.

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PARALLEL SESSIONS

HIGH RISK NEUROBLASTOMA BIOMARKERS LEADING TO THERAPY OR23 – OR30

OR23

BONE MARROW MONITORING BY AIPF – A PROGNOSTIC TOOL FOR HIGH RISK PATIENTS OVER 18 MONTHS OF AGE AT DIAGNOSIS

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Background: Stage 4 neuroblastoma (NB) still requires reliable therapy response criteria. The present pilot study aimed to examine the usefulness of the bone marrow (BM) monitoring during induction therapy using a highly specific and sensitive technique for tumour cell detection and verification.

Methods: BM samples from 80 Austrian stage 4 patients (age range: 0 to 239 months; median observation time: 8.2 years; 325 BM specimens) recruited in 3 consecutive prospective studies (A-NB87/94 and HR-NBL1/SIOPEN) between 1989 to 2010 met the inclusion criteria: availability and evaluability of BMs at diagnosis and given time points during treatment, investigation of at least 3x10e6 mononuclear cells and genetic information on the primary tumour. The BMs were tested with a fully automatic fluorescence based device combining GD2 based immunocytology and molecular-cytogenetic analyses of identical cells (automatic immunofluorescence plus FISH, AIPF).

Results: BM clearing after induction chemotherapy was achieved by 47 patients (58.8%) and was significantly associated with 5-year event-free survival (EFS) and overall survival (OS) in patients above 18 months at diagnosis (EFS: 66%+0.08 vs 21%+0.08; p=0.002; OS: 81%+0.07 vs 29%+0.09; p<0.001, Logrank test) but not in the 21 patients less than 18 months (EFS and OS for both: 86%+0.09 vs 57%+0.19; p=0.113). MNA was associated with BM clearing (p=0.02, Fisher's Exact Test). In the age group between 18 and 60 months, BM clearing was even more strongly linked to outcome (EFS: 72%+0.09 vs 14%+0.09, p<0.001; OS: 86%+0.06 vs 22%+0.11, p<0.001).

Conclusion: In Austrian stage 4 patients, BM clearing showed a statistically significant correlation with outcome in the age group over 18 months with a prognostic impact particularly pronounced between 18 and 60 months. This pilot study highlights accurate BM monitoring as an important tool for risk assessment currently under stringent evaluation within the ongoing HR-NBL1.5/SIOPEN study.

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OR24

Identification of ultra-high risk neuroblastoma by gene expression-based classification

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Background: High-risk neuroblastoma may consist of two distinct subgroubs: patients who benefit from current therapy protocols and patients in whom treatment will fail ("ultra-high risk neuroblastoma"). Prediction of the clinical course in these patients, however, has been difficult until now. In this study, a data driven machine learning approach was used to predict clinical outcome in high-risk neuroblastoma by transcriptomic characteristics.

Method: Gene expression microarray data for a cohort of 183 high-risk neuroblastoma samples were assembled (MYCN amplified, n = 89, MYCN non-amplified, n = 93, and one heterogeneous). Patients who had encountered an event within 36 months after diagnosis were labeled as unfavourable, whereas patients who survived event-free for >36 months were labeled as favourable (favourable, n = 55, unfavourable, n = 111, non-labeled, n = 17). This cohort was randomly divided into a balanced training (n = 111) and a test set (n = 55). Classifier training was done stepwise. In the first step simulated annealing was performed to select sets of features with high cross-validation accuracy. In the second step, a SVM ensemble was build based on the feature sets obtained in the first step.

Results: A gene expression-based ensemble classifier was defined comprising 385 genes. In the labeled test set, patients were classified with an accuracy of 74.6%. Survival analysis of the independent set (labeled and non-labeled, n = 72), revealed a clear separation of the respective survival curves (p=0.0152). Patients predicted to be favourable (n = 19) had a 5-year OS of 76.4% (CI = [0.584, 0.999]), and patients predicted to be unfavourable (n = 53) had a 5-year OS of 42.5% (CI = [0.298, 0.606]).

Conclusion: Our data indicate that patients who benefit from current therapies can be distinguished from "ultra-high risk" patients by specific transcriptomic signatures.

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OR25

Prominent Nucleolar Formation and N-myc/C-myc Protein Expression in Undifferentiated Neuroblastoma: Immunohistochemical study indicates the worst prognosis for the patients with C-myc positive tumors – A Report from the Children's Oncology Group

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Background: After analyzing157 Neuroblastoma, Undifferentiated subtype (NBUD) cases enrolled on the COG study (1/1/2000-8/31/2010), we previously presented their clinicopathological characteristics (2011 Spring Meeting, Society for Pediatric Pathology): They were rare (4.5% of all cases), classified into the Unfavorable Histology group, frequently diagnosed over 18 months of age (75%) with clinical stage 4 (82%), and associated with MYCN amplification (83%), diploid pattern (63%), and 1pLOH (72%). Patients with NBUD had a poor prognosis and their survival rates (48.4±5.0% 3-year EFS, 56.5±5.0% 3-year OS) were not significantly influenced by clinical factors and chromosomal abnormalities. Surprisingly, however, MYCN-amplified tumors had a significantly (p=0.0248) better 3-year EFS (53.4±5.6%) than MYCN-non-amplified tumors (31.7±11.7%).

Study Design: Among 157 cases, 68 tumors (48 MYCN-amplified, 20 MYCN-non-amplified) were available for immunostaining for N-myc and C-myc protein. Results were analyzed with presence or absence of prominent nucleoli of the tumor cells and prognosis of the patients.

Results: MYCN-amplified tumors often had prominent nucleoli (39/48, 81%) and expressed N-myc protein (42/48, 88%). C-myc protein expression was almost exclusively found in MYCN-non-amplified tumors, and significantly associated with prominent nucleoli: 8/11 tumors were positive with prominent nucleoli vs. 8/9 were negative without prominent nucleoli (p<0.009). One MYCN-amplified tumor expressed both proteins. The patients with C-myc protein positive tumors had a significantly poor prognosis (N=8, 12.5±11.7% 3-year EFS) compared to the patients with N-myc protein positive tumors (N=39, 49.9±17.7% 3-year EFS) and with both C-myc/N-myc protein negative tumors (N=15, 70.0±17.1% 3-year EFS) (p=0.0029).

Conclusions: C-myc protein expression indicated the worst prognosis of the patients in NBUD. Results of this study are prompting us to perform systematic nuclear and immunohistochemical analysis on neuroblastoma tumors in other subtypes, and also to critically investigate molecular mechanism of C-myc expression, besides N-myc expression, leading to aggressive behavior of this disease.

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OR26

Tandem high-dose chemotherapy (HDC) with Thiotepa and Mel-Bu and autologous stem cell transplantation (ASCT): the way to improve very high risk neuroblastoma patients prognosis ?

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Background: The administration of Bu-Mel and ASCT has been recently demonstrated to allow the best prognosis for patients with high-risk neuroblastoma (HR-NBL-1 SIOPEN Study). However, patients with less than a partial remission after 2 lines of conventional chemotherapy and adolescents are considered as very high risk (VHR) patients.

Methods: We developed an intensified HDC strategy with 2 courses of HDC to improve VHR patients prognosis. The first course consisted in thiotepa (300 mg/m2/d x3) followed by ASCT. In absence of major toxicity or disease progression, a 2nd course of melphalan (140 mg/m2) Busulfan (600 mg/m2) and ASCT was administered 2 months later.

Results: From April 1986 to April 2009, 22 patients (12 males, 10 females), median age 3.5 y (0.9-15.9) entered this programme. 20/22 had less than a partial remission after conventional chemotherapy. 2 were adolescents with a metastatic CR, 1 had a MYCN amplified tumour. Thiotepa-related toxicity was mainly digestive with a grade >2 mucositis and diarrhoea in 14 and 16 patients, respectively. Hospitalisation duration was 25 days (19-49). Mel-Bu was administered in 18 patients since 4 patients had a progressive disease after thiotepa. Toxicity was digestive with a grade >2 mucositis and diarrhoea in 13 and 7/18 patients, respectively and hepatic with 6/18 hepatic veno occlusive disease. Toxic related death occurred in 1 patient due to alveolar haemorrhage. The 3-year EFS survival is 42.6% (24-64).

Conclusions: This intensified HDC strategy in VHR patients seems to be feasible and to improve survival. It will be compared to a combined mIBG-Mel-Bu strategy in the future VHR neuroblastoma European Protocol.

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OR27

Haploidentical Natural Killer Cells plus Monoclonal Antibody 3F8 for Resistant High-Risk Neuroblastoma: Preliminary Results of an Ongoing Phase I study

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Background: Natural killer (NK) cell-mediated antibody-dependent cellular cytotoxicity (ADCC) is a potent mechanism of 3F8 activity against neuroblastoma (NB). KIR and HLA genotypes define NK activity and are key prognostic markers in 3F8-treated patients. NK-cells are depleted by standard NB chemotherapy, but when rescued by allogeneic NK transfusions, can be optimized for anti-NB cytotoxicity by selecting NK donors for maximum NK ADCC: from either licensed NK-cells responding to "missing self" or from unlicensed NK-cells responding to "missing ligand".

Methods: We initiated a phase I study of the combination of haploidentical NK-cells and anti-GD2 antibody 3F8 for the treatment of refractory or recurrent high-risk NB (www.clinicaltrials.gov NCT00877110). The primary objective was to determine the maximum tolerated NK-cell dose (MTD). Secondary objectives included assessing anti-NB activity and its relationship to KIR/HLA genotypes, NK function, and NK chimerism. Eligibility criteria included availability of ≥2x106 CD34+ autologous cells/kg. Patients received a lymphodepleting regimen of high-dose cyclophosphamide, topotecan and vincristine (days 1-3) prior to infusion (day 5) of NK-cells isolated from donor leukopheresis using a process of CD3-depletion (to <2x104 CD3+ cells/kg) followed by CD56-enrichment. 3F8 (20mg/m2/day) was administered on days 8-12. NK-cell dose-escalation occurred in the absence of dose-limiting toxicity (DLT). Cytoreduction-related side effects were not considered DLT.

Results: Ten patients have been treated thus far: 8 at dose-level 1 (1-4.99x106 CD56+cells/kg) and 2 at dose-level 2 (5-9.99x106 CD56+cells/kg). MTD has not yet been reached. One patient at dose level 1 developed DLT: grade 3 vomiting and hypertension. No other >grade 2 unexpected therapy-related toxicities were encountered. Neither GvHD nor myeloablation requiring stem cell rescue was observed. 2 patients achieved complete response.

Conclusion: Preliminary results from this first-in-human trial of NK-cells plus antibody against solid tumors suggest that the combination is safe following cytoreduction and may be effective for some patients with high-risk NB.

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OR28

Dose individualisation of 13-cis-retinoic acid in high-risk neuroblastoma patients based on pharmacological exposure – a national UK study

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Background: 13-cis-retinoic acid is an established part of high-risk neuroblastoma treatment. We have previously shown significant variation in 13cisRA pharmacokinetics, with many patients achieving potentially sub-therapeutic exposures. The current study investigated the feasibility of pharmacokinetic dose individualisation to achieve 13cisRA plasma concentrations >2μM in all patients.

Methods: 13cisRA (160mg/m2 or 5.33mg/kg/day for <12kg) was administered to 70 children and plasma concentrations determined at 0-6h on day 14 of course 1. Dose increases of 25-50% were implemented on course 2 for patients who achieved peak plasma concentrations <2μM, with concentrations again analysed on course 2.

Results: 13cisRA was extracted from capsules and administered with food in 39 patients and by nasogastric tube in 17 patients, with 14 patients able to swallow capsules. Peak plasma concentrations on course 1 ranged from 0.42-11.2μM, with 23 patients (33%) failing to achieve concentrations >2μM. Dose increases were carried out in 20/23 patients, with concentrations >2μM achieved in 18 patients (90%) at higher dose levels. The patient cohort included 9 patients <12kg, receiving a reduced dose of 5.33mg/kg, with 6 (67%) of these patients failing to achieve plasma concentrations >2μM. Dose increases implemented in all 6 of these patients led to concentrations >2μM and were well tolerated. All 14 patients who swallowed 13cisRA capsules achieved plasma concentrations >2μM, as compared to 21/39 (54%) and 12/17 patients (71%) when the drug was extracted and mixed with food or administered via nasogastric tube, respectively. A mean peak plasma concentration of 4.3±2.5μM was observed in patients able to swallow capsules versus 2.4±1.4μM for patients extracting the drug and mixing the contents with food (p=0.0076).

Conclusions: Data indicate the feasibility of 13cisRA dose individualisation and suggest that reduced dosing should not be implemented for children <12kg. Increased dose levels may be beneficial for children unable to swallow 13cisRA capsules.

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OR29

High-dose 131I-MIBG treatment incorporated into tandem HDCT/autoSCT for high-risk neuroblastoma: Preliminary results of SMC NB-2009 study

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Background: The strategy using tandem HDCT/autoSCT for high-risk neuroblastoma in which TBI was incorporated into the second HDCT/autoSCT (SMC NB-2004 study) demonstrated a very encouraging survival rate. However, most survivors experienced significant short- and long-term toxicities associated with tandem HDCT/autoSCT, particularly TBI. Therefore, we incorporated high-dose 131I-MIBG treatment into the second HDCT/autoSCT instead of TBI from 2009 (SMC NB-2009 study) to reduce toxicities without jeopardizing survival rate. Otherwise, there was no difference in treatment plan between the two studies.

Methods: From 2009 to 2010, 24 consecutive patients with high-risk neuroblastoma were assigned to receive tandem HDCT/autoSCT after 9 cycles of induction chemotherapy. CEC (carboplatin + etoposide + cyclophosphamide) and TM (thiotepa + melphaln) with (for stage 4) or without (for stage 3) high-dose 131I-MIBG treatment were used as the first and second HDCT regimen, respectively. High-dose 131I-MIBG (12 or 18 mCi/kg) was infused on day -21 of the second HDCT/autoSCT. Local radiotherapy, differentiation therapy with 13-cis-retinoid acid, and immunotherapy with IL-2 were given after tandem HDCT/autoSCT. Hematologic recovery, acute toxicities during the second HDCT/autoSCT, and survival rate were compared between NB-2004 and NB-2009 studies.

Results: All but 2 patients who died from myocarditis during the first HDCT/autoSCT completed tandem HDCT/autoSCT as scheduled. There was no significant immediate toxicity during 131I-MIBG infusion. There was no significant difference in hematologic recovery between the two studies. The duration of high fever was shorter (P = 0.003) and frequencies of grade 3/4 stomatitis and diarrhea were lower in NB-2009 study than in NB-2004 study (P = 0.031 and 0.032, respectively). There was no difference in 2-yr EFS (83.7 +/- 5.3% versus 80.4 +/- 9.0%, P = 0.805).

Conclusions: High-dose 131I-MIBG treatment incorporated into tandem HDCT/autoSCT was feasible and acute toxicities were less severe in NB-2009 study than in NB-2004 study.

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The role of surgery in the treatment of stage 4 neuroblastoma patients older than 18 months of age

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Background: The impact of primary tumor resection on outcome of stage 4 neuroblastoma patients is still discussed controversial.

Methods: Stage 4 neuroblastoma patients of the prospective clinical trial NB97 were included in this analysis when they were 18 months or older at diagnosis. In addition to clinical data in the trial database, operation notes and imaging reports were reviewed by two independent investigators. Image defined risk factors were assigned retrospectively. The extent of tumor resections was correlated with local control rate and outcome.

Results: A total of 278 patients were included. The median observation time was 9.1 years. The presence of image defined risk factors did predict the extent of tumor resection at first (p<0.001) and most radical operation (p<0.001). No patient died from surgery. Prior to chemotherapy, complete resection was achieved in 6.1% of patients, incomplete resection in 5.0%, and biopsy or no surgery was done in 88.5%. The extent of initial resection had no impact on event free survival (p=0.207), local progression free survival (p=0.195), and overall survival (p=0.351). During induction chemotherapy, 54.7% of patients finally underwent complete resection of the primary tumor, 30.6% incomplete resection, and 13.3% had only biopsy or no surgery of the primary tumor. The extent of best resection had also no impact on event free survival (p=0.877), local progression free survival (p=0.299), and overall survival (p=0.778). Moreover, multivariate analysis including the variables "complete vs. incomplete/no resection", "MYCN normal vs. amplification", "high-dose chemotherapy with stem cell transplantation no vs. yes", and "radiotherapy of primary tumor site no vs. yes" demonstrated no independent impact of surgery on event free survival and overall survival.

Conclusion: In metastatic neuroblastoma patients older than 18 months at diagnosis, surgery of the primary tumors site has no impact on outcome.

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PARALLEL SESSIONS

SIGNALING, APOPTOSIS, AND TELOMERES OR31 – OR38

OR31

Mitochondrial Bcl-2 family dynamics define therapy response and resistance in neuroblastoma

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Background: Patients with neuroblastoma (NB) succumb to chemoresistant disease due to deregulated mitochondrial apoptosis. BH3 functional profiles of NB mitochondria infer Bcl-2 protein addiction patterns and predict sensitivity to the Bcl-2/-xl/-w antagonist ABT-737 in vitro. We now directly identify the heterogeneous BH3 protein:Bcl-2-family member interactions responsible for survival in NB cell lines and tumors, and define their role in relapsed disease and in vivo response to ABT-737.

Methods: Coimmunoprecipitation (CoIP) of NB cell lines and primary tumors identified operative BH3:Bcl-2 protein interactions. NB xenografts (XG) were treated with ABT-737 alone or in combination with non-curative cyclophosphamide (CPM). CoIP, mitochondrial cytochrome c release, whole cell mitochondrial JC1 release, Bax/Bak oligomerization, and 2D mitochondrial proteome analyses were performed on isogenic paired NB cell lines from the same patient tumor at diagnosis and at relapse post-chemotherapy (n=5 pairs) to characterize resistance mechanisms.

Results: Viable NB cell lines and primary tumors at diagnosis are primed for death with the BH3 protein Bim bound to either Bcl-2 or Mcl-1. ABT-737 has single agent activity against Bim:Bcl-2 primed XGs with durable complete responses achieved with ABT-737 and CPM combination, even for highest-risk NBs with MYCN amplification and ALK mutation, but not against Bim:Mcl-1 XGs. In vitro selection for ABT-737 resistance showed this occurs through increased Mcl-1 and transition from Bim:Bcl-2 to Bim:Mcl-1 priming despite abundant Bcl-2. Lastly, we demonstrate that therapy resistance in relapsed NB is not mediated by upregulated Bcl-2 homologues nor loss of Bim priming, but by repressed Bak/Bax activation at the mitochondria.

Conclusions: These studies provide a classifier that can identify NBs susceptible to Bcl-2 antagonists such as ABT-737, define Mcl-1 as the principal mediator of ABT-737 resistance at diagnosis, and isolate the therapy resistant phenotype to the mitochondria that correlates with the profound multi-agent resistance seen clinically at relapse.

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OR32

A synthetic-lethal siRNA screen identifies the spliceosome as a key Mcl1 regulator and therapeutic target in neuroblastoma

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Background: Neuroblastomas (NB) are tonically primed for death with activated Bim (not Puma or Bid) sequestered to specific pro-survival Bcl2-homology proteins, blocking apoptosis. ABT-737 (ABT) is a small molecule Bcl2 antagonist that completely regresses NB xenografts that have a Bim:Bcl2 profile (in vitro IC50 20 nM). ABT does not antagonize Mcl1, so NBs with a Bim:Mcl1 profile are resistant in vitro (IC50 >2 uM) and in vivo, but potent ABT activity is restored following siMcl1.

Methods: We therefore undertook a synthetic-lethal screen to identify Mcl1 regulators using a 98 gene deubiquitinating enzymes (DUB) siRNA library, as Mcl1 is highly regulated via ubiquitin-mediated processes. We treated Mcl1-dependent NB cells (IMR5 and NLF) with siRNA targeting each DUB and ABT at a sublethal concentration (200 nM), measured viability by ATP content, and sought synthetic-lethal interactors.

Results: We identified 3 targets (PRPF8, UBL5, PSMD14) that demonstrated synthetic lethality (Z<-3) that were selective for Mcl1-dependent NBs, verified by measuring ABT IC50 (>10-fold decrease with siRNA), and validated as specific interactors (>10-fold IC50 decrease for multiple independent siRNAs for each target), and could be rescued with expression of an siRNA-resistant plasmid. Knock-down (kd) for each decreased Mcl1 function, while UBL5 and PRPF8 (decreased) and PSMD14 (increased) Mcl1 protein. PRPF8 and UBL5 are members of an alternative spliceosome complex, along with SART1. SART1 kd phenocopied PRPF8 and UBL5 kd, and kd of all three together markedly potentiated synthetic-lethality in Mcl-dependent NBs but was non-toxic to Bcl2-dependent NBs.

Conclusions: We identified multiple members of a single alternative spliceosome complex (PRPF8, UBL5, SART1) that support Mcl1 activity in NB. Our work provides a novel target to restore ABT activity, while demonstrating the robustness of this functionally-based synthetic-lethal screen to identify modulators of Mcl1.

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OR33

CASZ1 suppresses neuroblastoma growth by recruiting epigenetic modifier NuRD and restoring Rb activity

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Background: The zinc finger (ZF) transcription factor, CASZ1 is a neural fate-determination gene. It's also a chr1p36 neuroblastoma (NB) tumor suppressor gene which suppresses tumor growth through reprogramming gene expression. How CASZ1 regulates gene transcription remains unclear. Since epigenetic regulation controls developmental programs during embryogenesis and is dysregulated during tumorigenesis, we postulate that CASZ1 reprograms gene expression through epigenetic mechanisms to suppress tumor growth.

Methods: CASZ1 binding proteins were identified by immuno-precipitation (IP) and mass spectrometry (MsSpec). The effect of CASZ1 and its variants on cell growth was assessed by soft agar colony formation and tumor growth in mice.

Results: MsSpec results indicate CASZ1 binds to the nucleosome remodeling and histone deacetylase (NuRD) complex. NuRD subunits (HDAC1/2, MTA1/2/3, MBD3 and LysineSpecificDemethylase1 (LSD1)), with high levels of HDAC1 and LSD1 linked to drug resistance and poor prognosis respectively in NB. Co-IP of CASZ1 mutants demonstrated that the CASZ1 N-terminus (AA 31-185) binds all of NuRD subunits except LSD1 which binds the ZF region. CASZ1 fused to an artificial DNA-binding domain (GAL4DBD) caused a 4-fold repression of transcription (5xUAS-luciferase assay). Treatment with an HDAC inhibitor blocks CASZ1 transcriptional repression indicating NuRD is required for CASZ1 transcriptional activity. In NB, CASZ1 inhibits in vitro and in vivo tumor growth yet deletions of NuRD or LSD1 binding regions significantly lose xenograft growth-inhibiting activity. Restoration of CASZ1 activates the Rb pathway causing a 60% decrease in pRb phosphorylation and 80% reduction of E2F transcriptional activity. Since LSD1 promotes cancer cell cycle progression by deactivating Rb pathway, our result indicates that CASZ1 restores Rb activity by suppressing LSD1.

Conclusions: CASZ1 recruits chromatin regulators NuRD and LSD1 to regulate gene transcription and suppress NB growth. Thus loss of CASZ1 in neuroblast precursors contributes to dysregulation of the epigenome and a failure to implement a differentiation program.

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OR34

RNA helicase A is essential for 1p36 gene KIF1Bβ tumor suppression in neuroblastomas.

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Background: Developmental apoptosis of neuronal precursors is crucial in determining the final number of terminally differentiated cells. During neural development, cells undergo apoptosis as growth factors such as NGF becomes limiting. Abnormal NGF signaling or aberrant developmental apoptosis is implicated in pediatric nervous system tumors. Several genes act upon a developmental apoptotic pathway that is activated when NGF becomes limiting for neuronal progenitors and requires KIF1Bβ. KIF1Bβ is necessary and sufficient for neuronal apoptosis during NGF withdrawal. KIF1Bβ maps to 1p36.2, a region that is frequently deleted in neural crest-derived tumors including neuroblastomas.

Methods: Large-scale immunoprecipitation followed by mass spectrometry, cloning and mutagenesis studies, apoptosis assays, immunofluorescence, lentiviral expression or shRNA-based studies, siRNA silencing, RNA-SEQ, RT-PCR, immunohistochemistry, NGF withdrawal experiments, patient samples and mouse xenograft models are the key methods used.

Results: We identified a transcriptional basis for KIF1Bβ-induced cell death, which requires a RNA/DNA helicase known as RNA helicase A (DHX9). KIF1Bβ interacts with DHX9 to promote translocation of cytoplasmic DHX9 into the nucleus, resulting in transcription of apoptotic XIAP-associated factor 1 (XAF1). Transcription-impaired or nuclear localization-impaired DHX9 is unable to potentiate KIF1Bβ-induced cell death. Knockdown of DHX9 also protects from KIF1Bβ-induced cell death whereas KIF1Bβ negative mutant is unable to translocate cytoplasmic DHX9 into the nucleus. Furthermore, silencing of XAF1 protects from KIF1Bβ-induced cell death. In addition, a genome-wide shRNA library loss-of-function screen revealed a DHX9-interacting transcription factor ZIC2 that is deemed crucial for KIF1Bβ-induced apoptosis. This further suggests a DHX9-dependent transcriptional program initiated by KIF1Bβ to induce apoptosis in neuroblastomas.

Conclusion: Recent literature strongly pointed to KIF1Bβ as a bonafide tumor suppressor. Our findings provide a mechanistic understanding of this role, whereby KIF1Bβ interacts with cytoplasmic DHX9 leading to its accumulation in the nucleus to initiate a unique transcriptional signature that includes apoptotic effectors such as XAF1.

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OR35

LSD1 histone demethylase expression interferes with stress response and DNA repair in a p53-dependent manner in human neuroblastoma cell lines

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Background: Aberrant regulation of histone demethylases is an inherent feature of many cancer types. In neuroblastoma (NB), expression of the histone-modifying enzyme, lysine-specific demethylase 1 (LSD1), correlates with poor prognosis and is inversely correlated with differentiation in neuroblastic tumors. LSD1 also demethylates nonhistone proteins including the p53 tumor suppressor, thus regulating p53 activity. We hypothesized that LSD1 modulation might affect the cellular response to cytotoxic treatment and DNA double-strand break (DSB) repair in a p53-dependent manner.

Methods: The effects of siRNA-mediated LSD1 knockdown on cell viability and the cell cycle after treatment with cytostatic agents or ionizing radiation were assessed by proliferation assays and FACS analysis, respectively. Capacity for DSB repair following LSD1 knockdown was assessed by counting γH2AX-positive foci. LSD1-mediated regulation of gene expression of essential DSB repair genes was dissected by analyzing gene expression data and using quantitative ChIP-PCR.

Results: Cell cycle analysis in p53 mutant and MYCN-amplified NB cells showed an increase of apoptosis and G2 phase prolongation upon LSD1 knockdown. Lower expression of LSD1 correlated with elevated sensitivity to cisplatin, doxorubicin and etoposide in these cells. LSD1 knockdown caused sensitization to ionizing radiation only in p53 mutant cells, and DSB repair capacity was altered with LSD1 expression. LSD1 expression correlated inversely with expression of the DSB repair core factors, XRCC5, XRCC6 and Ligase IV in SY5Y cells. ChIP-PCR showed that H3K4 methylation increases with decreasing LSD1 expression and that the XRCC5 promoter is a direct target of LSD1.

Conclusions: Our data provide first evidence that the LSD1 histone demethylase is involved in the cellular response towards cytostatic treatment or ionizing radiation in NB cells and in maintaining the malignant phenotype by interfering with DSB repair. Further analysis of histone demethylases and their regulation might help to develop new strategies in improving prognosis and therapy of neuroblastoma.

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OR36

Alternative Lengthening of Telomeres (ALT) in neuroblastoma tumors

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Background: Cancer cells acquire unlimited proliferation by avoiding telomere shortening through the activation one of two telomere maintenance mechanisms: telomerase or homologous recombination-based ALT. While telomerase has been widely examined in neuroblastoma (NB), ALT has not been definitively studied due to the lack of an ALT activity assay until recently.

Methods: The C-circle (CC) assay, the first rapid ALT activity assay, detects CC (extrachromosomal partially single-stranded telomeric circles) in ALT cells. A novel telomere qPCR-based method was developed to measure telomeric content and CC levels using 30ng tumor DNA.

Results: In a cohort of 90 NB (53 high-risk (HR); 37 non-HR), we found 12% (n=11) of tumors to be ALT+(CC+). MYCN amplification was present in 0% of ALT+ and 32% of ALT- tumours (P=0.03). ALT was most prevalent (25%) in HR/MYCN-non-amp NB. CC+ tumors had greater telomeric content than CC- tumors (median 61 vs. 9; P<0.0001), consistent with the ALT phenotype of long telomeres. However, not all tumors with long telomeres were CC+; only 36% with long telomeres were CC+. Age at diagnosis was significantly older for ALT+ than ALT- NB (median 4.3 vs. 1.6yr; P<0.0001). For HR/MYNC-non-amp tumors, 5-yr OS for ALT+ group (n=7) was 0% (6 deaths; 1 patient lost to follow-up at 2.8y) and 52% for ALT- group (P=0.15). 5-yr OS for MYCN-amp group (n=25) was 27%. For non-HR/CC+ tumors (n=4), only 1 patient with the highest CC levels died. Time from diagnosis to death was also longer for ALT+ than ALT- NB (median 2.9 vs. 1.0yr; P=0.003).

Conclusions: We provide the first definitive evidence of ALT in NB tumors. CC, a reliable marker of ALT activity, is prognostic in NB. HR/ALT+ tumors have very poor outcome and may be considered for novel therapies. Follow-up of ALT+ tumors needs to be tailored for late events.

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OR37

LONG OR UNCHANGED TELOMERES PREDICT RECURRENCE IN NEUROBLASTOMA

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Background: Telomere maintenance is one of the hallmarks of cancer. How telomere length (TL) is regulated in neuroblastoma is not completely understood. Here we have investigated the prognostic value of telomere length focusing on localized relapsing neuroblastoma tumors.

Methods: Ninety Italian and eighteen Spanish primary neuroblastoma patients (fourteen stage 1, twenty-two stage 2, twenty-eight stage 3, thirteen stage 4s, and thirty-one stage 4 tumors) diagnosed from 2000 to 2008 were studied. Thirty-nine of whom had relapsed. Altogether, localized neuroblastoma samples were 64/108. TL was investigated by quantitative fluorescence in situ hybridization (Q-FISH) that allows to analyze individual cells in paraffin embedded tissues. The fluorescence intensity of chromosome 2 centromere was used as internal control to normalize TL values with ploidy.

Results: In all cases tested, short telomeres were predictive of favorable prognosis, whereas long or unchanged telomeres were predictive of poor outcome. There was a significant correlation between long or unchanged TL and relapse ($p<0.0001$) in all stages. Relapsed cases: N= 39; median = 538.1 (1°-3° q: 473.8-567.6), not relapsed cases: N= 69; median = 388.9 (1°-3° q: 354.6-534.8). When localized neuroblastoma cases were considered, EFS and OS were significantly higher in patients with short vs long or unchanged telomeres ($p=0.0007$ and $p=0.0004$, respectively). All patients belonging to the latter group relapsed in 24 months. There was heterogeneity in TL into individual tumors. In particular, 34 tumors contained in variable proportions two subpopulations of cells with differing TL. The presence of elongated telomeres in a subset of neuroblastoma cells (at least 50%) predicted recurrence of disease.

Conclusions: TL predicts tumor recurrence in neuroblastoma patients with localized disease. Identification of a relevant proportion of NB tumor with two subsets of cells with different TL opens up new perspectives in the study of telomere biology as prognostic tool for neuroblastoma.

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OR38

Manipulating the Rac/Rho GTPase signalling in Neuroblastoma cells restores neuritogenesis and inhibits proliferation.

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Background and Methods: The genomic landscape of neuroblastoma is starting to unravel through whole genome sequencing of large groups of tumors. We discovered neuritogenesis gene alterations to be one of the new molecular defects in high stage neuroblastoma. Although few recurrent amino-acid-changing mutations are found, we identified an enrichment of affected genes involved in the gene ontology category "regulation of GTPase activity" (Molenaar et al., Nature, in press).

Results: Detailed investigation of this gene category showed a list of GEFs (guanine nucleotide exchange factors) and GAPs (GTPase activating proteins) mutated in high stage neuroblastoma. The mutated GEFs and GAPs specifically regulate Rac and Rho proteins, which play a major role in control of neuritogenesis in neuronal cells. Rac signalling stimulates axon extension and guidance, while Rho signalling causes collapse of neuronal growth cones and axon retraction. The mutations in GEFs and GAPs identified in neuroblastoma are such that signalling through Rac is inhibited and Rho signalling is activated. As a result, neuronal growth cone collapse would be stimulated in neuroblastoma. Rho-activation and the resulting inhibition of neuritogenesis might therefore drive neuroblastoma formation. Rho signalling is mediated by the downstream effector Rho kinase (ROCK), which can be inhibited with small molecule drugs. Inhibition of ROCK in neuroblastoma cell lines resulted in an induction of neuronal outgrowths and inhibition of proliferation. In some cases treatment of neuroblastoma cells in combination with Retinoic Acid showed a synergistic differentiating effect.

Conclusions: We identified multiple alterations in GTPase-regulating genes in high stage neuroblastoma leading to either activation of Rho or inactivation of Rac. Restoration of the balance of Rac/Rho signalling can stimulate neuritogenesis, induce differentiation of neuroblastoma cells and therefore constitutes a means to inhibit progression of neuroblastoma.

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PARALLEL SESSIONS

CLINICAL AND BIOLOGICAL RISK FACTORS AND MRD

OR39 – OR46

OR39

Quantification of bone marrow disease in high risk neuroblastoma patients by anti-GD2 immunocytochemistry – impact on survival. A SIOPEN High Risk Study.

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Background: Bone marrow infiltration frequently occurs in patients with metastatic neuroblastoma and is routinely analysed with trephine biopsies and conventional cytomorphology performed on bone marrow smears before and after chemotherapy. The SIOPEN High Risk Study includes a prospective analysis of tumor cell infiltration using anti-GD2 immunocytochemistry (ICC) at diagnosis, after induction chemotherapy, prior to myeloablative therapy and at the end of treatment.

Objective: (1) to evaluate the prognostic impact of the number of infiltrating tumor cells in bone marrow at diagnosis and after induction chemotherapy and (2) to compare the results of anti-GD2 ICC on bone marrow aspirates with those of conventional cytomorphology and trephines.

Methods: Mononuclear cells were prepared from 400 bone marrow aspirates, stained with anti-GD2 and evaluated according to international consensus guidelines (Beiske et al, BJC, 2009). Not only the numbers of GD-2-positive tumor cells, but also the numbers of investigated bone marrow cells were recorded. Time to death (overall survival OS) and time to progression (EFS) were analyzed using the Kaplan-Meier method. ROC analysis was applied to evaluate possible cut-offs for continuous predictors.

Results: We analyzed the prognostic impact of tumor cell infiltration at similar levels of sensitivity, i.e. in samples containing comparable numbers of investigated bone marrow cells. ROC analyses of the sensitivity and specificity of various numbers of tumor cells to predict EFS and OS showed that only BM samples containing more than 2E+06 bone marrow cells provided prognostic information. At diagnosis, the number of infiltrating GD2-positive tumor cells was prognostic of EFS ($p=0,005$) and OS ($p=0,028$) as it was for bone marrow positivity by conventional cytomorphology (EFS $p=0,035$, OS $p=0,031$) and trephines (EFS $p<0,001$, OS $p=0,011$). However, after induction, only ICC results were statistically significantly prognostic of OS ($p=0,044$) while cytomorphology and trephines were not. Prediction of EFS by ICC at the end of induction was borderline
significant ($p=0,06$), but could be improved when both ICC and trephine data were taken into account ($p=0,004$).

Conclusion: Compared to cytomorphology and trephines, only anti-GD2 ICC is predictive of OS after induction chemotherapy. The strongest prediction of EFS was at this time point achieved by combining data from ICC and trephines. It is mandatory to analyse more than 2E+06 bone marrow cells in order to obtain a prognostically informative result. The ICC assay may be helpful to identify patients at ultra-high risk when the clinical criteria for this subgroup are clarified.

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OR40

Clinical Relevance of Positive Marrow MRD following 2 Cycles of Anti-GD2 Immunotherapy

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Background: With the proven clinical benefit of anti-GD2 antibody therapy in eradicating minimal residual disease (MRD), it is imperative to determine the appropriate time to attain prognostically significant MRD measurements.

Methods: 169 children with high-risk stage 4 neuroblastoma (1988-2008) in first remission were enrolled in consecutive adjuvant protocols using anti-GD2 monoclonal antibody 3F8±GM-CSF±13-cis-retinoic acid following induction chemotherapy. Their pretreatment and post-cycle#2 bone marrows (BM) were tested for MRD using a marker panel (B4GALNT1, CCND1, ISL1, and PHOX2B) by quantitative(q) RT-PCR. MRD positivity was defined as any one of 4 markers being positive, and negativity as all 4 markers being negative. Survival probabilities were estimated using Kaplan-Meier method and compared by the log-rank test.

Results: 86% (145/169) of patients had histologic BM involvement at diagnosis. Although histology was negative in all pre-immunotherapy BM (Pre-MRD), 33% were positive by molecular markers. Pre-MRD marker status (negative vs positive) was not prognostic for PFS (51±5% vs 49±7%) or OS (60±5% vs 59±8%). In contrast, marker status after 2 cycles of immunotherapy (Post-MRD), at a median of 3.1 months from start of treatment, was highly prognostic of PFS (63±4% vs 3±3%) and OS (72±5% vs 8±7%), p<0.001, irrespective of treatment era or immunotherapy regimen. Among patients whose Post-MRD was negative, 36% relapsed as defined by INSS-INRC criteria (relapse pattern being 54% non-BM, 24% CNS, and 22% BM plus other sites). 90% of early relapses (<18m from start of treatment) among patients with negative Post-MRD occurred outside the BM. When late relapses (n=23) were excluded from analysis, PFS and OS for negative Post-MRD patients were 74±4% and 81±4%, respectively.

Conclusions: The optimal time to achieve early prognostic MRD measurements by qRT-PCR is to test post-cycle#2 BM during immunotherapy. Molecular detection is less predictive when relapse involves non-BM sites or when relapse is ≥18m after treatment.

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OR41

Prospective Evaluation of MYCN Gene Amplification Status using Serum DNA of Neuroblastoma Patients

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Background: We previously showed that serum-based evaluation of MYCN gene amplification status (MNA status) can be used to determine the risk classification. Here we analyzed serum MNA status prospectively using patients' sera obtained before the initial diagnosis.

Patients and Methods: A total of 59 patients with NB were enrolled in the study. Sera were collected prior to tumor biopsy. Contaminating WBC was immediately removed to prevent their interference with determination of DNA-based MNA status. The serum MYCN/NAGK ratio was assessed for all cases; MNA was judged when the ratio was 5.0 or higher. Biopsied tumor samples were assessed by I-FISH.

Results: The serum MYCN/NAGK ratio was assessed for all cases. Of the 59 patients, 42 received primary biopsy before initial treatment, seven received delayed primary biopsy after the start of treatment, and 10 did not receive a biopsy. Thus far, 49 tumor samples were available for the assessment of tumor MNA status. Obviously, for the 49 patients that had both serum and tumor samples analyzed, the serum MNA status completely matched with those using tumor samples assessed by I-FISH (Mann-Whitney U test; p<0.001). Seventeen patients who did not received a primary biopsy were determined their risk classifications based on their age, INSS stage and serum MNA status, and decided on a course of treatment. Importantly, four of 17 patients who were judged "low-risk" NB and met the entry criteria were enrolled onto a "wait-and see" study without a biopsy during the course.

Conclusion: Our data demonstrate that serum-based MNA status could predict tumor MNA status precisely, and has clinical benefits in predicting risk stratification for the patients who have some problems to obtain their tumor samples. Now we are trying to apply this preoperative evaluation of serum MNA status to the new "wait-and-see" study for the low-risk NB patients.

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OR42

Combining gene set signatures and public databases identified a 20-gene predictor of survival in stage 4 neuroblastoma

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Background: Gene expression profiling using microarray technologies provides a powerful approach to predict the outcome of disease. A number of gene signatures which could classify tumors and predict clinical outcomes of neuroblastoma (NB) has been published. These gene signatures share a low number of genes.

Methods: We developed and validated a specific-stage 4 signature using the genes included in three published signatures (Oberthuer et al., 2006, Asgharzadeh et al., 2006, Vermeulen et al., 2009), and gathered by Medline search (100 papers analyzed) and by reanalysis of public microarray data from retinoic acid-treated NB cells (GSE9169). Four hundred genes were selected for further analyses. To find the optimal combination of genes able to predict survival, Cox regression and area-under-the curve (AUC) analyses were performed in two independent microarray datasets (GSE2446 and R2: microarray analysis and visualization platform (http://r2.amc.nl)) composed of 102 and 40 patients with stage 4 NB, respectively.

Results: A 20-gene signature, including genes only from the published signatures and Medline search, resulted to be an independent predictor of survival. The survival prediction ability of this signature was validated in four independent public microarray datasets of stage 4 NB (Kaplan Meier; P=9.1x10-17, P=2.0x10-3, P=1.0x10-4, P=6.5x10-10). The AUC analysis demonstrated that this 20-gene predictor carefully prognosticated patients' outcome better than other three published gene signatures in three datasets. Finally, in the same datasets, the 20-gene signature increased the prediction value of the well-known clinical markers such as age and MYCN status (increase of AUC= 6.1%, 12.9%, 10%).

Conclusions: We have generated a reliable 20-gene predictor with a constant ability to foresee, across diverse datasets, survival in patients with stage 4 NB. This could be useful to identify patients whose tumors are characterized by a less or more aggressive behavior, and thereby an appropriate therapy.

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OR43

Outcome Analysis of Non-high-risk Neuroblastoma Patients Enrolled on Children's Oncology Group Trials P9641 and A3961

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Background: Patients with non-high-risk (low- and intermediate-risk) neuroblastoma generally have excellent event free (EFS) and overall (OS) survival with current therapy. However, within this heterogeneous population, there are patients that may benefit from further refinement of therapy.

Methods: Survival tree regression analysis was performed in 1407 patients enrolled on P9641 and A3961 with OS the primary endpoint. Univariate Cox proportional hazards models determined statistically significant prognostic factors. Secondary analysis of cases with MYCN non-amplified, non-high-risk neuroblastoma classified patients by age, INSS stage, Shimada histology and genomic features to determine 5-year EFS and OS. Favorable genomics were pre-defined as hyperdiploid tumors without 1p or 11q loss of heterozygosity (LOH). Those with LOH at 1p or 11q or diploid DNA index were defined as unfavorable. Patients without genomic data were excluded.

Results: Overall, ploidy and genomic features were statistically significant predictors of survival. In the secondary analysis, patients <18 months of age with stage 2 or 3, favorable histology tumors with favorable genomic profiles (n=58) had 5-year EFS and OS rates of 92.9±3.9% and 100%. Patients with stage 2 and 3 tumors, unfavorable histology and unfavorable genomic features (n=38) had EFS of 68.4±8.0% and OS of 78.4±7.0%. Patients <12 months of age with stage 4 disease and either unfavorable histologic or genomic features (n=88) had EFS of 69.1±5.4% and OS of 88.5±3.8%.

Conclusions: Excellent outcomes in non-high-risk neuroblastoma patients with favorable histologic and genomic features suggest further reduction in therapy is possible in this cohort while maintaining survival and decreasing side effects. Select patients with unfavorable features have suboptimal OS and require modified therapy. Histologic and genomic criteria can identify patients with non-high-risk neuroblastoma that may benefit from refined therapy. We plan to test these hypotheses in a Phase 3 cooperative group clinical trial for patients with non-high-risk disease.

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OR44

Highly Sensitive Quantitation of Neuroblastoma Cells in Bone Marrow with a Five-Gene TaqMan® Low Density Array Assay Identifies Patients at High-Risk for Disease Progression after Myeloablative Consolidation Therapy

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Background: Assessing the response of neuroblastoma cells in bone marrow (BM) to therapy with a highly sensitive and quantitative assay may provide warning of relapse. The TaqMan® Low Density Array (TLDA) platform was used to 1) identify five genes that are strongly expressed by neuroblastoma but not by normal hematopoietic cells; 2) compare TLDA and immunocytology assays for quantifying tumor cells in bone marrow; and 3) assess the ability of the TLDA assay to predict event-free survival (EFS).

Methods: Expression of CHGA, DCX, DDC, PHOX2B, and TH (neuroblastoma genes) and of B2M, GAPDH, HPRT1, and SDHA (housekeeping genes) was quantified with a TLDA assay. Results are reported as positive/negative for tumor cells and as the geometric mean Cycle Threshold expression of the five genes (DG=detection gene score). Patients in studies CCG-321P3, CCG-3891, and 91LA6 had not developed progressive disease at 3 and 9 months after myeloablative therapy when BMs were obtained.

Results: Neuroblastoma genes are strongly expressed by untreated neuroblastoma tumors (n=25) and multi-drug sensitive/resistant neuroblastoma cell lines (n=23) but not detectibly or weakly by BM (n=20), peripheral blood mononuclear cells (n=25), or peripheral blood stem cells (n=15) from normal adults. Sensitivity is 81% for detecting one tumor cell per 10e-6 normal cells. TLDA and immunocytology results were highly correlated when immunocytology was positive (r=-.93, p<.001), but TLDA identified tumor cells when immunocytology was negative. Tumor cells were detectible in 87% and 76% of BMs at 3 (n=46) and 9 (n=38) months after myeloablative therapy, and subsequent EFS correlated with tumor load (P=0.02 at 3 and <0.001 at 9 months). At these same times, DG<37 vs. DG=40 predicted 1.8- and 3.7-fold increases in disease progression.

Conclusion: This 5-gene TLDA assay provides a sensitive and quantitative test for neuroblastoma cells in bone marrow that identifies patients at high-risk for disease progression.

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OR45

Factors that contribute to inferior survival of low-risk stage 2B neuroblastoma patients: A Children's Oncology Group study.

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Background: We have previously reported that patients with low-risk (LR) INSS stage 2B neuroblastoma (NBL) who are >18 months of age at diagnosis and who have unfavorable histology or diploid tumors have significantly inferior event-free (EFS) and overall survival (OS) rates compared to other LR NBL patients. We studied these patients to determine if the degree of surgical resection affected outcome and to identify the biologic factor(s) that most significantly contributed to lower EFS.

Patients and Methods: Of 915 eligible LR NBL patients on P9641, 237 patients had MYCN non-amplified INSS stage 2B disease. We examined their age at diagnosis, degree of initial tumor resection, tumor histopathology, ploidy, and 1p and 11q status. Survival tree regression analysis was performed using a Cox proportional hazards model, identifying the statistically significant factor(s) that had the highest hazard ratio (HR).

Results: Within the 237 stage 2B patients (3-year EFS: 85%±3%), only ploidy and histology were significant in univariate analysis. Degree of resection was not associated with outcome. Ploidy was the most highly prognostic factor (n=222; p=0.003; HR=2.7); within the hyperdiploid patients, no factors were significant. Within diploid patients (3-year EFS: 72%±7%), histology was prognostic (n=52; p=0.006; HR=5.9). Within unfavorable histology (3-year EFS: 54%±11%), 11q was prognostic (n=11; p=0.03; HR=12.0). All three patients with 11q LOH had an event.

Conclusions: Ploidy, histology and the status of 11q are powerful prognostic factors for patients with INSS stage 2B NBL. Although image defined risk factors have not been assessed in these patients, tumor biology and patient outcome indicate that they likely have International Neuroblastoma Risk Group L2 tumors. The lack of impact of surgical resection on outcome suggests that more intensive chemotherapy treatment regimens will be required to improve EFS in stage 2B patients with unfavorable histology and genetics.

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Neuroblastoma in Older Children, Adolescents and Young Adults:

A Report from the International Neuroblastoma Risk Group Project

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Background: Neuroblastoma in older children and adolescents has been reported to have an indolent phenotype and poor outcome, but little is known about the clinical and biological characteristics that distinguish this rare subgroup. We sought to determine if an optimal age cut-off exists that defines indolent disease and if currently accepted prognostic factors and treatment approaches are applicable to older children.

Methods: Using data from the International Neuroblastoma Risk Group, among patients ≥18 months old (n=4,027), monthly age cut-offs were tested to determine the effect of age on survival. The prognostic effect of baseline characteristics and autologous hematopoietic cell transplant (AHCT) for advanced disease was assessed within two diagnostic age cohorts; ≥5-<10 years (n=730) and ≥10 years (n=200).

Results: Older age was prognostic of poor survival, with outcome gradually worsening with increasing age at diagnosis, without statistical evidence for an optimal age cut-off ≥18 months. Among patients ≥5 years, factors significantly prognostic of lower event-free survival (EFS) and overall survival (OS) in multivariable analyses were INSS stage 4, MYCN amplification and unfavorable histology. Although only 9% of patients ≥10 years had MYCN amplification, those with stage 4 disease had particularly dismal long-term outcomes (10y EFS: 3%±3%; 10y OS: 5%±5%). Among stage 4 patients ≥5 years, AHCT provided a significant EFS and OS benefit (5y EFS: 28%±5% versus 13%±2%; 5y OS: 42%±6% versus 22%±3%). Following relapse, patients in both older cohorts had prolonged OS compared to those ≥18 months-<5 years (p<0.0001).

Conclusions: Despite indolent disease and infrequent MYCN amplification, older children and adolescents with advanced disease have poor survival, without evidence for a specific age cut-off. Our data suggest that AHCT provides survival benefit in older children with advanced disease. Novel and consistent therapeutic approaches are required to more effectively treat these patients.

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PARALLEL SESSIONS

NEW IDEAS

OR47 – OR56

OR47

Post-chemotherapy Image Defined Risk Factors assessment is a useful tool to predict surgical risk.

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Background: Image Defined Risk Factors (IDRFs) are used for pre-treatment local staging in the new INRG Staging System. We hypothesized that this approach is also appropriate after neoadjuvant chemotherapy to plan surgery and predict surgical morbidity.

Methods: From 2009 to 2011, 91 patients were operated for neuroblastic tumor in a single institution by two surgeons. Among them, 42 were operated after neoadjuvant chemotherapy according to ongoing SIOPEN protocols or treatment recommendations and included in this study. IDRFs were assessed both at diagnosis and preoperatively on CT scan and/or MRI. The quality of resection was evaluated per-operatively and by imaging one month post-surgery.

Results: Median age at diagnosis was 28.6 months [4-191]. Locations were adrenal (n=21), paravertebral (n=14) and perivascular (n= 7). INRGSS stage was L2 (n=14), M (n=26) and MS (n=2). Ten tumors were MYCN amplified. Chemotherapy resulted in a decreased number of IDRFs in 17/42 cases (40%). Such a decrease was observed in 42% (12/28) of M or Ms patients and 28% (4/14) of non-metastatic patients and in 52%, 31% and 0% of adrenal, paravertebral and perivascular locations, respectively. Invasion of renal pedicles was not modified by chemotherapy whereas encasement of major vessels was reduced in about 50%. Preoperative IDRFs were well correlated to surgical difficulties in 86% (36/42) of cases. Total macroscopic resection was achieved in 79% (33/42) but no link was observed between IDRF number and quality of resection. No surgery-related mortality was observed.

Conclusions: Post-chemotherapy IDRFs assessment is a valuable tool to evaluate local effect of chemotherapy and to anticipate surgical difficulties and should be further correlated to local response, quality of resection and survival in a larger cohort.

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OR48

The prognostic value of the SIOPEN skeletal scoring method in high-risk stage 4 neuroblastoma by semi-quantitative I-123-mIBG scintigraphy.

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Aim: To evaluate whether the skeletal disease pattern imaged with I-123-mIBG scintigraphy at diagnosis and after induction chemotherapy could predict event free survival (EFS) in patients (pts) with stage 4 high-risk neuroblastoma.

Material and methods: A panel of 8 nuclear medicine experts in 4 groups reviewed of 357 pts treated on the HR-NBL1/SIOPEN trial with high quality mIBG scans at diagnosis and post induction chemotherapy (Rapid COJEC) showing I-123-mIBG avid primary tumors at diagnosis. Skeletal mIBG uptake was evaluated using the SIOPEN scoring system: and scaled from 0 (no uptake) to a maximum of 6 (diffuse infiltration of the bone) yielding a maximum of 72 in 12 sections of the body. Focal disease (1 to 3 discrete individual lesions, score 1 to 3) was distinguished from diffuse involvement (4 to 6, according to extent). Primary tumor and soft tissue lesions were not included in this analysis.

Results: At diagnosis, 56 pts (15.7%) had negative (31 pts) or up to 3 lesions (25 pts), 64 pts (17.9%) moderate (score 4-17) and 237 (66.4%) extensive diffuse metastases (score ≥18). EFS was calculated using a cutoff score of 3. The 3-yr EFS for 56pts with scores ≤ 3 at diagnosis was 54%(±0.07) and for 301 pts with a baseline score >3 is 33%(±0.03) , (p= 0.017). After Rapid COJEC 341 pts were evaluable: 218 pts (63.9%) with a score <3 had a better 3-yrs [5-yrs] EFS of 44%(±0.04)[34%(±0.04)] when compared to 123 pts (36.1%) with score >3 (p= 0.002) and an 3-yrs[5-yrs] EFS of 22%(±0.04)[13%(±0.04)]

Conclusions: The SIOPEN score shows significant predictive value at diagnosis and after Rapid COJEC induction treatment using a cutoff point of 3. The latter was an eligibility criterion to proceed to high dose therapy.

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OR49

COUP-TF1, a transcription factor associated with the norepinephrine transporter, is essential for in vitro retinoic acid response in high-risk (HR) neuroblastoma

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Background: Neuroblastomas (NB) can take up the norepinephrine analog, 131I-Meta iodobenzylguanidine (MIBG). Results from a HR NB trial, A3973, showed patients with MIBG positive NB at diagnosis had worse survival compared to those with MIBG negative NB (3 year OS: 65% vs 85%; Parisi, M, 2010). We hypothesize that pathways involved in MIBG uptake affect therapy response and resistance in NB.

Methods: Published microarrays of primary HR NBs (GSE19724, GSE3960) were interrogated for gene expression changes based on norepinephrine transporter (NET1) expression, as this neural transporter has been implicated in MIBG uptake. Genes significantly altered were confirmed by immunoblot and assessed for effects on survival and therapy response in vitro.

Results: Twenty-one genes showed >3 fold change in expression between NET1-low- and NET1-high-expressing tumors. One gene increased in NET-low-expressing NB was the chicken ovalbumin upstream promoter1 gene, COUP-TF1 ($p<1.6x10-5$), a transcription factor necessary for embryonic neural maturation and retinoic acid (RA) induced differentiation in lung cancer. Gene expression arrays containing prognostic data confirmed NBs with high COUP-TF1 had superior survival versus those with low COUP-TF1 (OS: 90% vs 25%, $p < 4.5 x 10-6$, Wei, et. al.). In vitro, NBs expressing high COUP-TF1 were very sensitive to RA and NBs lacking COUP-TF1 were resistant. RA treatment increased COUP-TF1 expression in NB cell lines coincident with RAR induction, suggesting COUP-TF1 is a critical downstream effector of RA induced differentiation.

Conclusions: 13-Cis-RA is standard of care maintenance therapy for HR NB and RA resistance is common. COUP-TF1 expression is associated with improved patient survival and our results suggest this may occur through its role in retinoic acid response. Investigations are ongoing to validate the inverse relation of COUP-TF1 to MIBG uptake. Therefore, efforts to therapeutically enhance COUP-TF1 expression could be critical to improving survival for patients with high risk NB.

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OR50

Massively parallel transcriptome sequencing in Neuroblastoma reveals specific mRNA splicing patterns controlled by RNA binding splicing factors

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Alternative splicing events have crucial roles in tumorigenesis and progression, and are nearly universal in human genes. High-throughput RNA sequencing (RNA-seq) experiments allow us to quantify all genes and their isoforms across samples. By whole transcriptome sequencing analysis of 29 stage 4 neuroblastoma (NB) tumors, we discovered an alternative splicing signature for MYCN amplified stage 4 tumors compared to MYCN non-amplified 4S tumors. Pathway analysis demonstrated that splicing perturbed genes held enriched roles in cancer hallmarks biological functions including cell death, cell proliferation, apoptosis, cell movement and immune response. In particular, splicing factors such as RBFOX, CELF, and hnRNP families were differentially expressed between tumor subgroups. The splicing factors regulatory motif sequences were also enriched in adjacent introns of alternatively spliced exons. RNa-seq of MYCN induction and knockdown experiments on NB cell lines showed evidence of MYCN regulation of some of the splicing factors causing the induction of alternative splicing patterns in the cell lines. This study systematically examined and compared the splicing programs in stage 4 NB subgroups. Moreover, it demonstrated that in addition to MYCN's well characterized role in transcriptional regulation, it regulates splicing events by controlling the expression of splicing factors.

In summary massively parallel transcriptome sequencing in neuroblastoma identified key alternatively spliced genes and pathways contributing to the malignant phenotype in MYCN amplified tumors. Many of these represent prognostic biomarkers and potential targets for therapy.

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OR51

Whole-genome sequencing yields tumor-specific DNA rearrangements as targets for minimal residual disease detection in neuroblastoma

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Background: PCR-based detection of minimal residual disease (MRD) in neuroblastoma is presently based on tumor specific RNA markers. Neuroblastoma tumors are marked by copy number changes of large chromosomal domains. We investigated whether chromosomal aberrations, detected in primary tumor, can be used as tumor-specific MRD DNA markers.

Methods: To identify patient specific chromosomal fusions we used the data from paired end whole genome sequencing (WGS) of a series of 87 stage 4 neuroblastoma tumors of all stages (Molenaar et al, Nature 2012). For six stage 4 neuroblastoma patients we selected the chromosomal fusions with the most extensive evidence from the sequence analysis of the tumor. Primers were designed to result in a tumor specific PCR product. Sensitivity for each target was tested by diluting tumor DNA in control DNA. Aberrations were quantified in blood and bone marrow at diagnosis and during treatment using real-time quantitative (RQ-) PCR. To study clonal evolution for two stage 4 patients all rearrangements (13 and 14 aberrations, respectively) detected in primary tumor will be quantified in blood and bone marrow.

Results: For the six selected patients specific PCRs were set up for the unique chromosomal breakpoints (t(11;17) n=3; t(1;2) n=1; Deletion chromosome 5 n=1; t(1;6) n=1). A sensitivity of 10e-5 with a quantification range of at least 10e-4 was reached for all targets and all PCRs were highly tumor-specific. Tumor DNA could be readily detected and quantified in blood or bone marrow at diagnosis and during treatment for all six patients (infiltration levels ranging from > 10% at diagnosis to 0.001% during treatment). Results were comparable to neuroblastoma- specific RT-PCRs and more sensitive than immunocytology.

Conclusion: Chromosomal aberrations can be used as MRD targets, which is a highly tumor and patient specific method for MRD detection.

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MYCN-DNA vaccination suppresses growth of MYCN overexpressing neurobastloma in a syngeneic mouse model

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High-level expression of MycN protein, caused by amplification of the gene, characterizes a malignant phenotype of neuroblastoma (NB). Recent studies suggest that MycN might be a promising target for immunotherapy.

Here, we report the development of a MYCN-DNA vaccine (pMDV1), based on epitopes encoding for three peptides from the MycN protein sequence with high affinities to MHC class I. Effect and mechanism of this vaccine were tested in a novel syngeneic MYCN overexpressing NXS2 mouse-model.

To generate the mouse model, MYCN-cDNA was transfected into the murine NB cell line NXS2 and stable overexpression of MYCN was verified by qRT-PCR and western blot.

We analyzed the efficacy of MYCN vaccination on primary tumor growth and characterized the immune response. For this purpose, mice (n=9) were immunized by oral gavage of 5x108 salmonella typhimurium SL7207 (3x every 7 days) carrying pMDV1. Four days after the last vaccination, mice were challenged by s.c. injection of 5x106 NXS2-MYCN cells. Primary tumor growth was determined and splenocytes were isolated to further characterize the immune response by cytotoxicity assays and IFN-γ release.

Vaccination with pMDV1 resulted in a significant reduction of primary tumor growth compared to control groups. Furthermore, lymphocytes isolated from vaccinated A/J mice, but not control mice, effectively lysed NXS2-MYCN cells in contrast to wild type NXS2 cells. Splenocytes from vaccinated mice produced 5-fold higher amounts of IFN-γ after stimulation with irradiated NXS2-MYCN cells in contrast to controls. Importantly, lymphocytes from pMDV1 immunized mice specifically lysed MYCN-epitope primed non NB cells (SCK carcinoma) compared to untreated SCK cells, indicating antigen specificity.

In summary, we report effect and mechanism of a MYCN-DNA vaccine in a MYCN overexpressing syngeneic model. MYCN vaccination resulted in reduction of primary tumor growth and antigen specific target cell killing. These results provide a base line for further clinical development.

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OR53

Haploidentical stem cell transplantation and subsequent antiGD2 based immunotherapy for patients with relapsed metastatic neuroblastoma

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Pediatric patients with relapsed metastatic neuroblastomas have a poor prognosis and additional therapeutic strategies are warranted. We present an ongoing phase I/II-trial with subsequent immunotherapy with an anti-GD2mAb (CH14.18/CHO) after HLA mismatched, haploidentical stem cell transplantation (SCT). T and B-cell depleted stem cells from parental donors were used for transplantation in combination with Melphalan140mg/m², Thiotepa10mg/kg, Fludarabin160mg/m² and ATG-F. Infusions with CH14.18/CHOmAb were started on day 60-180 posttransplant: 6 cycles with 20mg/m²/day x 5; in cycles 4-6, 1x106 units/m² IL2 were given additionally. 17 patients with metastatic relapse were enrolled and received a total of 79 cycles. Disease status after SCT and prior to antibody infusions was: CR2 n=1; ³PR2 n=13; stable/progression n=3. Side effects: inflammation (pain 79, fever 63 and CRP elevation 57/79 cycles, which decreased by increasing cycle numbers); accommodation disturbance n=7, seizures n=2, loss of weight n=2; transient graft vs host disease (GvHD) grade II n=1. During antibody infusions, endogenous secretion of Interleukin 2 in the patients was increased (928U/ml prior vs 1690U/ml post, p<0.001), which resulted in a likewise significantly increased activation of Natural Killer (NK) cells (measured by CD69 expression: 3 vs 13% p<0.01). In 5/7 investigated patients, effective ADCC and complement mediated (CDC) anti-tumor effects against neuroblastoma cells were detectable in-vitro (85% specific lysis, E:T-ratio=20:1, BATDA-release). 9 patients finished the protocol so far and were evaluable after 6 cycles. 6/9 patients responded according to whole body MRI and/or MIBG scan and reached a CR (n=3) or improved their partial remission (n=3), whereas 3 patients progressed. Overall/progression free survival at 2 years was 75% and 40%. Conclusions: CH14.18 infusions after haploidentical stem cell transplantation appear to be feasible without increased risk of inducing GvHD. Preliminary results of our ongoing study suggest an anti tumor effect of the new, donor-derived immune system in-vitro and in-vivo.

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Aberrant activation of the RAS pathway confers resistance to targeted therapeutics in neuroblastoma

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Retinoic acid (RA) and the ALK inhibitor crizotinib are targeted cancer drugs being used or tested in the clinic to treat neuroblastoma (NB). Targeted cancer therapeutics provoke dramatic, but short-lived responses often due to the rapid emergence of drug resistance. Therefore it is critical to identify mechanisms of resistance to RA and crizotinib to improve treatment of NB.

Functional genetic screens provide a powerful tool to identify novel components and crosstalk of signaling pathways and can help to identify mechanisms of drug resistance in preclinical models of cancer. Using such approaches, we previously identified NF1 and ZNF423 as genes whose down-regulation suppresses the response to RA in NB. Loss of NF1 activates RAS-MEK signaling, which in turn represses ZNF423, a critical transcriptional co-activator of the retinoic acid receptors. As a follow up study to dissect the crosstalk between RAS and RA pathways, we identified that SPROUTY1 (SPRY1), a known inhibitor of RAS signaling, is a direct RA target gene. Importantly, downregulation of SPRY1 confers RA resistance through RAS activation similar to NF1 loss, which can be circumvented by the combination of RA and MEK inhibitor. Our data suggest that RA signaling inhibits RAS as part of a "feed forward loop" which prevents inhibition of RA signaling by RAS. This may have important implications for understanding how NB patients respond to RA-based therapies.

The recent identification of activating mutations in the ALK receptor tyrosine kinase in NB provides a highly promising drug target for this common childhood malignancy. NB cells with ALK mutations are extremely sensitive to ALK inhibitor crizotinib. We have performed genome-wide RNA interference (RNAi) based loss-of-function screens to identify genes whose knockdown confers resistance to ALK inhibitors in sensitive NB cell lines, and identified NF1 whose down-regulation suppresses the response to crizotinib. Our findings suggest that aberrant activation of RAS pathway confers resistance to ALK inhibition, and that combination of ALK and MEK inhibitors may be effective to overcome such resistance.

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OR55

Non-polyalanine repeat expansion mutations of the PHOX2B gene dysregulate Sox10 expression and cause the neurocristopathy in the autonomic nervous system

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Background: Neuroblastoma (NB) and Hirschsprung disease (HSCR) are the most common neurocristopathy affecting the sympathoadrenal and enteric ganglion cells, respectively. Although NB is a tumor and HSCR is a loss of neural crest cell-derivatives, NB and HSCR occasionally co-occur. This association is especially prominent in patients with congenital central hypoventilation syndrome (CCHS; a failure in autonomic control of breathing). The incidence of HSCR and NB is 500-1000 fold greater in CCHS patients than in the general population. Recent genetic studies in CCHS patients have identified heterozygous mutations in the PHOX2B gene. PHOX2B is a paired homeodomain transcription factor and is essential for the development of the cells constituting the autonomic neural circuits. Interestingly, isolated CCHS almost exclusively carry polyalanine repeat expansion mutations, whereas patients with CCHS-HSCR-NB association harbor non-polyalanine repeat expansion mutations (hereafter referred to as NPARM).

Methods: To understand how NPARM PHOX2B affects the development of sympathoadrenal and enteric ganglion cells, we generated knockin mice carrying NPARM PHOX2B.

Results: Embryos with NPARM PHOX2B developed to term but died soon after birth due to the lack of spontaneous breathing. Moreover, these newborn mice displayed colonic aganglionosis and impaired differentiation of the sympathetic ganglia, thus recapitulating at least partially the features of CCHS-HSCR-NB association. In NPARM PHOX2B embryos, enteric and sympathetic ganglion progenitors showed sustained Sox10 expression, failed to proliferate properly and underwent biased differentiation toward glial lineage. NPARM PHOX2B reduced transactivation of wild type PHOX2B on the DBH promoter, which is a target of PHOX2B, in a dominant-negative fashion. Moreover, NPARM converted the transcriptional effect of PHOX2B on a Sox10 enhancer from repression to transactivation.

Conclusions: These data collectively reveals that NPARM PHOX2B as a combined dominant-negative and gain-of-function mutation, and demonstrate that Sox10 regulation by Phox2b plays a pivotal role in the development and pathogenesis of the autonomic ganglia.

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OR56

Neuroblastoma stage 4S is a multifocal stem cell disease of defected neural crest precurser cells

Nagoshi N, et al. Cell Stem Cell 2008; M.M. van Noesel, Lancet oncol 2012

Background: Neuroblastoma stage 4S (NB4S) is considered a metastasized neuroblastoma of infancy, although the pattern of tumor spread (often both adrenals, skin, liver, minimal invasion bone marrow) differs considerably with stage 4 neuroblastoma (4NB; one primary tumor, lymph nodes, bone, bone marrow). NB4S' favorable outcome and high rate of spontaneous tumor regression also contrasts sharply with 4NB. The combination of aberrant tumor sites and high regression rate suggests a different etiology than a metastasizing neuroblastoma. Study of the migration pattern of neural crest cells directs to a model in which NB4S is a multifocal disease of migrating neural crest cells.

Results: Neuroblastoma tumors arise from early neural crest progenitor cells. Neural crest stem cells (NCSC) not only proliferate and differentiate, but also migrate from the dorsal NC to their target organs. The target organs include adrenal glands (chromaffin cells), the sympathetic ganglia (side chain, enteric ganglia) and melanocytes in the skin. Recently it was shown that NCSC also enter the bloodstream and travel through the liver and invade the bone marrow and form a small population of hematopoietic stem cells (1). In E14,5 mouse embryo's NCSCs were shown in all segments of the fetal liver and at E18,5 NCSCs appear in the bone marrow. These data reveal that NCSCs migrate through, and populate sites in the embryo that coincide exactly with tumor locations in NB4S: adrenal glands, liver, skin and a small population in the bone marrow. I hypothesize a model in which NB4S tumors originate from defected pre-migratory NCSCs (2). During migration, these defected NCSCs are seeded in different NC organs and grow tumor nodules of undifferentiated NCSC. In this model, tumor regression seems a delayed activation of normal, developmentally regulated apoptosis.

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PARALLEL SESSIONS

GENOMICS

OR57 – OR64

OR57

Discovery of rare variants in TP53 associated with neuroblastoma

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Background: A neuroblastoma (NB) genome-wide association study (GWAS) has identified several common variants as susceptibility alleles, but much of the heritability remains undefined. Rare single nucleotide variants (SNVs) and copy number variants (CNVs) may explain a substantial proportion of this "missing heritability".

Methods: We imputed genotypes for 2,101 NB cases and 4,202 genetically matched controls of European ancestry using IMPUTE2 and a multi-population reference panel from the 1000 Genomes Project. For replication, we analyze imputed genotypes in an African American cohort of 365 cases and 2,491 controls. A third independent cohort from Italy of 350 cases and 780 controls is genotyped using a PCR-based approach for further replication. In parallel, rare CNVs greater than 0.5 Mb in size are tested for association with NB in a discovery set of 2,083 cases and 6,146 controls. Replication of rare CNV associations is planned in a set of 1,500 cases and 6,000 controls.

Results: We first focused on a rare germline variant in TP53 recently shown to confer susceptibility to cutaneous basal cell carcinoma, prostate cancer, and colorectal adenoma (Stacey, Nature Genet 2011). rs78378222, located in the polyadenylation signal of TP53, was significantly associated with NB in the discovery set of individuals of European ancestry (minor allele freq (MAF): 2.69% cases, 1.32% controls; P = 6.41 x 10-9; OR: 2.05, 95% CI: 1.57-2.68), and was the top NB associated SNV in the region within the African American replication cohort (MAF: 1.05% cases, 0.02% controls; P = 7.6 x 10-5; OR: 4.83, 95% CI:1.92-12.16). We then surveyed the entire TP53 locus, and identified a second rare SNV, rs35850753, located in the 5′ UTR of a short form of TP53, highly associated with NB: European Americans: (MAF: 3.60% cases, 1.93% controls; P = 2.20 x 10-9; OR: 1.89, 95% CI: 1.51-2.37) African Americans: (MAF: 1.23% cases, 0.03% controls; P = 2.61 x 10-3; OR: 3.18, 95% CI: 1.43-7.04). PCR-based replication of both SNVs in an Italian cohort is ongoing. Genome-wide identification of additional rare variants is currently in the initial replication stage of evaluation.

Conclusions: Rare SNVs in the 5′ and 3′ UTR of TP53 are associated with neuroblastoma. Ongoing work will likely uncover additional rare variants contributing to disease susceptibility.

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OR58

Whole exome sequencing of 59 neuroblastomas with different genomic subgroups, P1a or Ss, has revealed distinct pattern of mutations and pathways

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Previously, we have established the genomic subgrouping system based on array CGH analysis for the risk classification of neuroblastoma (NB). According to their chromosomal aberration profiles, NB patients are divided into three genomic groups: silent (S), partial gains and/or losses (P) and whole gains and/or losses (W). Each group is further segregated into subgroups based on their clinical outcomes together with genomic signatures including MYCN amplification, 1p loss, 11q loss and 17q gain. Of these, P1a subgroup is characterized by 1p loss and MYCN amplification with an 8-year survival rate (SR) of about 30%, while Ss subgroup almost without any chromosomal aberration has an overall 8-year SR more than 80%. In this study, we focused on these two subgroups to identify novel somatic mutations linking to the different outcomes. An analysis of 130 whole exome sequences including 59 tumor/normal pairs (P1a:17, Ss:22, others:20) was performed using Illumina platform to an average sequencing depth ≥75X. A wide diversity of 746 non-silent somatic single nucleotide variations (SNVs) was observed in the dataset. 223 novel high-confidence coding somatic SNVs were found in 40 tumors: among those, 17 P1a tumors had multiple, but diverse somatic SNVs in each tumor (2 to 33 per tumor; total 121), while 10 in 22 Ss tumors had a limited number of those (total 17). Intriguingly, pathway analysis by David with KEGG database indicated that a part of these novel somatic SNVs were concentrated in cancer-related signaling pathways. Among those, 9 genes were involved in MAPK pathway, which mainly existed in P1a tumors [35.3% (6/17) in P1a vs. 4.5% (1/22) in Ss, p=0.016]. Ongoing efforts will validate SNV candidates using an independent platform, in a larger scale sample set that includes other genomic group cases as well.

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OR59

Whole Genome Sequencing Revealed Recurrent ATRX Somatic Mutations Correlated with Age at Diagnosis and Telomere Length among Patients with Stage 4 Neuroblastoma

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Background: Metastatic neuroblastoma is diagnosed over a wide age range from birth through adolescents to adulthood. Older age at diagnosis is well recognized to be associated with worse patient outcome.

Methods: We performed whole genome DNA sequencing using diagnostic tumors and their matched germlines obtained from 40 patients with stage 4 neuroblastoma. Age groups at diagnosis included infants (0-<18 months), children (18 months-<12 years), and adolescents and young adults (>=12 years). To confirm the findings from this discovery cohort, validation testing using tumors from an additional 64 patients was also performed. Formalin-fixed paraffin-embedded tumor tissues were used for immunohistochemistry and fluorescent in situ hybridization. Telomere lengths were analyzed using the whole genome sequencing data, quantitative polymerase chain reaction, and fluorescent in situ hybridization.

Results: We identified mutations in the ATRX gene which strongly correlated with age at diagnosis (p<0.001), with 44% (14/32) of tumors from patients in the adolescent and young adult group, 17% (9/54) of tumors from children, and 0% (0/18) of tumors from infants. ATRX mutations were associated with the absence of ATRX protein in the nucleus and with long telomeres (p<0.001). ATRX mutations were mutually exclusive of MYCN amplification. No DAXX mutations were found.

Conclusions: ATRX mutations were associated with older age at diagnosis, present in children and young adults with stage 4 neuroblastoma. Since ATRX is part of a multi-protein complex that plays a role in regulating ATP-dependent chromatin remodeling, nucleosome assembly, and telomere maintenance, these mutations may be important in the oncogenesis of a subset of neuroblastoma.

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OR60

Segmental Chromosome Aberratons In Localized Resectable Neuroblastoma Without Mycn Amplification Have A Strong Prognostic Impact In Patients Diagnosed Over The Age Of 18 Months But Not In Younger Patients

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Background: Data from the Localized Neuroblastoma European Study Group Trial (LNESG1, 94.01, 1995-99) indicated the prognostic impact of segmental chromosome aberrations (SCA) in localized resectable neuroblastomas (NB) without MYCN amplification (MNA) treated by surgery alone to be age-dependent. To test this result, non-MNA tumors from an independent cohort of patients from the Children's Oncology Group (COG) with comparable treatment were analyzed.

Methods: Diagnostic tumor samples of 123 LNESG1 patients were analyzed by pangenomic (aCGH) and/or multilocus techniques (MLPA) and FISH. All genetic data were quality controlled by the SIOPEN Biology Group. 91 COG tumor samples from patients with localized resectable stages were investigated by MLPA.

Results: Pangenomic/multilocus (besides FISH) data were available for 67 LNESG1 and 70 COG tumors. SCA were found in 35.8% of LNESG1 tumors and 25.9% of COG tumors. For those tumors considered as having no segmental changes only complete data sets for 1p, 1q, 2p, 3p, 4p, 11q, 17q were taken into account. 17q gain was the most frequently found aberration (20.7% in LNESG1 and 20.6% in COG), followed by 11q aberrations in the COG (12.5%) and 1p/1q aberrations in the LNESG1 cohort (8.8%). In both, EFS and OS was significantly associated with SCA if age was over 18 months at diagnosis (EFS/OS, LNESG1: 60% vs 100% for both; EFS/OS, COG: 44% vs 100% and 56% vs 100%; p<0.001 for all). Such a correlation was not observed for patients below 18 months.

Conclusion: The observed diverging age-dependent prognostic significance of SCA in LNESG1 was confirmed by the COG cohort and could indicate the need for different age appropriate therapeutic strategies in localized resectable non-MNA NBs inspite of similar tumor genetics. Moreover, the patients' age at diagnosis is possibly not only a surrogate marker for favorable tumor biology/genetics but also points to an unresolved biological phenomenon.

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OR61

Characterization of the neuroblastoma transcriptome by RNA deep-sequencing: A study of the Sequencing Quality Control (SEQC) consortium

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Background: Gene expression studies using microarrays have demonstrated that transcriptomic patterns of neuroblastoma are closely associated with distinct clinical and biological phenotypes, and may serve as accurate prognostic markers. This collaborative study is part of the Sequencing Quality Control (SEQC) project and aims at the comprehensive characterization of the neuroblastoma transcriptome on the basepair-level as a basic resource for gene expression-related research in neuroblastoma.

Methods: A cohort of 498 primary, pre-treatment neuroblastomas (stage 1, n=120, stage 2, n=77, stage 3, n=63, stage 4, n=184, stage 4S, n=54) was investigated. cDNA libraries were constructed according to the TruSeq RNA Sample Preparation protocol, followed by cluster generation and paired-end sequencing with a read length of 2x 90 to 100 bp on the Illumina HiSeq 2000 platform.

Results: To generate a comprehensive transcriptome database of neuroblastoma, a total of 30,577,822,900 reads corresponding to roughly 3 Tbp were sequenced from 498 primary neuroblastoma samples (mean, 61,401,251 reads per sample). After purity filtering, a mean of 59,272,504 reads per sample remained. Of these, 48,749,265 reads (82.25%) could be mapped to the human reference genome, and 39,090,767 reads (65.95%) were mapped to coding genes. We identified a mean of 16,749 expressed genes per sample corresponding to 87.86% of the well-annotated genes deposited at the UCSC database. First analyses of these data aim at clinical outcome prediction of neuroblastoma patients in comparison to gene expression-based classification using microarrays, and determination of expressed sequence variants that are relevant to neuroblastoma pathogenesis.

Conclusions: We have generated a unique database of the neuroblastoma transcriptome using next-generation sequencing technologies. Our data will enable the establishment of general guidelines for the evaluation of prognostic biomarkers based on RNA deep-sequencing information, and will serve as a valuable resource for addressing relevant biological and clinical questions in neuroblastoma research.

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OR62

Genome-wide massively parallel sequencing to characterize genomic rearrangements in neuroblastoma: from unbalanced translocations to chromothripsis

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Background: Neuroblastoma (NB) is characterized by a great genetic heterogeneity. Although well described by cytogenetic and array studies, most genomic rearrangements observed in NB samples remain uncharacterized at the DNA level and the mechanisms underlying genetic instability in this pediatric cancer are still incompletely understood.

Methods: In the present work, we investigated somatic rearrangements in two NB cell lines and two primary tumors using paired-end sequencing of mate-pair libraries.

Results: In cell lines, this approach allowed the characterization of inter-chromosomal rearrangements corresponding to unbalanced translocations previously detected by spectral karyotyping and array-CGH as well as intra-chromosomal rearrangements. Complementary experiments documented these structural variants to the base-pair level. In one cell line, mate-pair analysis unexpectedly revealed a huge number of somatically acquired rearrangements between two regions of chromosomes 2p and 3p. This observation was reminiscent of the recently described phenomenon of chromothripsis and prompted us to investigated two primary NB tumors presenting with shattering of a specific chromosome, previously detected by array-CGH. Mate-pair analysis confirmed complex rearrangements targeting a single chromosome, consistent with the model of massive genomic rearrangements in a single cellular catastrophe. Analysis of breakpoint junctions revealed frequent micro-homologies at the junction points both in cell lines and tumors. In a subset of cases, more complex rearrangements with template insertion of fragments of nearby sequences at the junction were observed.

Conclusions: Genome-wide massively parallel sequencing provides a powerful approach to get insights into the mechanisms that underlie NB genetic instability. Our results indicate that the recently described mechanisms of fork stalling and template switching (FoSTeS) and microhomology-mediated break-induced replication (MM-BIR) may account for genomic rearrangements in NB. They also provide evidences demonstrating that chromothripsis, previously identified in different tumor types may occur in a subset of NB cases.

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OR63

Paired-end whole genome sequencing identifies chromothripsis in high stage neuroblastoma

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Background: Despite various efforts, the underlying cause of Neuroblastoma remains largely unknown. As cancer is considered to be a genetic disease, characterization of the cancer genome may shed light into this often lethal disease.

Methods: We performed paired-end whole genome sequencing of 87 neuroblastoma tumors from all INSS stages, together with their respective normal lymphocyte material. Comparison of normalized read depth of the tumor/normal pair was represented as high resolution CGH information, while paired-end information was used to identify somatic structural variations.

Results: Characterization of somatic structural variants within all stages of neuroblastoma shows that the number of events is much higher in high stage (ST3 / ST4) than in low stage (ST1 / ST2 / ST4S) tumors. We also discovered that in 18% of high stage tumors a single chromosome displayed a large amount of variants, resembling the recently defined chromothripsis. Prognosis of the patients with chromothipsis was poor. Amongst the chromothripsis tumors, a number of known target genes were gained or amplified, such as the MYCN, and CDK4 genes. Interestingly, in a single case the chromothipsis arised around the MYC (c-MYC) gene, leading to its amplification. Chromosome 5 was affected in a number of patients, although the target in these cases remains elusive. We also identified a germline mutation in p53 in one of the samples, a characteristic which has recently been implicated in chromothripsis cases of medulloblastoma as well.

Conclusion: Using whole genome sequencing, we were able to characterize 87 neuroblastoma samples at the structural variant level and identified the presence of chromothripisis in a substantial amount of high stage neuroblastoma. This recently identified mechanism sheds new light into the genesis of neuroblastoma and may expose novel options for treatment.

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OR64

Genetic evolution of neuroblastoma is characterized by new chromosome breakpoints

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Background: In neuroblastoma (NB), relapse can be associated with new chromosome breakpoints (BPs), but few data precisely describe relapse-specific genetic alterations.

Methods: To characterize relapse-specific genetic events, SNP6 (Affymetrix®) analysis of 22 paired diagnostic and relapse NB samples was performed. For one patient, genetic evolution was studied by whole-genome paired-end sequencing of constitutional (C), diagnostic (D) and relapse (R) tumor material (30x haploid coverage).

Results: SNP6 analysis revealed new small interstitial alterations, extensive segmental alterations, or both, at relapse for 6, 1 and 12 cases, respectively (median: 12 new BPs). In 11 cases, BPs observed at diagnosis were not found at relapse. Gene ontology analysis of the smallest regions of overlap at relapse identified the histone-methyltransferase pathway as most frequently targeted by these alterations.

In the deep-sequenced C/D/R case, among predicted structural variants, 21% were D-specific, 16% were common to D and R, and 63% were R-specific, most concerning small interstitial deletions. Three R-specific inter-/intrachromosomal rearrangements could be experimentally validated: a 5q34/11q24.3, a 2q37/12p13 and a 11q22.3/11q25 translocation; breakpoints in the AX747213, SLC6A13, and PDGFD and OPCML genes, respectively.

Among single nucleotide variants (SNVs), 30% were D-specific, 50% common to D and R, and 20% observed only in R. Non-synonymous SNVs predicted to be deleterious were, amongst others, the ALK Y1278S mutation in D and R, and one R-specific mutation, in the GRIK2 gene.

Interestingly, allele ratios indicated 12 isodisomic chromosomes at diagnosis, with three additional chromosome arms showing copy-neutral LOH at relapse. The heterozygous allele ratio for some mutations showed that they occurred after isodisomerisation.

Conclusion: These data support the hypothesis of an abnormality in DNA maintenance/repair leading to variable chromosome BPs in NB evolution. The observation that some genetic alterations of D were not observed in R support the hypothesis that relapse occurred from a more ancestral clone.

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PARALLEL SESSIONS

EXPERIMENTAL THERAPIES (RX I)

OR65 – OR72

OR65

Propranolol as a novel treatment for neuroblastoma

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Background: In order to identify novel therapies for relapsed neuroblastoma (NB) we performed a high-throughput screen with the Prestwick library (Prestwick Chemical, Inc) of FDA approved drugs using IMR-32 and SKNAS NB cell lines. In addition to chemotherapies with known efficacy in NB we identified additional active compounds including propranolol hydrochloride, a non-selective beta-blocker that competitively inhibits epinephrine and norepinephrine on β1 and β2-adrenoreceptors. Since previous studies have demonstrated that catecholamines and their metabolites increase proliferation of cancer cells and patients with NB have elevated serum and urinary catecholamines we hypothesized that propranolol may inhibit NB tumour growth.

Methods: IC50, proliferation, and viability were determined using Alamar blue, BrdU incorporation and Trypan blue exclusion assays. Apoptosis was determined by Caspase 3/7 ELISA and cleaved-PARP immunoblots. In vitro synergy with chemotherapies was assessed using Chou-Talalay index calculations.

Results: Propranolol and other β2 but not β1 specific antagonists, inhibited the growth and proliferation of more than 10 NB cell lines (with differing MYCN and p53 status) in a dose-dependent manner. In addition, propranolol induced apoptosis (caspase 3/7 activation and increased PARP cleavage) that was blocked by caspase inhibition, and propanolol was synergistic with the irinotecan metabolite SN-38. SKNAS xenografts of mice treated with propranolol had decreased rate of growth and lower tumour volume. The protein levels of the pro-apoptotic p53 paralogue TAp73β and its downstream target genes PUMA, NOXA and p57kip2 increased following propranolol treatment.

Conclusions: The β2 antagonist propranolol inhibits NB in vitro and in vivo at doses similar to those used to treat pediatric patients with hypertension and hemangiomas, supporting its potential use in combination with chemotherapies for patients with relapsed NB. Studies are ongoing to determine whether propranolol inhibition of NB requires the β2 receptor and/or p53 family proteins.

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Anticancer Compounds that simultaneously suppress NFKB and activate p53 are highly effective at delaying tumor development and progression in the TH-MYCN mouse model of neuroblastoma

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Background: We have reported that the anti-malarial drug, quinacrine (QC), has significant in vivo anti-tumor activity (Cell Cycle, 2009, 8,1,). We have since identified Curaxins, a new class of similar, but more potent compounds, whose anti-cancer activity results from chromatin-trapping of the Facilitates Chromatin Transcription complex (Science Transl. Med, 2011, 3,1). These novel nongenotoxic agents, like QC, cause simultaneous p53 activation and NFkB inhibition, and result in tumor cell death. We have now studied the effects of QC and one of the most active curaxins, CBLC137, in the TH-MYCN mouse model of neuroblastoma.

Methods: Cohorts of homozygous MYCN mice (n=10), either at time of weaning or with a small palpable tumor, were treated with CBLC137 or QC alone, or combined with chemotherapeutic drugs.

Results: Oral CBLC137 treatment of mice post-weaning caused significantly delayed tumor development compared to untreated controls (P=0.007). While QC alone had no significant effect on tumor progression, and did not enhance cisplatin or VP16 activity, strong anti-tumor activity was observed when QC was combined with cyclophosphamide (CPM) (4/10 QC/CPM-treated vs 0/10 CPM-treated long-term survivors, P<0.001). The effect of CBLC137 was even more dramatic. CBLC137 as a single agent significantly extended survival (median time to death from start of treatment = 31.0+/-9.9, days versus controls = 3.0+/-0.2 days; P<0.0001), and was as effective as cisplatin or CPM single-agent treatment. When CBLC137 treatment was combined with CPM, survival was dramatically increased (8/10 long-term tumor-free mice) compared to CBLC137 alone (P<0.0001, 0/10 alive) or CPM alone (P=0.0002, 1/10 alive).

Conclusions: These are the most impressive results we have ever observed for any therapeutic protocol in the MYCN homozygous mouse model. The combination of CPM with a curaxin compound appears to be a highly promising new treatment approach for refractory neuroblastoma.

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Volasertib (BI 6727), a second generation Polo-like kinase 1 (Plk1) inhibitor, is an active agent in preclinical neuroblastoma mouse models

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Background: Polo-like kinase 1 (Plk1) is highly expressed in unfavorable neuroblastoma (NB) and in NB cell lines, and a first generation PLK1 inhibitor BI 2536 showed considerable efficacy in NB cell lines and xenografts (Ackermann et al Clin Can Res 2011; Grinshtein et al Can Res 2011), making it an attractive new molecular target for therapy. Volasertib (BI 6727) is a first in class, potent and selective agent that induces mitotic arrest and apoptosis by targeting Plk1, and has been reported to be well-tolerated with clinical activity in a Phase II clinical trial for urothelial cancer (ASCO 2011). It has an improved pharmacokinetic profile as compared to BI 2536.

Methods: NB cell lines CHLA-15, CHLA-20, CHLA-90, SH-SY5Y, NUB-7, SK-N-BE(2), SK-N-AS, IMR-32 and BE(2)C cells, and the primary line NB153 were treated with various concentrations of Volasertib in vitro for 72 hours. Cell viability was measured by Alamar Blue assay. Volasertib was assessed in NB xenografts in NOD/SCID mice, using weekly dosages of 10mg/kg, 15mg/kg, 20mg/kg, 30mg/kg and 40mg/kg. The cell lines tested in xenografts were CHLA-20, SK-N-BE(2) and NB249, a primary tumor-initiating cell line from a bone marrow metastasis that is devoid of EBV by Q-PCR.

Results: In vitro, IC50 values for NB cell lines were between 20nM (SK-N-AS, IMR-32, 153) and 1uM. With CHLA-20 and SK-N-BE(2) xenografts, Volasertib, administered at 10mg/kg twice weekly, caused significant inhibition of tumor growth without affecting body weight. With NB249 xenografts at 30mg/kg weekly dosage, Volasertib completely suppressed tumor growth and increased lifespan, accompanied by no weight loss or toxicity after the first week of administration.

Conclusion: Volasertib showed strong anti-tumor activity on neuroblastoma cells in vitro and in vivo, which warrants further investigation in pediatric phase I solid tumor clinical trials.

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4-HPR (fenretinide) sensitizes human neuroblastoma cells for ch14.18-mediated NK cell and complement killing

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Background: 4-HPR is a synthetic retinoid that induces apoptosis in cancer cells mainly through the accumulation of ceramides. We tested the hypothesis whether 4-HPR and ch14.18 would work additively in NK cell-mediated (ADCC) and complement-mediated killing (CDC) of highly drug-resistant human neuroblastoma (NB) cells.

Methods and Materials: Six GD2+, drug-resistant human NB cell lines were treated with sub-toxic concentrations (2.5 and 5μM) of 4-HPR and analyzed for GD2 expression by flow cytometry and in ADCC and CDC assays with anti-GD2 antibody ch14.18/CHO and human expanded NK cells as well as donor-derived PBMCs. Additionally, granzyme B (GrB) and perforin production by human PBMCs co-incubated with 4-HPR or control-treated NB cells was determined. Further, we analyzed the effect of 4-HPR on GD2 expression in s.c. NB tumors in NOD/SCID mice. For this purpose, tumor-bearing mice were treated with 4-HPR Lym-X-Sorb powder by oral gavage. Tumor cells were isolated, GD2 expression levels were determined and cells were employed ex vivo in ADCC assays.

Results: Interestingly, treatment of NB with 4-HPR resulted in an increase of GD2 expression in vitro and in vivo. This correlated with a significantly enhanced susceptibility of treated NB cells towards ch14.18-mediated CDC and ADCC, both by expanded NK cells and freshly isolated PBMCs from several different donors. Finally, we found that donor-derived PBMCs produce significantly higher amounts of GrB and perforin upon co-incubation with 4-HPR-pre-treated NB cells compared to controls.

Conclusion: We can show here for the first time that 4-HPR treatment increases ADCC and CDC by human NK cells and complement towards drug-resistant human NB cells, thereby providing an important baseline for the combination of 4-HPR and passive immunotherapy with ch14.18 in future clinical trials.

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In vitro and in vivo validation of ABT263 and YM155; new targeted compounds in apoptotic signaling.

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Background: The intrinsic apoptotic pathway is at the core of cell faith determination. As a rule this signaling is strongly impaired in any tumor type. In neuroblastoma several aberrations in apoptotic signaling have been described. The BIRC5 (Survivin) gene is located at chromosome 17q in the region that is frequently gained in high risk neuroblastoma and the BCL2 gene shows significant over-expression.

Methods: We used high throughput DNA, mRNA expression and protein data to validate targets in the apoptotic signaling cascade. The BIRC5 and BCL2 gene were further tested as potential drug target using various interference techniques and targeted compounds. The YM155 (BIRC5 inhibitor) and ABT263 (BCL2 inhibitor) compounds were both tested for in vitro and in vivo efficacy.

Results: BIRC5 is strongly over-expressed in neuroblastoma tumor samples, which correlates to a poor prognosis. Targeted knock down of BIRC5 with shRNA's triggers an apoptotic response through mitotic catastrophe. YM155 drug response curves showed IC50 values in the low nM range which resulted in targeted BIRC5 inhibition and an apoptotic response. Nine out 23 cell lines were relatively resistant to YM155 with IC50 values >200nM, although in the same cells shRNA mediated knock down of BIRC5 caused massive apoptosis. Affymetrix mRNA expression data revealed that ABCB1 (MDR1) caused resistance to YM155. Testing of the YM155 compound in a subcutaneous neuroblastoma xenografts model showed a strong tumor inhibitory response.

BCL2 mRNA and protein levels were increased in the majority of neuroblastoma tumors compared to normal tissues and also compared to other malignancies. Most neuroblastoma cell lines lack this high BCL2 expression. Only two neuroblastoma cell lines (KCNR and SJNB12) show BCL2 expression levels representative for neuroblastoma tumors. Lentiviral shRNA mediated silencing of BCL2 in KCNR and SJNB12 resulted in massive apoptosis, while cell lines with low BCL2 expression were insensitive. Identical results were obtained by treatment of the neuroblastoma cell lines with the small molecule BCL2 inhibitor ABT263, which is currently in phase1/2 trials in adults. Combination assays of ABT263 with most classical cytostatics showed strong synergistic responses. Subcutaneous xenografts of a neuroblastoma cell line with high BCL2 expression in NMRI nu/nu mice showed a strong response to ABT263.

Conclusion: We conclude that BIRC5 and BCL2 are viable drug targets in subsets of neuroblastoma tumors. The YM155 and ABT263 compounds are currently further developed for potential clinical use.

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Targeting the Hedgehog signal transduction pathway at the level of GLI inhibits neuroblastoma cell growth in vitro and in vivo

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Background: Hedgehog (HH) signaling is an important regulator of embryogenesis that has also been associated with the development of several types of cancer. HH signaling is characterized by Smoothened (SMO)-dependent activation of the GLI transcription factors, which regulate the expression of several genes critical for proper cell growth during development. Neuroblastoma has recently been shown to express high levels of HH signaling molecules.

Methods: Cytotoxic activity of HH inhibitors was studied in cell viability assays. The molecular mechanisms were characterized using cell- and molecular biology techniques. A xenograft study in mice was performed to validate the therapeutic effects and toxicity in vivo.

Results: Using specific compounds blocking SMO (cyclopamine and SANT1) or GLI1/GLI2 (GANT61) activity revealed that inhibition of HH signaling at the level of GLI was the most efficient target in reducing neuroblastoma growth. The GANT61 sensitivity positively correlated to the expression levels of GLI1 and negatively to MYCN expression in the neuroblastoma cell lines tested. Additionally, GANT61 downregulated GLI1, C-MYC, MYCN and Cyclin D1 expression, and induced apoptosis of neuroblastoma cells, in-line with the reduction of cell numbers elicited by GLI1 knockdown. Furthermore, GANT61 enhanced the effects of chemotherapeutic drugs used in the treatment of neuroblastoma in an additive or synergistic manner and significantly reduced the growth of established neuroblastoma xenografts in nude mice.

Conclusions: Taken together this study suggests that inhibition of HH signaling is a highly relevant therapeutic target for high-risk neuroblastoma lacking MYCN amplification and should be considered for clinical trials testing.

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A phase I study of bolus and metronomic cyclophosphamide with zoledronic acid and bevacizumab in children with recurrent or refractory neuroblastoma: a New Approaches to Neuroblastoma Therapy consortium trial

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Background: Microenvironment modification aims to affect local tumor conditions such as angiogenesis or access to cytokines. We are conducting a phase I trial to determine the feasibility of combining conventional dose cyclophosphamide (CTX) with microenvironment modifiers, zoledronic acid (ZA), bevacizumab (BV) and metronomic CTX in children with refractory NB.

Methods: Bolus CTX and ZA (4mg/m2) were administered q28 days with daily oral CTX (25 mg/m2/day). BV (10mg/kg q14 days) was initiated with course 2. Fully evaluable subjects received 2 doses of bolus CTX and ZA, BV, and >80% of planned oral CTX.

Results: 16 patients (14 male), median age 10.2 (4.3 – 17.2 years), have enrolled. The median number of cycles completed is 2 (range 1-12), with 59 cycles delivered; 6 patients had >4 cycles. Six patients initially received CTX 500mg/m2 of which 5 were evaluable for dose determination. There were 4 dose limiting toxicities (DLT) in 3 patients: Grade 3 hepatic transaminase elevation (n=2, both with pre-existing Gr2 ALT), and Gr4 bone pain / Gr3 osteonecrosis at a site of known metastasis (n=1). After eligibility modification for ALT, 7 patients received CTX 375mg/m2. Five were fully evaluable for toxicity; there were no DLTs. The study has re-escalated to 500mg/m2. Other Gr3/4 toxicities at least possibly related include lymphopenia (n = 3), neutropenia (n = 3), thrombocytopenia (n = 2), ALT (n=1), AST (n=1), nausea (n=1) and hyponatremia (n = 1). Mean c-telopeptide decreased from 1.15 ULN (range 0.04-2.69) to 0.4 ULN (range 0.04-1.97; n=10, p =0.001), and mean n-telopeptide from 3.01 ULN(range 0.39 – 9.57) to 0.74 ULN (range 0.12 – 1.54; n=9, P=0.076). Patterns of change in angiogenic and immunologic cytokines, circulating endothelial and progenitor cells will be discussed.

Conclusions: Combining CTX with ZA, BV and with metronomic CTX is feasible with evidence of microenvironmental effects.

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Two step-Phase II study of imatinib mesylate in pediatric patients with unresponsive or relapsing metastatic neuroblastoma

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Background: Primary endpoints were to determine clinical response and dose-limiting toxicity of continuous daily oral administration of imatinib mesylate in patients with relapsing or unresponsive metastatic neuroblastoma. Secondary endpoints were to obtain pharmacokinetics (PK) data and to evaluate molecular analysis for TH expression as surrogate response biomarkers (PD).

Methods: Twenty-four children were enrolled in two steps with 10 and 14 patients, respectively. For each patient, a starting dose of 340 mg/sqm per day was administered for the first 4 weeks; then, if no major toxicity occurred, the dose was escalated up to 600 mg/sqm per day for a maximum of 12 cycles (four weeks per cycle). PK evaluations were performed during the first three cycles. Molecular analysis was performed at study entry up to tumour progression.

Results: Grade 3 toxicity occurred in few patients during dose escalation and no grade 4 toxicity was observed. Seven major responses (29%) were obtained with 5 complete responses lasting up to 52 months and 2 partial responses lasting up to 29 months. Two additional patients (8.3%) had stable disease up to 8 months. The average plasma AUC exposure at steady-state for imatinib at doses 340-600 mg/sqm was 82.3 μg*hr/mL, being consistent with that seen in adult leukemia patients receiving 1000 mg per day (82.5 μg*hr/mL). The active metabolite (CGP74588) to imatinib ratio appeared to be similar between different age groups. Low or absent levels of bone marrow (BM) TH expression at study entry significantly correlated with a better clinical response.

Conclusions: Continuous oral administration of imatinib mesylate at 600 mg/sqm per day at maintenance was well-tolerated. Imatinib was active in the subset of children with absent or low BM infiltration, as assessed by molecular analysis. In this subset of neuroblastoma patients the association of imatinib with chemotherapy may be worth further investigations.

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OR73

Curie and SIOPEN scoring in stage 4, high risk neuroblastoma. A report from the Metastatic Imaging Working Group of the International Neuroblastoma Response Criteria (INRC) Committee

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Two MIBG scoring systems are currently in use, Curie scoring (CS) in Children's Oncology Group (COG) trials and SIOPEN scoring in SIOPEN trials. Both scoring methods have reported utility in predicting outcome in high risk disease.

Methods: MIBG scans from 280 patients enrolled on COGA3973 were examined at diagnosis and post-induction using the CS system. MIBG scans from 357 patients enrolled on HR-NBL1/SIOPEN were examined at similar time points, using the SIOPEN system. Curie scoring evaluates MIBG scans over 10 anatomic regions, 9 osseous and 1 extra-osseous, with regions scored 0-3(maximal score=30). SIOPEN scoring evaluates MIBG scans over 12 skeletal regions, with the regions scored 0-6(maximal score=72). The major difference in the two systems is the inclusion of primary extraosseous disease in Curie scoring, with extraosseous disease excluded in SIOPEN scoring. In the current analysis, all patients exhibited stage 4, MIBG avid disease.

Results: At diagnosis, SIOPEN scores (on HR-NBL1/SIOPEN) were predictive of outcome, with a 3-yr EFS=0.54±0.07 for patients with a SIOPEN score ≤3 at diagnosis, the EFS highly significant when compared to other cut-points, p=0.034. In contrast, no differences in outcome were noted based upon CS at diagnosis in COGA3973, at any cut-point. Post-induction, the optimal cut-points were a CS=2 (COGA3973) and a SIOPEN score=3(HR-NBL1/SIOPEN). Outcomes were poor for patients with post-induction CS>2 in COGA3973 (3-yr EFS: 0.15±0.05) and SIOPEN scores >3 in HR-NBL1/SIOPEN (3-yr EFS:0.22±0.04). Improved outcomes were noted for post-induction CS≤2 in COGA3973 (3-yr EFS:0.44±0.04) and SIOPEN scores ≤3 in HR-NBL1/SIOPEN (3-yr EFS:0.44±0.04).

Conclusions: In both COGA3973 and HR-NBL1/SIOPEN, post-induction MIBG scoring was highly predictive of outcome. A CS>2 or a SIOPEN score >3 post-induction were each associated with 3-yr EFS≤22%. Given the inherent difference in scoring methods, with Curie scores inherently lower than SIOPEN scores, the post-induction findings in the two studies are strikingly similar.

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The CURIE and the SIOPEN mIBG-scoring systems equally predict outcome in patients with stage 4 neuroblastoma: Results of the Cologne Inter-score Comparison Study

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Background: I-123-mIBG scintigraphy is an established imaging method in neuroblastoma. Semi-quantitative scoring systems have been used to assess metastatic disease and response to chemotherapy. We present the results of the comparison between the recently developed SIOPEN score and the established CURIE score.

Methods: We retrospectively scored 185 mIBG-scans of 71 patients >1 year of age with stage 4 neuroblastoma from the trial NB97 according to both scoring methods (CURIE: 9 skeletal regions plus soft tissue, score 0-30; SIOPEN: 12 skeletal regions, score 0-72). MIBG scans were done at diagnosis (n=71), after 4 cycles (t2; n=55) and after 6 cycles of chemotherapy (t3; n=59).

Results: Scoring results were highly correlated between both methods (Pearson's correlation coefficient: at diagnosis: r=0.97; t2: r=0.99; t3: r=0.98, all p<0.001). Interobserver reliability was very good for both (Pearson's correlation coefficient: CURIE: r=0.98, SIOPEN: r=0.99). A CURIE score ≤2 as well as a SIOPEN score ≤4 (=best cut-offs) at diagnosis was correlated to a significantly better event-free (EFS) and overall (OS) survival compared to higher scores (CURIE: EFS: p=0.01, OS: p=0.01; SIOPEN: EFS: p=0.01, OS: p=0.02). At t2, best cut-off was at a score=0 for both methods. OS but not EFS was significantly better for mIBG-negative patients compared to those with any residual mIBG-positive metastases (CURIE EFS: p=0.32, OS: p=0.02; SIOPEN: EFS: p=0.22, OS: p=0.02). At t3, there was no significant difference in survival between mIBG-negative patients and patients with residual mIBG-positive metastases (CURIE EFS: p=0.13, OS: p=0.10; SIOPEN: EFS: p=0.21, OS: p=0.23).

Conclusions: Higher mIBG scores at diagnosis predicted a worse outcome for patients with stage 4 neuroblastoma. Occurrence of any residual mIBG-positive metastases after 4 cycles of chemotherapy was correlated with a worse overall survival. The CURIE and the SIOPEN score were equally reliable and predictive.

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OR75

Norepinephrine transporter (NET) protein, but not mRNA, expression is correlated with metaiodobenzylguanidine (MIBG) avidity in newly diagnosed neuroblastoma (NB) patients: A Report from the Children's Oncology Group (COG)

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Background: 123I-MIBG is routinely used for the diagnostic evaluation of NB. Preclinical studies demonstrate that NET is critical for active uptake of MIBG by NB. Limited clinical data have confirmed these findings. We evaluated the correlation between NET expression with MIBG uptake and other clinical features.

Methods: Patients on COG protocols A3961 and A3973 for treatment of intermediate or high-risk NB with available tumor mRNA and/or paraffin embedded tumor were included. Quantitative reverse transcription PCR was performed using commercially available probes for NET (N=82), with results normalized to PGK1 expression. NET-17 antibody was used to perform immunohistochemistry (IHC; N=61), with results scored semi-quantitatively for intensity (0-3+) and percent of cells positive for NET. Twenty-seven patients with mRNA and 23 patients with paraffin embedded tumor had centrally reviewed scans to assess MIBG avidity.

Results: The full cohort studied (N=82) had a median age of 11.9 months and 62% had high-risk disease. Among the 27 patients with centrally reviewed scans, the median NET mRNA expression level for the 19 patients with MIBG avid tumors was 12.9% (range 1.6-73.7%), versus 5.9% (range 0.6-110.0%; p = 0.31) for the 8 patients with non-avid tumor. The percent of tumor cells with NET protein expression by IHC correlated with MIBG avidity. The median percent expression was 50% (range 0-100%) in MIBG avid patients compared to 10% (range 0-80%) in MIBG non-avid patients (p = 0.027). There was a trend suggesting higher NET IHC intensity scores among patients with MIBG avid tumors (p = 0.06). MYCN amplified tumors (N=13) had lower NET protein expression compared to non-amplified tumors (N=48; 10% vs. 50%; p = 0.0002).

Conclusions: NET protein, but not mRNA, expression in NB tumor correlates with MIBG avidity. MYCN amplified tumors have lower NET protein expression, which may be an important biomarker of 131I-MIBG therapeutic effectiveness.

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Image-defined risk factors in localized thoracic neuroblastoma

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Background: The INRG staging system discriminates between L1 or L2 localized tumors depending on absence or presence of image-defined risk factors (IDRFs) at diagnosis. Referring to this new staging system, we retrospectively assessed initial imaging of localized thoracic neuroblastoma (NB) and ganglioneuroma (GN).

Methods: Imaging series (MRI or CT) done at diagnosis and available at the trial office of patients with localized thoracic NB or GN were retrospectively reviewed. IDRFs according to Brisse 2011 were assessed and correlated to extent of resection and INSS stage.

Results: Imaging series of 66 patients (52 NB, 14 GN) were available for central review. In 12 (18%) patients no IDRF was present, 21 (32%) exhibited one, 13 (20%) two and 20 (30%) ≥3 IDRFs, resulting in 12 (18%) patients with L1-disease (7 NB, 5 GN) and 54 (82%) with L2-disease (45 NB, 9 GN). In 26 patients (39%), the tumor extended within two body compartments (cervico-thoracic: n=21 (32%); thoraco-abdominal: n=5 (8%)). Encasement of any relevant vessel was present in 30 patients (45%), lower mediastinal tumor in 23 (35%), intraspinal tumor invading >1/3 of spinal canal in 13 (20%), encasement of brachial plexus roots in 13 (20%) and compression of trachea and/or principal bronchus in 9 (14%). According to INSS, 14 patients (21%) had stage 1 (5=L1, 9=L2), 33 (50%) stage 2 (7=L1, 27= L2) and 18 (27%) stage 3 (18=L2) disease. While 21 patients started chemotherapy after diagnosis, and observational strategies were applied in 13 patients, initial resection was aimed for in only 32 patients. Complete resection was achieved in 4 of 7 L1-tumors and 11 of 25 L2-tumors.

Conclusion: In this retrospective cohort of thoracic neuroblastoma, a high proportion had L2-tumors. About ¾ of INSS stage 1 or 2 tumors were upgraded to INRG L2. Therapeutic strategies should avoid overtreatment in these patients.

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OR77

The role of imaging in detecting relapse in patients with Neuroblastoma. Can post-therapy surveillance programs be simplified?

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Introduction: 25-35% of patients with Neuroblastoma (NBL) will relapse. Surveillance is costly and potentially results in high cumulative doses of radiation. Currently children with high risk NBL undergo approximately 5-10 CT and 5-7 MIBG scans during treatment and up to 10 routine CT and 7 MIBG following treatment (surveillance).

Methods: We reviewed all cases of relapsed NBL at Sick Kids between January 2000 and December 2011 to determine how relapses were detected and determine whether routine surveillance investigations can diagnose relapses without using regular CT/MR imaging.

Results: 183 children with NBL were treated during the study period. 50/183 (27%) relapsed (median age 3.54 years, range 0.04-8.30 years). 42/50 (84%) were initially treated on high-risk protocols (COG, POG, CCG), 3 were treated on intermediate-risk protocols and 5 were observed following resection (low-risk). Median time from diagnosis to relapse was 1.20 years (range 0.18-6.66 years). 32/50 relapses were detected by scheduled surveillance investigations and18/50 due to new onset symptoms. 37/50 had new lesions visible by MIBG at relapse. Of the remaining 13, 5 recurrences were detected by elevated urinary catecholamines (UCats), 2 by bone scan, 1 by Ultrasound (US) and 1 by CXR. Two patients relapsed at initial sites of disease and did not have a concurrent MIBG and two patients had relapse diagnosed by CT due to symptomatology – one initially diagnosed with Stage 4, non-MIBG avid disease and the other due to signs of raised intracranial pressure.

Conclusions: Relapsed disease was detected in almost all patients by MIBG scan, UCats and CXR/US alone, supporting the reduced use of CT imaging in post therapy surveillance, thereby reducing cumulative radiation doses. The intensity of post-therapy surveillance may also be guided by initial disease risk stratification. A small sub-group of patients, (including intraspinal residual disease) may require 3-D imaging as part of post therapy monitoring.

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OR78

Phase I Study of 131I-MIBG with Vincristine and Five Days of Irinotecan for Patients with Relapsed or Refractory Neuroblastoma

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Background: 131I-metaiodobenzylguanidine (MIBG) is a targeted radiopharmaceutical active in neuroblastoma. A previous study (NANT N04-06) demonstrated that MIBG (18 mCi/kg) could be combined with vincristine and irinotecan (20 mg/m2/dose for 5 days/week x 2 weeks) as a radiation sensitizer. However, 25% of courses were associated with grade 3 diarrhea. To reduce the incidence of diarrhea, the current phase I study evaluated MIBG together with vincristine and 5 days of higher-dose irinotecan, a standard schedule in recent pediatric trials.

Methods: Patients < 30 with relapsed or refractory MIBG-avid neuroblastoma were eligible. Prior MIBG therapy was allowed if > 6 months from prior MIBG and < 18 mCi/kg cumulative prior dose. Patients received cefixime on days -1 to +6. Irinotecan (50 mg/m2/dose IV) was given on days 0-4 with vincristine (2 mg/m2) on day 0. MIBG was given on day 1 and peripheral blood stem cells were given on day 13. Two MIBG dose levels (15 and 18 mCi/kg) were planned, with 6 patients per dose level if < 2 patients had dose-limiting toxicity (DLT) per dose level.

Results: 12 patients received 17 courses of therapy. No first course DLTs were seen at 15 mCi/kg, though one patient had grade 3 dose-limiting diarrhea in a second course. At 18 mCi/kg, 1 patient had protocol-defined first course DLT with transient asymptomatic grade 3 hyperamylasemia. Myelosuppression and diarrhea were the most common toxicities. 65% of courses had diarrhea of any grade, though only 6% of courses had grade 3 diarrhea. The objective response rate was 25% (2 complete responses and 1 partial response).

Conclusions: MIBG at doses of 18 mCi/kg with vincristine and 5 days of irinotecan is tolerable and active, with less severe diarrhea. This regimen has been incorporated into an ongoing COG pilot trial for patients with newly diagnosed high-risk neuroblastoma.

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OR79

Long-term outcome of MATIN, a schedule of high-administered activity Iodine 131 meta-iodobenzylguanidine and topotecan in neuroblastoma: A SIOPEN study

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Purpose: MATIN is a molecular radiotherapy schedule of Iodine-131 meta-iodobenzylguanidine (131I-mIBG) and topotecan in neuroblastoma. It incorporates higher than conventional administered activities of 131I-mIBG, coupled with topotecan as a radiosensitiser, and autologous haemopoietic support. This study evaluates the toxicity and long-term outcome of MATIN in a European multi-centre setting.

Patients and Methods: Patients with refractory or relapsed high-risk neuroblastoma with stem cells available were eligible. MATIN delivers two administrations of 131I-mIBG prescribed to give a whole body radiation dose of 4 Gy, with concomitant topotecan. Autologous stem cells were returned around two weeks after the second mIBG administration.

Results: MATIN has been used 5 European centres in 69 patients with neuroblastoma, 44 male, 25 female, median age 6 years. 46 patients had refractory or progressive disease without prior myeloablative therapy (MAT), 23 had relapsed after MAT.

Toxicity: In 2 patients, the full treatment was not given because of adverse events. There was 1 treatment-related death. 5 patients failed to regain normal platelet counts.

Further therapy: In 61% of refractory patients, further potentially curative treatment including MAT was delivered. In six patients, repeat MATIN was given. Survival: For all patients, three year event free survival (EFS) was 0.16 (+-0.05) and overall survival (OS) was 0.26 (+- 0.06). For patients without prior MAT, EFS and OS were 0.25 (+-0.07) and 0.37 (+-0.09). For relapsed patients, EFS was 0 and OS 0.07 (+-0.07). These differences are statistically significant (P=0.002).

Conclusions: The MATIN schedule has an acceptable morbidity and mortality profile in a group of neuroblastoma patients with a very poor prognosis. In very high-risk patients with refractory disease, MATIN enabled further, potentially curative, treatment to be given resulting in encouraging survival rates. The MATIN schedule will be further evaluated in a randomised trial.

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OR80

Treatment of children over the age of one year with unresectable localized neuroblastoma without MYCN amplification: results of the Siopen Study

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Background: Treatment for children >1 year old with unresectable, localized neuroblastoma without MYCN amplification varied in Europe with different intensity and duration of chemotherapy, and sometimes the addition of radiotherapy. This study recommended discontinuing treatment after surgery and chemotherapy irrespective of the completeness of surgical excision.

Methods: Previously untreated children >1 year of age with a localized, unresectable neuroblastoma as defined by the presence of one or more surgical risk factors (SRF), without MYCN amplification were eligible. Following biopsy, six courses of chemotherapy were given, four before and two after surgical resection. Courses alternated with carboplatin 200mgs/m2 and etoposide 150mgs/m2 each daily for three days, with cyclophosphamide 300mgs/m2 on days 1-5, adriamycin 30mgs/m2 on days 4 and 5, and vincristine 1.5mgs/m2 on days 1 and 5. Survival analyses were performed using an intention-to-treat approach and estimated by the Kaplan-Meier method; differences between groups were assessed by the log-rank test.

Results: One hundred and sixty patients were evaluable. According to INPC, 47.5% had favorable, and 52.5% unfavorable histology. Chemotherapy was well tolerated and reduced the number of SRF by a third. 86.3% of patients underwent second surgery with 45.7% complete excision rate, and 28.3% minimal residual. There was one surgical death. Thirty-eight patients experienced progression or relapse and 19 eventually died. At five years EFS was 76.4% and OS 87.6%. A significantly higher EFS and OS were observed for younger patients for those with favorable histology, and normal LDH. The extent of surgical resection made no significant difference to survival.

Conclusions: Children with favorable INPC prognosis have an excellent outcome without radiotherapy and can be treated with reduced intensity chemotherapy. Those with unfavorable INPC prognosis have a significantly poorer outcome diminishing further with increasing age, and these patients require treatment additional to that used in this study.

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OR81

An epidemiological view on neuroblastoma trials over 30 years: Is there any progress?

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Background: Epidemiological childhood tumor registration covering > 95% of all diagnosed patients, five consecutive trials for all stages of neuroblastoma, and a data base with a treasure of 98% long-term follow-up data provide a unique opportunity to investigate generalized outcome results as obtained in Germany. Have all the efforts paid off for the patients?

Methods: The outcome data of all neuroblastoma patients in Germany diagnosed between 1980 and 2009 were related to age-specific population figures. Inclusion criterion was diagnosis of neuroblastoma and trial participation.

Results: Of 3570 patients diagnosed until 2009, 3425 were included (96%). They participated in one of the trials NB79,85,90,97,2004. 50.9% had localized neuroblastoma, 10.5% stage 4S and 38.6% stage 4 disease. The median age at diagnosis was 1 year 3 months and stable over time with an exception of the first decade (1 year 8 months 1980-9). 12.6% of the patients had MYCN amplified tumors (1990-2009). The age-specific incidence rates increased for infants from 1980 until 1995. During the period of neuroblastoma screening a distinct, but reversible peak of incidence was observed for the group of 1 to <2 year children (screening at 1 year of age). The age groups of 2,3,4, and 5-14 years showed no trend for the incidence. The cumulative population based mortality observed 5 and 10 years after diagnosis decreased for all age groups significantly for all age groups by 30% (p=0.0018). The 5 year survival after neuroblastoma increased significantly for all ages and all age groups (from 50.8% 1980-84 to 73.5% 1990-95, 75.0% 2000-4, test for trend p<0.0001).

Conclusion: Except for the screening period and the infants, age specific incidence rates have not changed since 1980. We observed a decrease in population based mortality and an increase in survival However, much room for improvement in subgroups remains.

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PARALLEL SESSIONS

EXPERIMENTAL THERAPIES (RX II) & IMMUNORX

OR82 – OR90

OR82

Signal Transduction and Activator of Transcription and Environment Mediated Drug Resistance in Neuroblastoma

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Background: Drug resistance is a major cause of failure in cancer treatment. There is recent evidence that in addition to genetic factors, the tumor microenvironment and in particular the bone marrow, is a source of drug resistance by providing cancer cells with a sanctuary against the injuries of chemotherapy.

Methods: Eight drug sensitive human neuroblastoma cell lines and 2 murine cell lines were examined in co-cultures with bone marrow-derived mesenchymal cells (MSC) and peripheral blood (PB) monocytes for their sensitivity to chemotherapeutic agents. Bone marrow biopsies from patients with stage 4 disease were examined by immunohistochemistry. Statistical analysis was performed using the ANOVA test and a two-tailed unpaired student 't' test.

Results: We observed that when co-cultured in the presence of MSC, neuroblastoma cells became resistant to drugs like etoposide and melphalan but that resistance was reversed upon pharmacological inhibition of STAT3 activation or STAT3 knock down by siRNA. Activation of STAT3 in neuroblastoma cells was not constitutive but occurred through a paracrine production of interleukin-6 and its agonistic soluble receptor sIL-6R produced by MSC and PB monocytes and by ciliary neurotrophic growth factor (CNTF). STAT3 activation upregulated several survival proteins including survivin, Bcl-xL, Mcl-1 and XIAP. Consistent with a central role for STAT3 in human neuroblastoma progression and drug resistance, we demonstrated elevated expression of phospho STAT3, Bcl-xL and survivin in neuroblastoma patient bone marrow samples infiltrated with high number (>10%) of tumor cells when compared to samples with low level of infiltration (<10%).

Conclusions: These data demonstrate that activation of STAT3 by the bone marrow microenvironment in neuroblastoma leads to drug resistance. Considering the recent evidence that STAT3 is a major contributor to inflammation and a target for therapeutic intervention, our data suggest that targeting STAT3 in combination with chemotherapy could be of therapeutic value in the treatment of patients with high risk neuroblastoma.

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OR83
Preclinical studies support therapeutic evaluation of targeting IL-6/JAK/STAT3 pathway in Neuroblastoma
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Background: Recently, IL-6 was identified as a poor-prognosis marker in NB patients. Levels of IL-6 were significantly increased in high-risk patients with poor outcomes. The rs1800795 IL-6 promoter SNP, correlates with high IL-6 levels and is an independent, poor prognostic factor for EFS and OS in high-risk NB patients. Declerck's group showed IL-6 promotes survival and protects NB in BM from drug-induced apoptosis by activating STAT3. Thus targeting the IL6/JAK/STAT3 signaling may represent a new therapeutic approach for NB.

Method: We evaluated the anti-tumor effect of AZD1480 using standard models in NB in vitro and in vivo.

Results: AZD1480 decreased cell viability in 6/6 NB cell lines in vitro (median IC50 =1.17uM, ranging 0.42-5.1uM). AZD1480 (0.5uM) blocked constitutive and IL-6 induced STAT3 activation. AZD1480 induced cell growth inhibition with increases in G2 and subG1. Mechanistically, the AZD1480-induced inhibition of STAT3 was consistent with reduction of STAT3 targets, CyclinD1, 2, 3 and CDC25A that regulate cell cycle and anti-apoptotic targets Bcl-2 and Survivin. AZD1480 induced a significant increased Caspase3/7 activity, suggesting it has both anti-cell proliferative as well as pro-apoptotic activities. In vivo studies showed AZD1480-treated mice bearing NB tumor xenografts had longer survival than untreated mice at 21 days (untreated vs. treated, median survival: SY5Y, 19 days vs. notreached, P =0.02; KCNR, 15 days vs. notreached, P<0.0001; AS, 10 days vs. 21 days, P= 0.056). AZD1480 was more effective in inhibiting growth in SY5Y and KCNR xenografts with little effect on AS xenografts. KCNR and SY5Y tumor xenografts of AZD1480-treated mice in showed inhibition of STAT3 phosphorylation and reduction of its downstream-targets compared to tumors from untreated mice.

Conclusion: Our study provides demonstrated in vitro and in vivo anti-tumor activity of AZD1480, in pre-clinical NB tumor models and provides a compelling rationale for the evaluation of AZD1480 in NB patients.

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OR84a
Inhibiting Monocyte/Macrophage-Neuroblastoma Cell Interactions with Sorafenib Increases Tumor Cell Response to Cyclophosphamide and Topotecan
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Background: New therapeutic strategies based upon understanding the tumor microenvironment may improve the efficacy of chemotherapy and, as a result, long-term survival for patients with high-risk neuroblastoma. We showed that tumor associated monocytes/macrophages (TAMs) and cytokines (IL-6/IL-6R, IL-7, IL-10, IL-8, VEGF, TNFα, and TGFβ1) are prominent in high-risk neuroblastomas. We hypothesize that TAM-neuroblastoma interactions promote tumor growth and interfere with the efficacy of targeted and non-targeted chemotherapeutic agents. Our specific aims were to 1) characterize TAM-neuroblastoma interactions; and 2) determine if sorafenib, a multi-kinase inhibitor, suppresses these interactions and improves efficacy of cyclophosphamide-topotecan chemotherapy.

Methods: The effect of TAMs upon tumor cell growth was determined in vitro with co-cultures (Boyden chamber; 15 cell lines tested) and in vivo with subcutaneous co-injection of monocytes and tumor cells into NOD/SCID mice (3 cell lines tested). Using co-cultures, tumor cell DNA synthesis was determined by flow cytometry, pathway activation by Western blotting, and cytokine secretion by Luminex® assay. NOD/SCID subcutaneous and disseminated tumor models were used to determine effects of sorafenib and cyclophosphamide-topotecan upon growth of luciferase expressing neuroblastoma cell lines.

Results: Co-culture and co-injection into NOD/SCID mice of monocytes with neuroblastoma cells markedly enhanced tumor cell growth. Co-culture increased tumor cell DNA synthesis, phosphorylation of STAT3, PI3K (110α, 110γ, p85, p55), AKT, mTOR, S6, 4E-BP1, and IKKβ in both tumor cells and TAMs, and secretion of IL-6, IL-7, IL-10, CCL2, TNFα, and VEGF>10-fold. Under these same conditions, sorafenib significantly inhibited tumor cell growth, DNA synthesis, protein phosphorylation, and cytokine secretion. Tumor cells co-injected with monocytes were most effectively eliminated by sorafenib with cyclophosphamide-topotecan compared to sorafenib or cyclophosphamide-topotecan alone (P=0.001). Similarly, mice with disseminated neuroblastoma were most effectively treated with sorafenib with cyclophosphamide-topotecan (P=0.0024).

Conclusions: Chemotherapy with cyclophosphamide-topotecanis improved by sorafenib, which inhibits pro-tumor interactions of TAMS and neuroblastoma cells.

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OR84b
Inhibiting Monocyte/Macrophage-Neuroblastoma Cell Interactions with Lenalidomide Increases Tumor Cell Response to Cyclophosphamide and Topotecan
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Background: New therapeutic strategies based upon understanding the tumor microenvironment may improve the efficacy of chemotherapy and, as a result, long-term survival for patients with high-risk neuroblastoma. We showed that tumor associated monocytes/macrophages (TAMs) and cytokines (IL-6/IL-6R, IL-7, IL-10, IL-8, VEGF, TNFα, and TGFβ1) are prominent in high-risk neuroblastomas. We hypothesize that the milieu created byTAM-neuroblastoma interactions promotes tumor growth and interferes with the efficacy of targeted and non-targeted chemotherapeutic agents. Our specific aims were to 1) characterize TAM-neuroblastoma interactions; and 2) determine if lenalidomide, an immunomodulating drug, inhibits these interactions and improves efficacy of cyclophosphamide-topotecan chemotherapy.
Methods: The effect of TAMs upon tumor cell growth was determined in vitro with co-cultures (Boyden chamber; 15 cell lines tested) and in vivo with subcutaneous co-injection of monocytes and tumor cells into NOD/SCID mice (3 cell lines tested). Using co-cultures, tumor cell DNA synthesis was determined by flow cytometry, pathway activation by Western blotting, and cytokine secretion by Luminex® multiplex assays. The NOD/SCID model was used to determine effects oflenalidomide and cyclophosphamide-topotecan upon growth of luciferase expressing neuroblastoma cell lines.
Results: Co-culture and co-injection into NOD/SCID mice of monocytes with neuroblastoma cells uniformly and markedly enhanced tumor cell growth. Co-culture increased tumor cell DNA synthesis, phosphorylation of STAT3, PI3K (110α, 110γ, p85, p55), AKT, mTOR, S6, 4E-BP1, and IKKβ in both tumor cells and TAMs and secretion of G-CSF, IL-10, IL-1RA, IL-6, IL-7, CCL5, TNFα, and VEGF>10-fold. Under these same conditions, lenalidomide significantly inhibited tumor cell growth, DNA synthesis, protein phosphorylation, and cytokine secretion. Tumor cells co-injected with monocytes were most effectively eliminated by lenalidomide with cyclophosphamide-topotecan compared to lenalidomide or cyclophosphamide-topotecan alone (p=0.01).
Conclusions: Chemotherapy with cyclophosphamide-topotecan is improved by lenalidomide, which inhibits pro-tumor interactions of TAMS and neuroblastoma cells.
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OR85
GRHL1 inhibits tumorigenicity and is a prognostic marker in neuroblastoma

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Background: The state of histopathological tumor differentiation strongly impacts neuroblastoma biology and the course of disease. Here we aimed to decipher the underlying molecular mechanisms controlling tumor cell differentiation that have remained enigmatic.

Methods: Transcriptional changes in an in vitro differentiation model were analyzed via gene expression profiling in time-course. Clinical relevance of candidate genes was evaluated in primary tumors. Functional analyses were performed in preclinical neuroblastoma models.

Results: GRHL1, a highly conserved neuroectodermal developmental transcription factor with no prior defined role in oncology, was among the five strongest immediate early response genes genome-wide to pan-HDACi treatment. Expression remained up-regulated throughout the 120 h time-course. Different pan-HDACi, including those clinically approved, similarly induced GRHL1 in five cell lines and a primary sphere culture as well as neuroblastoma xenografts in mice, supporting GRHL1 induction as a common early event of HDAC inhibition in neuroblastoma cells. High GRHL1 expression in 380 neuroblastomas was prognostic for favorable event-free and overall patient survival and significantly correlated with localized disease stage, young age, favorable Shimada/INPC histology, MYCN single copy and 1p wildtype status, and a favorable PAM transcriptional profile. These findings were confirmed in two independent cohorts of 102 and 88 neuroblastomas. Immunohistochemistry revealed strong nuclear GRHL1 expression in favorable, but no expression in unfavorable tumors. Enforced GRHL1 expression in three neuroblastoma cell lines strongly suppressed anchorage independent colony formation in soft agar, and distinctly attenuated xenograft growth in mice, suggesting that GRHL1 targets tumorigenic pathways.

Conclusions: GRHL1 is more active in favorably prognostic, differentiated tumors than in poor prognostic, aggressive tumors. Functional relevance is provided by the strong tumor-suppressive effects caused by GRHL1 in different preclinical neuroblastoma models. From a therapeutic perspective, triggering GRHL1 expression by pharmacological inhibition of HDAC activity may tip the scales in favor of a favorable phenotype.

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OR86
Fenretinide and Vorinostat combination therapy for neuroblastoma

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Background: Retinoids are an important component of therapy at the stage of minimal residual disease for advanced neuroblastoma. However, 40-50% of patients treated with 13-cis-retinoic acid still relapse, indicating necessity for more effective retinoid therapy. Fenretinide (4-HPR) and vorinostat (SAHA), were utilised in early phase single agent paediatric oncology trials, but only stabilised disease.

Methods: We used human neuroblastoma and medulloblastoma cell lines, flow cytometry, gene-expression analyses, siRNA knockdown and xenograft tumour models to evaluate 4-HPR + SAHA combination treatment for therapeutic synergy and biomarkers of response.

Results: At clinically relevant concentrations of 4-HPR (1.33-3μM) + SAHA (0.22-0.5μM) the combination therapy exerted potent cytopathic effects in multiple neuroblastoma and medulloblastoma cell lines (combination index < 1). The proportion of apoptotic cells was markedly increased in neuroblastoma cells compared with non-malignant MRC-5 cells. The cytopathic effect of 4-HPR + SAHA was much greater than 13-cis-retinoic acid + SAHA. In vivo xenograft experiments of BE(2)-C cells injected into the flank of athymic nude mice treated with 4-HPR (1.45mg/kg, i.v.) + SAHA (35mg/kg, i.p.) also demonstrated therapeutic synergy. To identify biomarkers of sensitivity to 4-HPR + SAHA, we evaluated the transcriptional changes in neuroblastoma cells treated with the combination. The four candidate biomarker genes for which changes in expression level best correlated with the cytopathic responses were RARα, RARβ, Thymosin-beta-4-X (Tβ4), and Synaptogyrin-3 (SYNGR3). High expression of both Tβ4 and SYNGR3 was associated with a significantly better patient outcome by logrank analysis among 650 neuroblastoma patients. We performed siRNA knockdown of Tβ4 in neuroblastoma cells treated with 4-HPR + SAHA and found treatment synergy was lost without Tβ4 expression, indicating Tβ4 was necessary for the 4-HPR + SAHA cytopathic effect.

Conclusion: Our data suggest that 4-HPR + SAHA is an effective anticancer combination therapy in vitro and in vivo in neuroblastoma.

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OR87
Immunotherapy for Neuroblastoma by GD2-specific chimeric antigen receptor

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Background: Chimeric antigen receptors (CARs) combine, in a single molecule, the MHC-unrestricted specificity of monoclonal antibodies with potent T cell signalling. This technology allows the generation of large numbers of T-cells specific to any cancer antigen without requiring T-cell selection/expansion. T cells from relapsed neuroblastoma patients engineered to express a first generation CAR directed against GD2, were well tolerated and showed preliminary clinical activity in a phase I clinical trial (Pule et al, Nat Med, 2008).

Methods: We have made a series of refinements to CAR and adoptive transfer methodologies, and have performed preclinical evaluation prior to the opening of a follow-on study incorporating a second generation receptor and host lymphodepletion.

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 a CD28 co-stimulatory domain, and have codon-optimised the entire CAR sequences for improved expression. (2) We improved the vector cassette to include a Genomic Scaffold Attachment Sequence to create more homogenous bright expression, improving expression and suicide gene activity. (3) We have improved receptor stability and detection by FACS by substitution of a IgG1 hinge-CH2-CH3 spacer domain, and have limited potential detection by FcγR-expressing innate cells, by mutation of essential binding motifs. (4) To manage possible toxicity, we have developed and incorporated a novel co-expressed suicide gene comprising Rituximab-binding epitopes from CD20, which allows efficient elimination of cells expressing the CAR following administration of Rituximab.

We have demonstrated efficient and specific killing and proliferation in the presence of GD2-expressing neuroblastoma cells in vitro and in an immunoreplete mouse model.

Conclusions: We have been awarded funding for a follow up study using this CAR in which survival of infused T cells and clinical activity will be measured following lymphodepletion of relapsed neuroblastoma patients.

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OR88

Antagonizing polyamine homeostasis prevents tumor initiation and lethal progression in complementary models of murine and human neuroblastoma

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Background: Polyamines (PA) are essential cellular cations that mediate protein processing downstream of MYC. Expression levels of all PA pathway genes are significantly associated with neuroblastoma (NB) outcome (Haber, ANR 2012). We showed that inhibiting ODC1, rate-limiting for PA synthesis, delayed tumor onset in TH-MYCN mice and synergized with chemotherapy to treat established NBs (Cancer Res, 2008). This study investigated the potential benefit of targeting multiple steps in PA metabolism.

Methods: Colony formation, PA uptake and expression assays were performed on human and TH-MYCN NBs in vitro, with DFMO (ODC inhibitor), celecoxib (SAT1 inducer), SAM486 (AMD1 inhibitor) and AMXT1501 (PA transport inhibitor). In vivo models included NB xenografts and TH-MYCN mice.

Results: PA inhibition profoundly reduced clonogenicity of NB cells. Clonogenicity was restored with PA-supplemented media, supporting PA import as a relevant rescue mechanism. Combination therapy established synergistic combinations for in vivo testing. Pre-emptive treatment of NB-prone TH-MYCN mice with SAM486 and DFMO nearly abolished tumorigenicity ($P<0.01$ compared with DFMO), yet SAM486 did not augment efficacy of DFMO when treating established NBs. In contrast, celecoxib and DFMO synergized to augment cyclophosphamide (CPM) efficacy when treating established tumors, with extended survival compared with CPM alone or CPM/celecoxib in TH-MYCN mice ($p<0.001$) and XG models utilizing BE(2)-C or SK-N-SH cells ($P<0.01$). The addition of celecoxib/DFMO to CPM/topotecan (TOPO) similarly extended survival compared with CPM/TOPO alone, in both transgenic and XG models ($P <0.01$)

Conclusions: Targeting multiple steps in the PA pathway inhibited colony formation and tumorigenesis, and synergized with chemotherapy in complementary in vivo models of established NB. Optimal PA inhibition regimens, targeting synthetic enzymes and import pathways, warrant clinical investigation. A Phase 1 study with high-dose DFMO and celecoxib, with CPM/TOPO, is being opened within the NANT consortium.

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OR89

Phase I trial of a bivalent vaccine with escalating doses of the immunological adjuvant OPT-821, in combination with oral β-glucan for high-risk neuroblastoma (NB)

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Background: Gangliosides GD2 and GD3 are highly expressed on NB. Anti-GD2 antibodies mediate efficient lysis of NB cells in vitro and are active clinically. In the adjuvant setting, vaccines may help maintain antibody levels for months-to-years to eradicate dormant NB. Preclinical studies and clinical trials with adults show optimal induction of anti-GD2/GD3 antibody responses with vaccines comprising: 1) GD2 and GD3 structures stabilized by lactone formation (GD2L, GD3L); 2) GD2L and GD3L conjugated to the immunological carrier protein keyhole limpet hemocyanin (KLH); and 3) the immunological saponin adjuvant OPT-821 (natural form of QS-21). b-glucans have strong immune stimulant effects, including via binding to the iC3b-receptor widely expressed on leukocytes, which can enhance tumor-cell recognition by leukocytes.

Methods: To determine the maximally-tolerated dose of OPT-821 in a vaccine containing GD2L-KLH and GD3L-KLH, NB patients in >2nd complete/very good partial remission (CR/VGPR) could receive subcutaneous injections (weeks 1-2-3-8-20-32-52) of the vaccine if there was no dose-limiting toxicity (DLT) and/or relapse (Clinicaltrials.gov NCT00911560). A standard dose-escalation schema regarding OPT-821 was used. -glucan was started at week 6 (2 weeks on/2 weeks off). Extent-of-disease evaluations were every 10-to-12 weeks.

Results: The study enrolled 11 patients in 2nd and 4 patients in 3rd CR/VGPR. 13/15 patients received the entire protocol treatment including 12 who remain relapse-free at 16-31 (median 24) months and 1 who relapsed (single node) at 21 months. Two other patients had early focal relapses (2.3 and 4.6 months). Relapse-free survival was 87+9% at 12 months and 78+11% at 24 months. Overall survival is 100% (follow-up >14+ months). The treatment was well tolerated. Antibody responses against GD2 and/or GD3 were seen.

Conclusions: This consolidative immunotherapy holds promise for improving NB prognosis. No DTL was seen so the highest dose (the adult dose) of OPT-821 was selected for a proposed randomized phase II trial.

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OR90

Phase I Study of Anti-GD2 Humanized 3F8 (hu3F8) Monoclonal Antibody (MoAb) in Patients with Relapsed or Refractory Neuroblastoma (NB).

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Background: Mouse 3F8 (m3F8) was successfully humanized to the IgG1 subclass, retaining nM affinity, with a Koff ~10-fold slower than other anti-GD2 MoAbs. Longer retention of MoAb on tumor cell surfaces translated into more efficient antibody-dependent cellular cytotoxicity (ADCC) in vitro by ~10-100 fold. Since pain is the major side effect and is mediated through complement activation, selective improvement of ADCC may allow lower MoAb dosing while retaining anti-tumor efficacy with less pain.

Methods: A phase I study (http://www.clinicaltrials.gov , NCT01419834) of standard 3+3 design (12 dosage levels, 0.06 mg/kg to 3 mg/kg) was initiated to define the maximum tolerated dose (MTD) of hu3F8. Secondary objectives were defining hu3F8 pharmacokinetics (PK) in the first cycle, measuring pain side effects and anti-tumor activity. Hu3F8 was given intravenously over 30 minutes, 2 doses/cycle, 7 days apart; cycles were repeated every 21 days x 12 cycles. Eligibility included patients ≥1 and <22 years at diagnosis with resistant NB, adequate organ function, and negative serum human anti-hu3F8 antibody (HAHA) titer.

Results: The 12 patients treated to date had no dose limiting toxicities (DLT) and their pain thus far was less compared to that from m3F8. 8 of 12 patients had previous antibody response to m3F8, and 2 developed positive HAHA, while the rest of the patients were HAHA negative even after repeated hu3F8 cycles (3-6 cycles total). PK showed expected dose-dependent increases in Cmax and Cmin (mean[SD]), levels >IC50 of NB killing in vitro.

Conclusions: Hu3F8 has been well tolerated thus far. The Hu3F8 serum levels of PK analysis, the relative lack of pain side effect and the absence of HAHA were encouraging. This dose-escalation study is ongoing.

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POSTERS
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POB001 – POB124

POB001
Midkine and Alk signaling in sympathetic neuron proliferation and neuroblastoma predisposition
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Background: Neuroblastoma (NB) is the most common extracranial solid tumor in childhood and arises from cells of the developing sympathoadrenal lineage. Activating mutations in the gene encoding the ALK tyrosine kinase receptor predispose for NB. However, the normal function of Alk and the identity of the ligand activating Alk during sympathetic ganglion development were not known.

Methods: To identify the role of endogenous Alk signaling we analyzed the expression of Alk and candidate ligands Midkine and Pleiotrophin in embryonic chick sympathetic ganglia. Gain- and loss-of-function experiments were carried out in cultures of proliferating sympathetic neurons and in vivo, in the chick embryo. Effects on proliferation and target gene expression were analyzed.

Results: Forced expression of wildtype ALK as well as of NB-related constitutively-active ALK mutants in cultures of proliferating immature sympathetic neurons results in a strong proliferation increase whereas Alk knockdown and pharmacological inhibition of Alk activity decrease proliferation. Alk activation upregulates NMyc and trkB and maintains Alk expression by an autoregulatory mechanism involving Hand2. The Alk-ligand Midkine (Mk) is expressed in immature sympathetic neurons. In vivo inhibition of Alk signaling by virus-mediated shRNA knockdown of Alk and Mk leads to strongly reduced sympathetic neuron proliferation. In vivo Mk overexpression results in increased proliferation and ganglion size.

Conclusions: These results demonstrate that sympathetic neurogenesis is controlled by Mk/Alk signaling. Aberrations of this mechanism, which lead to elevated and sustained Alk signaling and increased NMyc expression may underlie NB predisposition.

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POB002
Internalization and down-regulation of the ALK receptor in neuroblastoma cell lines upon monoclonal antibodies treatment.
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Activating mutations of the full length ALK receptor, with two hot spots at positions F1174 and R1275, have been characterized in sporadic cases of neuroblastoma. Using stably transfected NIH3T3 cells expressing mutated ALK at position F1174 or R1275, we recently established that the constitutive kinase of the mutated receptors impaired their trafficking, with retention in intracellular compartment. Strikingly intracellular miss-localized receptors appeared much less phosphorylated than the cell surface pool (Mazot et al. 2011). Here, we report similar basal patterns of ALK phosphorylation between the neuroblastoma IMR-32 cell line which expresses only the wild-type receptor (ALKWT) and the SH-SY5Y cell line which exhibits a heterozygous ALK F1174L mutation and expresses both ALKWT and ALKF1174L receptors. We demonstrated that this lack of detectable increased phosphorylation in SH-SY5Y cells is a result of intracellular retention and proteasomal degradation of the mutated receptor. As a consequence, in SH-SY5Y cells, plasma membrane appears strongly enriched for ALKWT whereas both ALKWT and ALKF1174L were present in intracellular compartments. We further explored ALK receptor trafficking by investigating the effect of agonist and antagonist mAb (monoclonal antibodies) on ALK internalization and down-regulation, either in SH-SY5Y cells or in cells expressing only ALKWT. We observe that treatment with agonist mAbs resulted in ALK internalization and lysosomal targeting for receptor degradation. In contrast, antagonist mAb induced ALK internalization and recycling to the plasma membrane. This study provided novel insights into the mechanisms regulating ALK trafficking and degradation, showing that various ALK receptor pools are regulated by proteasome or lysosome pathways according to their intracellular localization. The lack of detectable phosphorylation of the intracellular mutated receptor in SH-SY5Y cells may rely on a permanent dephosphorylation by specific tyrosine phosphatises. This point is currently under investigation.

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POB003
A novel mechanism of cell migration by NMyc through a direct transactivation of ALK gene in neuroblastoma
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Introduction: Human Anaplastic lymphoma kinase (ALK) has been identified as an oncogene mutated or amplified in neuroblastoma (NBL). However, the role of wild-type ALK in NBL is still largely unknown. For better understanding a molecular event associated with ALK in the pathogenesis of NBL, it is necessary to clarify how ALK gene contributes to NBL progression.

Methods: RT-PCR and Western-blot were performed to check mRNA and protein expression, respectively. ALK promoter activity was measured by dual luciferase assay. Chromatin Immunoprecipitation (ChIP) assay was used to analyze recruitment of NMyc onto the promoter region. WST-8 assay was employed to monitor cell proliferation. Cell migration and invasion assay was performed to analyze cell motility after ALK expression.

Results: From the analysis of NBL clinical samples, we have found that ALK expression was significantly high in NMyc amplified group ($n = 126$, $p < 0.01$). Consistent with this evidence, the developing tumors with NBL characteristics in NMyc-transgenic mice showed a high expression of ALK. Promoter analysis of ALK showed that NMyc was recruited to the upstream of the first exon, suggesting that ALK is a direct transcriptional target of NMyc. Functional assay revealed that overexpression of ALK enhanced NBL cell growth, migration and invasion. Moreover, these activities were suppressed by siRNA-mediated knockdown of ALK. On the other hand, the forced expression of NMyc enhanced cell migration. Of interest, the increased migration by NMyc expression was suppressed by siRNA-mediated knockdown of ALK, suggesting that the cell migration induced by NMyc is at least partly regulated by ALK expression.

Conclusion: Our results suggest that NMyc directly regulates ALK and NMyc function might through ALK in neuroblastoma. Our present findings explore the fundamental understanding of ALK in order to develop novel therapeutic tools by targeting ALK for aggressive NBL treatment.

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POB004
Complex genomic rearrangements within the ALK gene may lead to ALK activation in a subset of neuroblastoma samples
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Background: Activating mutations of the ALK gene, highly expressed in neuroblastoma (NB), an embryonal cancer of the peripheral autonomous nervous system, have been identified in sporadic and familial cases and are thought to be the primary mechanism of oncogenic activation of this receptor in this pediatric neoplasm. In several other human cancers, including a subset of anaplastic lymphomas, myofibroblastic tumors and lung carcinomas, translocations leading to fusion proteins containing the tyrosine kinase domain of ALK and involving various partners, have been shown to result in ALK activation and oncogenesis. Here, we addressed the possibility that ALK activation may occur through genomic rearrangements in a subset of NB cases.

Methods: We used high-resolution array-CGH combined to capture/paired-end sequencing experiments, mass spectrometry analysis and FISH experiments to search for ALK rearrangements.

Results: various types of rearrangements were fully characterized, including partial gains or amplifications, in several NB cell lines and primary tumors. In one cell line, we described a genomic rearrangement associated with an amplification of the ALK locus, leading to the expression of a mRNA variant encoding a protein of 170 kD lacking part of the extra-cellular domain encoded by exons 4 to 11. This variant protein was highly phosphorylated suggesting increased activation of the receptor. Analysis of genomic DNA from the tumor at diagnosis and relapse revealed that the ALK gene was amplified at diagnosis but that the rearranged ALK allele was observed at the relapse stage only, suggesting that it may be implicated in the aggressiveness of the tumor.

Conclusions: Whereas the functional consequences of some of the described rearrangements targeting the ALK gene remain to be elucidated, these results indicate that genomic alterations within the ALK gene may occasionally constitute an alternative mechanism to ALK point mutations resulting in ALK receptor activation.

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POB005

Flotillin-1 is a novel ALK binding protein which regulates ALK singnaling through receptor endocytosis.

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Background: Recent studies revealed that amplification of anaplastic lymphoma kinase (ALK) and series of oncogenic mutations of ALK are potent oncogenic factors of neuroblastoma. To investigate the role of ALK in oncogenesis of neuroblastoma, we investigated the specific downstream signaling molecules of ALK in neuroblastoma .

Results: We established stable transfectants of FLAG-tagged wild-type and oncogenic mutants of ALK in TNB1 neuroblastoma cells, and cell lysates of each transfectants were subjected to two-step immune-affinity purification using anti-FLAG M2 antibody and anti-phosphotyrosine antibody (4G10). Purified samples were subjected to silver staining followed by identification using mass-spectrometry. Through the analysis, we identified Flotillin-1 (FLOT1), a plasma membrane protein known to be involved in endocytosis, as the tyrosine-phosphorylated binding partner of ALK. Immunoprecipitation in NB-39-nu neuroblastoma harboring amplified ALK revealed that FLOT1 is a binding partner of ALK and tyrosine phosphorylation of FLOT1 is ALK-dependent. Interestingly, oncogenic ALK mutants showed less binding affinity to FLOT1 than wild-type ALK in TNB1 cells. Knockdown of FLOT1 using specific siRNA induced accumulation of ALK to plasma membrane, causing activation of ALK and phosphorylation of its downstream signal molecules such as ERK, AKT, and STAT3. It was also observed that oncogenic mutants of ALK were significantly accumulated to the plasma membrane compared with wild-type ALK.

Conclusions: These results suggest that FLOT1 controls the amount of ALK protein at the cell surface through the regulation of receptor endocytosis. Decrease binding affinity of oncogenic ALK mutants to FLOT1 may cause the activation of ALK signaling which leads to poor prognosis of neuroblastoma cases harboring these mutations. Further studies on the biological roles of FLOT1 in ALK-mediated oncogenesis of neuroblastoma are currently in progress.

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POB006

NLRR1 binds to ALK and regulates its function in cell proliferation

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Background: We have previously reported that neuronal leucine rich repeat 1 (NLRR1), highly expressed in unfavorable neuroblastoma (NB), accelerates cell proliferation upon EGF and IGF treatments, whereas its function in other signaling molecules remains unknown. Anaplastic lymphoma kinase (ALK) has been found to contribute to malignant state of NB with activated point mutations or gene amplification. In the present study, we examined the functional regulation of ALK by NLRR1 in NB cells.

Methods: Immunoprecipitation was employed to check protein-protein interactions. NLRR1 function in cell proliferation was analyzed by WST-8 assay. Phosphorylation status of ALK and its mRNA expression level were evaluated by western blot and RT-PCR, respectively. To test the function of NLRR1 in ALK-inducing neuronal differentiation, neurite outgrowth assay was performed in PC12 cells.

Results: Immunoprecipitation revealed that NLRR1 physically interacted with ALK (wild-type, F1174L and R1275Q mutants) when co-expressed in HEK293 cells. The endogenous interaction was also confirmed in NB cell line, SMS-SAN cells, which highly express NLRR1 and ALK. Unexpectedly, NLRR1 overexpression suppressed cell growth in NB cells transiently expressing wild-type ALK, while the growth inhibitory effect was marginal in the mutant ALK expressing cells. Although the expression level of ALK was not affected by up-regulation of NLRR1, its phosphorylation status was reduced by NLRR1 expression in NB cell line, SH-SY5Y cells, which harbor mutant ALK. In neurite outgrowth assay, however, the expression of NLRR1 had no significant effect on ALK-inducing neuronal differentiation, suggesting that NLRR1 is involved in the regulation of ALK function mainly in cell proliferation.

Conclusions: Our results suggested a novel function of NLRR1 which physically interacts with ALK and negatively regulates its function in cell proliferation. The present study may lead us to the better understanding how membrane protein-protein interaction between NB-associated proteins regulates cell signaling in the development of NB.

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POB007

SH2 domain containing adaptor protein Shf, a favorable prognostic factor of neuroblastoma, inhibits ALK-induced oncogenic signals in neuroblastoma

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Background: Anaplastic lymphoma kinase (ALK), an oncogenic receptor tyrosine kinase, is frequently hyper-activated by its gene amplification or mutations in advanced neuroblastoma, while the contribution of its downstream signal regulation in neuroblastoma has not been elucidated. To address this issue, we investigated whether the Src homology 2 domain containing F (Shf) protein associated with ALK and modulated its function in neuroblastoma. Shf is an adopter protein that contains a SH2 domain and four putative tyrosine phosphorylation sites, and originally identified from our cDNA project.

Methods: Interaction and sub-cellular localization of Shf and ALK were analyzed by immunoprecipitation and immunostaining. We detected Shf and ALK mRNA expressions in 106 neuroblastoma primary samples using qRT-PCR. We performed overexpression and/or knockdown studies of Shf and ALK in neuroblastoma cell lines with wild-type or mutant ALK. In these cells, ALK downstream signal activation was assessed by western blotting, cell growth was measured by the WST-8 assay, and cell motility was evaluated by a migration and invasion assay.

Results: Shf was associated with ALK at the juxtamembrane region in the cell. The binding ability of activated F1174L ALK mutants with Shf was stronger than that of wild-type or other ALK mutants. In primary neuroblastoma, Shf was more highly expressed in the favorable patients than in the unfavorable group ($p<0.035$, n=106). High ALK and low Shf expression in patients were significantly correlated with an unfavorable prognosis ($p<0.03$, n=106). Knockdown experiments revealed that Shf negatively modulated ALK downstream Ras/MAPK and Jak/STAT pathways, cell growth, and the migratory and invasive abilities of neuroblastoma cells. ALK overexpression in Shf knockdown neuroblastoma cells further enhanced survival signal activation and cell growth.

Conclusion: The expression of Shf predicts the prognosis of neuroblastoma. Shf may suppress progression of neuroblastoma by inhibiting the ALK-mediated oncogenic signals.

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POB008

The ALK-F1174L activating mutation is tumorigenic in MONC-1 neural crest stem cells in an orthotopic murine model of neuroblastoma

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Background: Activating mutations of the anaplastic lymphoma receptor tyrosine kinase gene (ALK) were identified in both somatic and familial neuroblastoma. The most common somatic mutation, F1174L, is associated with NMYC amplification and displayed an efficient transforming activity in vivo. In addition, both AKL-F1174L and NMYC were shown cooperate in neuroblastoma tumorigenesis in animal models. To analyse the role of ALK mutations in the oncogenesis of neuroblastoma, ALK wt and various ALK mutants were transduced in murine neural crest stem cells (MONC1).

Methods: ALK-wt, and F1174L, and R1275Q mutants were stably expressed by retroviral infection using the pMIGR1 vector in the murine neural crest stem cell line MONC-1, previously immortalised with v-myc, and further implanted subcutaneously or orthotopically in nude mice.

Results: Both MONC1-ALK-F1174L and -R1275Q cells displayed a rapid tumour forming capacity upon subcutaneous injection in nude mice compared to control MONC1-MIGR or MONC1 cells. Interestingly, the transforming capacity of the F1174L mutant was much more potent compared to that of R1275Q mutant in murine neural crest stem cells, while ALK-wt was not tumorigenic. In addition, mice implanted orthotopically in the left adrenal gland with MONC1-ALK-F1174L cells developed highly aggressive tumours in 100% of mice within three weeks, while MONC1-Migr or MONC1 derived tumours displayed a longer latency and a reduced tumour take.

Conclusions: The activating ALK-F1174L mutant is highly tumorigenic in neural crest stem cells. Nevertheless, we cannot exclude a functional implication of the v-myc oncogene used for MONC1 cells immortalisation. Indeed, the control MONC1-Migr and MONC1 cells were also able to derive subcutaneous and orthotopic tumours, although with considerable reduced efficiency. Further investigations using neural crest stem cell lacking exogenous myc expression are currently on way to assess the exclusive role of ALK mutations in NB oncogenesis.

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POB009

ETV5 regulates cell proliferation downstream of the ALK signaling pathway

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Background: Activating ALK mutations are present in almost 10% of primary neuroblastomas (NB) and serve as new therapeutic targets for treatment. Clinical trials for small molecule ALK inhibitors have been initiated for NB and other ALK driven tumor entities. However, in many instances, tumors acquire resistance to small molecule inhibitors, illustrating the need for additional compounds directed against downstream target genes or alternative survival pathways. To achieve this goal, we analyzed aberrant ALK signaling to identify such vulnerable nodes for combined pharmacological targeting.

Methods: Transcriptome profiling was performed on 10 NB cell lines (ALK wild type, ALKF1174L, ALKR1275Q mutant or amplified) following NVP-TAE684 treatment. Data mining analysis and functional validation experiments were integrated to identify ALK driven functional cellular networks and aberrantly regulated downstream pathway components.

Results: Differential gene expression analysis allowed delineation of a 150-gene signature representative for high ALK activity in NB. This signature was significantly enriched for genes implicated in MAPK/ERK signaling, including several negative MAPK regulators, indicating strong ALK induced MAPK activity. In addition, genes implicated in neuronal differentiation and growth control were identified. We selected ETV5, known to be involved in neuronal fate decision and metastasis/invasion, for further investigation. RNAi-mediated ETV5 knock down showed drastic reduction in cellular growth measured in NB cells with activated ALK. Elevated ETV5 levels were apparent in human and mouse ALK positive NB. Remarkably, inhibition of ALK signaling in NPM/ALK positive lymphoma and EML4/ALK positive lung cancer also strongly reduced ETV5 expression.

Conclusion: We obtained for the first time a detailed picture of the transcriptional consequences of sustained ALK signaling in human and mouse NB cells. The MAPK driven ETV5 oncogene was identified as a robustly regulated ALK target in NB and other ALK activated cancers, thus offering new therapeutic targets for molecular therapy.

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POB010

Screening of a natural resource library for antitumor activities using midkine as an indicator

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Background: We previously found that the growth factor midkine (MK) is highly expressed in human neuroblastoma (NB), and its blood levels work as a prognosis factor (Ikematsu et al., Cancer Sci 99, 2008; Br J Cancer 88, 2003). The purpose of this research is to search for active antitumor ingredients from marine resources, using MK expressed by NB cells as an indicator.

Methods: We used a library of 700 ingredients of marine resources extracted from the sea near Okinawa, Japan. First, ingredients of the library were added to SK-N-SH cells, a NB cell line. The culture supernatants of both 24 h and 48 h after sample addition were collected. Next, the amounts of MK in the culture supernatants were measured by MK-ELISA, which we had developed. Ingredients which showed the reduction in MK production was applied to the 2nd screening. In the 2nd screening, MK production as well as cell viability was evaluated.

Results: We obtained 71 candidates of 700 after the 1st screening. Among 71 candidates, 8 ingredients exhibited the reduction of MK production and cell viability in the 2nd screening. These ingredients were derived from marine resources classified into sponges, echinoderm, and cnidarian.

Conclusions: The ingredients with antitumor activity could be seeds for developing therapeutics for NB.

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POB011

Enhanced Tumorigenicity of Neuroblastoma Cells after Retinoic Acid Treatment is Reversed by Telomerase Inhibition.

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Background: Relapse is the major cause of fatality in neuroblastoma (NB). The ability of tumor cells to self renew is controlled in part by telomere maintenance, and telomerase activity is predictive of tumor relapse in NB. We have previously demonstrated that telomerase inhibition by the Terc antagonist, Imetelstat, causes irreversible loss of the ability of NB tumor-initiating cells to self renew. Since all-trans retinoic acid (ATRA) is known to cause differentiation of human NB cells, we hypothesized that the combination of differentiation and telomerase inhibition may be effective for the suppression of NB self-renewal and tumorigenesis.

Methods: We treated multiple NB cell lines in culture and in mice with different regimens of ATRA and Imetelstat. Telomere length, telomerase activity and expression of hTERT, the catalytic subunit of telomerase, were analyzed upon and after treatment. NOD/SCID mice were inoculated with pretreated cells and monitored for tumor development.

Results: Although ATRA resulted in differentiation of NB cells in culture, population doublings of ATRA-treated cells increased dramatically after cessation of treatment. This resistant subpopulation of cells did not respond to subsequent treatment with ATRA and exhibited higher hTERT expression than the parental population, suggesting the acquisition of a more aggressive phenotype. Animals inoculated with ATRA-pretreated cells developed larger tumors in a shorter period of time when compared with control group ($p<0.01$). Imetelstat-pretreated cells failed to activate hTERT after subsequent ATRA treatment, and exhibited growth arrest even after cessation of therapy ($p<0.05$). These cells exhibited reduced tumorigenic potential in vivo ($p<0.05$), suggesting that NB self-renewal was suppressed.

Conclusions: Telomerase inhibition in combination with other NB directed therapies might prevent tumor recurrence, and that patients should be treated first with Imetelstat followed by ATRA. These observations are the basis of a Phase I trial with Imetelstat and ATRA to be led by our institution.

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POB012

Mitochondrial apoptosis is the preferential pathway in neuroblastoma by effect of Casiopeínas®.

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Background: Neuroblastoma is the main extra-cranial childhood tumor and is refractory to numerous anticancerigens. The Casiopeínas® (Cas) are Cu(II) coordination compounds with antitumoral effect in several tumors. The central core of Cas, might be responsible of the catalysis of reactive oxygen species (ROS) and therefore of the apoptosis. The objective of this work is to contribute to the action mechanism of Cas by means of identification of preferential apoptotic pathway in neuroblastoma.

Methods: CHP-212 and SK-N-SH cells were treated (2 h and 4 h respectively) with cisplatin, Cas IIgly, IIIEa, or IIIia. The inhibitory concentration media (IC50) was the working concentration. Cellular death was detected by LDH (24 h) and Annexin V/PI. Apoptotic molecules caspase-8, caspase-3, Bcl-2, Bax and cytochrome c were identified by cellular fractionation and Western blot. With MitoTracker™ was shown the mitochondrial damage by FACS. For oxidative stress we determine GSH by monoclorobimane and the ROS production with 2'7'-Dichlorofluorescein Diacetate by FACS. All determinations were developed by triplicated.

Results: The IC50 in CHP-212 were 8, 14, 21 and 68 µg/ml for Cas IIIEa, IIgly, IIIia and cisplatin respectively; whereas in SK-N-SH cells were 8, 10, 31 and 37 µg/ml for Cas IIgly, IIIEa, IIIia and cisplatin, respectively. The LDH assay showed 15% of citotoxicity for cisplatin (SK-N-SH) corroborated in both cell lines by Annexin V/PI where >90% cells treated with Cas died by apoptosis. With all treatments, the two cell lines showed cytoplasmic expression of caspase-3 and cytochrome c, Bax decreased, Bcl-2 increased, caspase-8 was absent, and mitochondrial activity decreased 15-20%. After the treatments, ROS increased and GSH decreased.

Conclusions: Casiopeínas® with lower concentrations than cisplatin, have an antitumoral effect on neuroblastoma by means of mitochondrial apoptosis as a consequence of the pro-oxidant environment.

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POB013

Determination of apoptosis and autophagy in neuroblastoma by Casiopeínas®.

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Background: Neuroblastoma is the most common extra-cranial solid tumor in children. Currently, the mainstay of neuroblastoma chemotherapy is combination with some traditional drugs, but usually is inefficient. Casiopeínas® is the generic name of a group of coordination complexes with a central copper atom bound to organic ligands. They were designed to be an alternative to cancer therapy by means of apoptosis. Controversy exist between autophagy itself may be a mechanism of caspase-and apoptosis-independent cell death or rather, is a self-limited process that protect cells from death by multiple mechanisms. Based in our previous results, where caspase-3 was expressed at 2 but not at 24 h, the objective of this study was determine if apoptotic and autophagic processes were present in a time kinetic.

Methods: CHP-212 cells were treated with the IC50 of 8, 14, 21 and 68 µg/ml of Cas IIIEa, IIgly, IIIia or cisplatin at 2, 10 and 24 h, respectively. Previous cellular fractionation, autophagic molecules BECN1 and MAP-LC3β, and apoptotic molecules caspase-3, caspase-7, Bcl-2, Bax and cytochrome C, were identified by Western Blot and analyzed with Image J® program. All determinations were developed by triplicated.

Results: Our results showed that autophagic molecules were expressed throughout the time; however, a peak was observed at 10 h. Meanwhile, apoptotic molecules showed their highest expression at 2 h and later they gradually decreased.

Conclusions: Treatment with Casiopeínas® can promote apoptosis at very early periods of time and autophagy is a secondary event that occurs later.

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POB014

Identification of novel candidate compounds targeting TrkB to induce apoptosis in neuroblastoma: in silico screening utilizing a grid computing technology

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Background: Neuroblastoma (NB) is one of the most frequent solid tumors in children and its prognosis is still poor. The neurotrophin receptor TrkB, expressing at high levels in high-risk tumors, is involved in defining the bad prognosis of the patients. However, the TrkB targeting therapy has never been real in the clinic.

Methods: We performed an in silico screening procedure utilizing an Autodock / grid computing technology in order to identify novel candidate compounds targeting the BDNF binding domain of TrkB. A grid computing technology links many individual computers, creating a large networking system with massive computational power that occasionally far suppresses the power of a handful of supercomputers (courtesy of IBM Co. Ltd.).

Results: As the first screening, a library of synthetic compounds including three million molecules was screened in silico. The top-ranked 60 compounds were further screened functionally for cytotoxicity by using NB cell lines. We have finally identified 7 low molecular weight compounds to kill NB cells by the IC-50 values of 0.05 to 5.0 µM. The TUNEL assay showed that these molecules induce apoptosis accompanied by p53 activation in NB cell lines. The candidate compounds and BDNF demonstrated a synergistic effect on cell growth, possibly suggesting the competition at the BDNF binding site of TrkB. The in vivo toxicity test (oral and intravenous administrations) using mice has not shown any abnormal sign. Interestingly, the candidate compounds decrease the phosphorylation levels of mitogen-activated protein kinases, suggesting that these compounds inhibit the signaling pathway downstream of TrkB.

Conclusions: In this study, we demonstrated that novel candidate compounds were effectively identified by an in silico screening, followed by in vitro assays. We propose that the candidate compounds affecting the downstream signaling pathway of TrkB could help developing a novel treatment and cure for childhood cancers including neuroblastoma.

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POB015

An investigation into the potential therapeutic benefit of targeting Skp2 in Neuroblastoma.

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Background: S-phase kinase-associated protein 2 (Skp2) is a member of an E3 ubiquitin ligase complex which targets several key regulators of the G1/S transition of the cell cycle. Skp2 is overexpressed in many cancers and has been implicated in tumourigenesis due to its ability to degrade the cyclin-dependent kinase inhibitor p27Kip1, promote S phase entry and induce growth. We identified Skp2 as a possible MYCN target gene in neuroblastoma (Bell et al, Cell Cycle 2007), and other groups have reported an increase in Skp2 transcript levels in aggressive MYCN- amplified neuroblastoma (Westermann et al, Clinical Cancer Res 2007). As MYCN amplification is a well-established poor prognostic marker we hypothesise that targeting Skp2 may be a potential therapeutic approach for neuroblastoma, especially the MYCN-amplified subtype.

Methods: The relationship between MYCN and Skp2 was investigated using the SHEP Tet21N-regulatable MYCN expression system and Skp2 siRNA. The effects of Skp2 knockdown on downstream proteins (p21, p27), cell cycle arrest and cell death were analysed using western blotting, flow cytometry, and the Caspase-3/7 glow assay, respectively.

Results: An increase in p27 and p21 levels was seen in SHEP Tet21N cells when MYCN expression was suppressed. Skp2 knockdown caused an increase in p21 levels in both non-MYCN¬ amplified SHSY5Y cells and MYCN amplified IMR-32 cells, with a lesser effect on p27 levels. A modest G1 arrest (p=0.05) was seen in IMR-32 cells 24 hours after Skp2 knockdown, with SHSY5Y cells showing a small but significant increase (p=0.0469) in caspase activity. Both cell lines had ~25% reduction in growth after 72 hours Skp2 knockdown compared to the scrambled siRNA control.

Conclusions: These data further establish the relationship between MYCN and Skp2. The effect of Skp2 knockdown on growth arrest and apoptosis suggest it would be a valid therapeutic target in neuroblastoma.

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POB016

A Large Tenascin C Isoform Promotes Micro-Vessel Remodeling After Treatment With Anti-Endothelial Cell Monoclonal Antibodies In An Orthotopic Model Of Human Neuroblastoma

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Background: In a recent study we identified Tenascin C (TNC)+ perivascular NB cells with a high degree of plasticity that served as progenitors of tumor-derived endothelial cells (TEC) (Pezzolo A. et al, Cell Res 2011). To investigate the correlation between the presence of TEC and tumor growth and gain new insight into anti-vascular treatment strategies, we targeted with monoclonal antibodies (mAbs) human TEC in orthotopic NB carrying mice.

Methods: HTLA-230 cells were injected in the adrenal gland of immunodeficient mice. To identify which domains of TNC was NB-associated we used a series of mAbs specific for different epitopes of this molecule. NB-bearing mice were treated with anti-hCD31 or anti-prostate specific membrane antigen (PSMA) monoclonal antibodies. Angiogenesis-related genes expression was investigated using PCR-arrays.

Results: We identified in perivascular NB cells a large TNC isoform containing the alternatively spliced FnIII domains A1, A2, A4, and B. The expression of this TNC isoforms was described in normal neural stem cells. TNC+ NB cells increased significantly in tumors from anti-hCD31 treated or anti-PSMA treated vs isotype-treated mice (p= 0.0012 and p< 0.0001, respectively). Anti-hCD31 or anti-PSMA mAb slightly reduced orthotopic NB growth but did not impact on survival. In vivo targeting of TEC with mAbs was followed by a significant increase in the proportions of both apoptotic and proliferating TEC, indicative of ongoing vascular remodeling. This latter process was fueled by trans-differentiation of TNC+ NB cells into TEC and involved epithelial-mesenchymal transition-like events occurring in mAb treated mice only. Thus, NB cells up-regulated mesenchymal proteins such as periostin and N-cadherin and down-regulated the epithelial marker E-cadherin. Prominent up-regulation of tumor-derived angiogenic genes expression occurred in anti-endothelial cell mAbs treated mice.

Conclusions: mAb therapy targeted to TEC is ineffective at curing human NB bearing mice. Alternative approaches are needed to hit angiogenesis and vascular mimicry.

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POB017

Whole-exome sequencing reveals second driver mutations in MYCN-amplified neuroblastoma

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Background: The genetic etiology of high-risk neuroblastoma has remained largely enigmatic. Some of these tumors bear amplification of the proto-oncogene MYCN, which is known to contribute essentially to the malignant phenotype. This study aimed at identifying additional somatic mutations that drive tumorigenesis of high-risk neuroblastoma using a whole-exome sequencing approach.

Methods: Whole-exome sequencing was performed on matched tumor-normal pairs of primary high-risk neuroblastomas (MYCN amplified, n=13; MYCN non-amplified, n=13). Somatic mutations were identified from the aligned sequencing data using a new variant caller, and were validated by Sanger sequencing or targeted re-sequencing on the Roche 454 platform.

Results: We detected a mean of 16 somatic variants per tumor, 12 of which were non-silent mutations. The mutation rate ranged from 0.06 to 0.97 per Mb of the coding genome, which is 2- to 32-fold lower than the average mutation rate reported in adult cancers. In addition, we observed a positive correlation (Spearman's rank correlation, r=0.50) between the patients' age at diagnosis and the mutation rate in the corresponding tumors. The overall mutation spectrum in high-risk neuroblastomas was heterogeneous: In the entire tumor cohort, non-silent mutations were detected in 326 genes, 317 of which were uniquely affected. We unexpectedly noticed, however, that 10 out of 13 MYCN-amplified tumors carried driver mutations in second bona fide cancer genes such as ALK, RAS and TP53. Additional mutations in these genes were detected in an independent set of 61 MYCN-amplified neuroblastomas, all of which occurred in mutually exclusive fashion.

Conclusions: Our findings point towards a previously unknown pathogenetic role of second major cancer signaling pathways in MYCN-amplified neuroblastoma, which may provide a rationale for targeted therapeutic interventions in these tumors. In addition, these results suggest that as few as two oncogenic events may be sufficient to establish malignant solid tumors in human.

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POB018

Genomic Heterogeneity within Neuroblastomas

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Aim: To analyze the variation in whole chromosome aberrations within neuroblastomas (NBs) and to create algorithms to model the process leading up to aneuploidy.

Background: NBs are tumours of the sympathetic nervous system, occurring predominantly in early childhood and accounting for 8-10% of all paediatric cancers. NBs exhibit extensive inter-tumour genetic heterogeneity, but the extent of intra-tumor diversity remains largely unexplored. Some tumours with favourable prognosis is characterised by multiple extra chromosomes, leading to hyperdiploid-triploid karyotypes.

Materials and Methods: We started by using SNP-array data to quantify the size of intra-tumoural subclones. Fluorescence in situ hybridization on tumour cell imprints and cultured NB wells was then used to assess copy number diversity in detail, primarily with respect to whole chromosome copy numbers. This was complemented by immunofluorescence-based detection of chromosome segregation errors to evaluate correlations between aneuploidy and mitotic defects and with in silico simulations of different mechanisms of chromosome missegregation.

Result: All cell lines as well as tumours showed exensive inter-cellular copy-number variations, with up to 75% and 73% of tumour cells showing non-modal chromosome numbers in primary tumours and cell lines, respectively. All cell lines also showed different types of aberrations during mitosis such as lagging, anaphase-bridges and, less commonly, multipolar mitoses in up to 3% of cell divisions.

Conclusion: NBs typically exhibit prominent intra-tumour genomic diversity, which may have a role in the chemotherapy resistance in some of these tumours. This diversity can to some extent be explained by errors of chromosome segregation at mitosis. Computer modelling showed that aneuploidy in NB can be recreated with the highest fidelity through a process of chromosome loss from the tetraploid level, fine-tuned by selection against cells with too many chromosome losses.

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POB020

No evidence for viral replication in deep transcriptome sequencing data of metastatic neuroblastoma with progressive stage 4 and regressive stage 4S

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Background: 15% of all cancers are assumed to be associated with infections of oncogenic pathogens. So far, investigations on the presence of viral replication signatures in neuroblastoma have not been reported. We generated transcriptome deep-sequencing data of regressive (stage 4S) and progressive (stage 4) neuroblastomas in order to identify such replicating viral agents.

Methods: An unbiased computational approach for investigating the replication of known and unknown viruses in human tumor samples was developed. Our method assigns deep sequencing reads from tumor samples to the human genome and all known viral genomes in parallel. mRNA splicing artifacts and close viral-human homologs are taken into account by a gapped and splicing-aware read aligner and by setting putative viral signatures in their context of close human and viral homologs. Whole-transcriptome deep sequencing reads were obtained from 7 stage 4S and 7 stage 4 neuroblastomas (all not MYCN-amplified). Three HPV-positive cervical cancer samples and one EBV-positive B-cell-lymphoma served as positive controls. All reads were mapped against the human genome and ~3900 known viral genomes, followed by de-novo transcriptome assembly of unmapped reads.

Results: Viral agents could unambiguously be identified within all positive control samples. Human transcripts with close viral homologs were correctly assigned to their human origin by our context-aware approach. In contrast, none of the neuroblastoma samples showed transcriptional activity unambiguously associated with any viral family. All ambiguous sequence reads in the neuroblastoma samples were more likely to represent human than viral transcripts. De-novo assembly of the unmapped reads followed by automatic sequence annotation did not result in any signatures likely to represent an unknown virus.

Conclusions: Based on this limited cohort, our results indicate that there are no viral agents actively replicating in whole-transcriptome deep-sequencing samples of progressive and regressive subtypes of metastatic neuroblastoma.

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POB021

Exome sequencing reveals few recurrent somatic mutations in neuroblastoma

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Background: Next generation sequencing allows systematic and cost-efficient analysis of the genomic landscape of cancer through detection of single nucleotide variations and indels. Here we have performed deep sequencing on the coding parts of the genome in order to determine the genetic background of neuroblastoma.

Methods: Whole-exome sequencing was performed on six paired neuroblastoma tumour/control samples and one additional tumour, representative of three major prognostic groups; MYCN-amplified, 11q-deleted and tumours with numerical chromosomal abbreviations only. Enrichment was made with Agilent SureSelect Human All Exon 50Mb, sequencing was performed on Illumina platforms with pair-end reads while alignment and variant calling was made with DNAnexus.

Results: At average 43 million mapped reads was generated per sample with a mean coverage of 16x. At average were 1100 novel protein variants called in tumour samples with minimum 10x coverage and >20% supporting reads. We found two somatic missense mutations in the ALK gene in two different tumours; the activating 3824G>A R1275Q mutation in exon 25 and a previously unpublished 1288T>A S430T mutation, located outside the tyrosine kinase domain. Screening of genes previously implicated in cancer showed heterozygous, non-silent variations for COL1A, FLT3, KTN, MLL3, NOTCH1 and XPC, indicating that neuroblastoma tumours have a heterogeneous mutation spectrum.

Conclusions: The finding of mutations in ALK gene is concordant with previous studies and further supports its contribution to neuroblastoma oncogenesis. Otherwise, neuroblastoma tumours displayed a heterogeneous mutation spectrum and additional studies of larger cohorts are required to elucidate the genetic contribution of the neuroblastoma pathogenesis.

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POB022

Whole-exome sequencing of advanced neuroblastomas

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Background: The molecular basis of neuroblastoma development and progression still remains poorly understood except for MYCN amplification and ALK mutations or amplification. The recent advances in high-throughput deep sequencing enable us to extensively screen the mutations occurring in cancer genomes. Using this technology, novel candidate genes have identified among adult and pediatric tumors. Thus, to understand the molecular pathogenesis of neuroblastoma, we undertook whole-exome sequencing of neuroblastoma genomes using next-generation sequencer.

Methods: Whole-exome sequencing was performed using DNA samples from three neuroblastomas and matched normal tissues (germline). All cases were diagnosed as stage 4, and two of which have MYCN amplification, simultaneously with an ALK mutation in one case. Coding regions were enriched by capture with the Agilent SureSelect kit and sequenced with 75-108 bp paired-end reads on Illumina GAIIx platform. After eliminating low quality data, the significance of mutation was evaluated by Fisher's exact test. The remaining candidates for somatic mutations which were not included in public and in-house SNP database were subjected to validation using Sanger sequencing.

Results: Total reads of three specimens (tumor/normal) were 76.4G/79.1G, 91.0G/89.4G and 231.0G/167.8G, respectively. Mean target coverage of each individual was at least 86.3, and 82 90% of all target bases achieved more than 19x coverage. In total, 17 somatic mutations were confirmed by Sanger sequence, of which 14 were missense, 1 nonsense and 2 frameshift mutations. There were no overlapping mutations among samples examined. A number of functionally-eligible target genes were included in the candidates, such as cell cycle related protein, phosphatase, deubiquitinating enzyme and RNA splicing factor.

Conclusions: Our results suggest that whole-exome sequencing is a powerful method to identify new gene targets involved in the pathogenesis of neuroblastoma.

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POB023

Detection Of Genes And Pathways Involved In The Development Of Aggressive Neuroblastoma Using Genome Copy Number Data

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Background: Neuroblastoma (NB) patients display two distinct types of genetic profiles, consisting of either numerical or segmental chromosome alterations. The latter type is generally associated with a poor prognosis. Such segmental chromosome alterations as loss of chromosomes 1p, 3p, 11q and gain of 1q, 2p, and 17q have been shown to be of prognostic impact. However, with a few exceptions, it is still unknown which genes are involved in NB progression.

Methods: We analyzed array CGH data for 270 NB patients present with segmental chromosome alterations. We explored the role of gene gains and losses in NB progression using local assessment of the differences in Kaplan–Meier curves with log-rank test (LRT) between groups of patients with different copy number status of each genomic region. We analyzed LRT q-values together with average copy number status (ACNS) of genomic regions. We selected genomic regions that corresponded to local extrema of LRT q-value profile and ACNS profile along the genome.

Results: We provide a list of genes, whose loss or gain has the highest predictive effect on the patient outcome. This list contains genes regulating chromatin during DNA repair, functioning in neurite outgrowth and axon guidance, etc. Interestingly, the only "important" gene detected on chromosome 2 was MYCN. ALK, also located on chromosome 2, did not come out from our analysis since we only studied whole gene amplifications and deletions.

Conclusions: We constructed a list of genes which are frequently lost or gained in NB and whose gain or loss status has the highest association with a poor outcome in their region. Biological validation of these genes shall give insights into mechanisms of NB progression. In future, the use of a classifier based on our gene list may be a step forward in personalized medicine strategy for treatment of NB patients.

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POB024

Identification of familial neuroblastoma associated genes by whole exome sequencing

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Background: Although neuroblastoma (NB) usually occurs as a sporadic tumor, a familial recurrence is observed. Interestingly, in a large pedigree (IGG-E), where ALK has been identified as a major NB predisposing gene, we previously reported a co-segregating region on chromosome 12p, suggesting that other predisposing and/or modifier genes are yet to be discovered. In this study, we sequenced the whole exome of two distantly related NB patients and two unrelated relatives from family IGG-E in order to identify novel mutations involving genes that might contribute to NB development.

Methods: Germline DNA samples were sequenced in paired-end mode on the Illumina HiSeq 2000, obtaining over 120 million short (100 bp) reads. After a quality control of sequencing data by FastQC, filtered sequences were aligned on the human reference genome (GRCh37, ENSEMBL database) using Novoalign software. SNPs were called with Samtools and reported in VCF format. The variants were annotated through the SeattleSeq Annotation database.

Results: Our analysis identified an average of 70,703 SNPs in the four samples. Of these, 7,263 were missense mutations, 77 were nonsense and 28 were in splicing sites. Preliminary analysis of results identified 6,999 SNPs shared by the two affected and not by the two unrelated individuals and 255 were not described in the dbSNP database. Giving priority to unknown missense mutations we found out 30 candidate genes over the genome, including ALK. Moreover, a total of 459 variations, shared only by the two NB patients map in the region we previously identified on chromosome 12p and involve 72 genes, including DYRK4, NTF3, PTPN6, DUSP16, PTPRO and LMO3.

Conclusions: Whole exome sequencing identified candidate genes that may contribute to NB development. We are now performing Sanger sequencing to confirm these mutations in all IGG-E family members as well as in sporadic NB cases and healthy unrelated individuals.

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POB025

R2: A Public User-friendly Website For integrated Analysis Of Expression Data And Associated Clinical Parameters In Neuroblastoma.

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Background: Microarray analyses have established gene expression profiles in neuroblastoma series and provided prognostic signatures. Such data also contain a blueprint of all pathways and genes relevant for neuroblastoma, but this information can be difficult to retrieve.

Methods: We designed a web-based program to facilitate functional analysis of mRNA expression data of neuroblastoma. Next to a series of 88 richly annotated neuroblastoma samples generated in our lab, the database also contains in addition, a range of other public neuroblastoma datasets and thousands of microarrays from other tumor types and normal tissues.

Results: The R2 program has a user friendly interface enabling a wide range of rapidly executed analyses. Any clinical or biological group can be analysed for differentially expressed genes (e.g. MYCN amplified vs. single copy). Also significant correlations of any gene (e.g. MYCN) with all other genes can be calculated. The results are graphically displayed and the obtained gene lists can be analysed for pathways and functional categories (Gene Ontology, KEGG mapping). Also microarray data from cell lines with ectopic expression of e.g. MYCN are included. MYCN-target genes can be identified and compared to the correlations found in the tumor series. In addition, such functional signatures can be used to classify patient cohorts. R2 can also calculate Kaplan Meier curves based on the expression of each gene, and scans for the strongest prognostic factors in any chosen subtype of neuroblastoma. Prognostically highly significant expression profiles were thus identified. All data are linked to external database like PubMed, KEGG and GeneCards.

Conclusions: R2 provides a valuable resource for high throughput data of Neuroblastoma. The R2 program and database has been used in a number of publications and is publicly accessible via http://r2.amc.nl. R2 will help researchers in identifying important genes and biological processes in neuroblastoma.

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POB026

Identification of molecular subgroups and pathways in pheochromocytomas and paragangliomas

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Background: Pheochromocytoma (PC) and paraganglioma (PG) are heterogeneous tumour types of the sympathetic nervous system that show several similarities to neuroblastoma. Around 32% of PC & PG display mutations in one of nine susceptibility genes; NF1, VHL, RET, SDHA, SDHB, SDHC, SDHD, SDHAF1, or SDHAF2. To date clinical, biochemical and histological features cannot reliably distinguish malignant from benign tumours and there is a need of better prognostic markers. The aim of the present study was to use a discovery-based approach to identify tumour subgroups and affected pathways at the molecular level.

Methods: Whole genome microarray gene expression analysis using Agilent 44K microarrays was performed for 57 primary PC/PG tumours. Principal Components analysis using the Omics Explorer software (www.qlucore.se) was used for subgroup discrimination.

Results: Our results identified three distinct tumour groups, with SDHB- (3 out of 4) and VHL-mutated tumours (1 out of 1) mainly represented in the first group (n=12). RET- (3 out of 5) and NF1-mutated tumours (2 out of 3) were overrepresented in the second group (n=39); all tumours of this group displayed very high RET expression. The third group (n=6) was small and spread; most tumours of this group displayed high expression of PDGFRA. Also a network modelling strategy is currently being utilized to identify downstream factors and pathways affected in subgroups of PC/PG. Expression groups will be verified by RNA-seq using overlapping and additional tumour sets. Differentially expressed genes will be validated by real-time PCR at the protein level by tissue microarrays.

Conclusions: Our preliminary data are in line with previous results, i.e. PC/PG tumours were divided into two expression groups: SDH /VHL- and RET/NF1-related tumours, respectively. By identification of dysfunctional pathways associated with different tumour subtypes, the findings of this study will hopefully contribute to personalized therapeutics for PC/PG patients.

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POB027

Impact of MDM2 SNP309 on the survival of neuroblastoma patients

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Background: Neuroblastoma is a pediatric tumor with typically a low percentage (<2%) of p53 mutations at diagnosis. Intact p53 suggests that the p53 pathway is under negative control of upstream or downstream components. MDM2 has been clearly shown to inhibit p53 function and this interaction can be disrupted using small molecule inhibitors such as Nutlin. Also, a particular polymorphism located within the MDM2 promoter region, SNP309, has previously been associated with increased cancer risk in some tumor entities or protection against certain other cancers.

Methods: Using TaqMan SNP genotyping, we evaluated the effect of MDM2 SNP309 on the survival and clinic-genetic characteristics in 400 neuroblastoma patients.

Results: No significant difference in overall survival was observed among neuroblastoma patients comprising all stages. However, the data from stage 4 patients reveal a statistically significant difference between the survival rates over time with patients being heterozygous (G/T) having the worse overall survival. The presence of the SNP was not associated with the timing of cancer onset or MYCN status.

Conclusion: We are currently investigating the relationship between the different allele situations, expression levels of MDM2 and survival of neuroblastoma patients.

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POB028

miR-137 exerts tumor suppressive functions mediated via downregulation of the epigenetic target enzyme LSD1 in neuroblastoma cells

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Background: The histone demethylase lysine-specific demethylase 1 (LSD1) is strongly expressed in neuroblastoma (NB), and its overexpression has been correlated with poor patient prognosis. Differentiation of NB cells resulted in down-regulation of LSD1 and siRNA-mediated LSD1 knockdown induced growth inhibition of NB cells. The microRNA miR-137 has been reported to be downregulated in several human cancers, and LSD1 was reported as a putative target of miR-137 in colon cancer. Based on these data, we hypothesized that miR-137 might have a tumor suppressive role in NB mediated via down-regulation of LSD-1.

Methods: MiR-137 and LSD1 expression was re-analyzed in pre-existing next-generation RNA sequencing and microarray expression data from 69 primary NBs. The effect of either LSD-1 knockdown or miR-137 re-expression in the NB cell lines, SHEP, IMR-32 and SK-N-BE was assessed on cell viability, proliferation and apoptosis. Reporter assays were performed to assess miR-137 targeting of LSD1.

Results: High levels of miR-137 expression in primary NBs were correlated with poor patient prognosis. Re-expression of miR-137 in NB cell lines increased apoptosis and decreased cell viability and proliferation. LSD1 was repressed by miR-137 in NB cells, and was validated as a direct target of miR-137 using reporter assays in SHEP and HEK293 cells. Furthermore, siRNA-mediated LSD1 knockdown phenocopied the miR-137 re-expression phenotype in NB cells.

Conclusions: We have shown that miR-137 directly targets LSD1 in NB cells, and activates cell properties consistent with tumor suppression. Therapeutic strategies focused on the re-expression of miR-137 in NBs could be useful to reduce NB aggressiveness.

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POB029

MicroRNA-125b regulates proliferation and differentiation of Neuroblastoma Cells by targeting Lin28b

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Background: Neuroblastoma is the most common solid tumor of pediatric malignancy. The tumor arises from the neural crest precursors of the sympathetic nervous system and represents aberrations of normal development programs. MicroRNA belongs to the short non-coding RNA family known to play important roles in development and tumorigenesis. MicroRNA-125b (miR-125b) is enriched in neural cells, and up-regulated during neuronal differentiation. However, the biological roles of miR-125b in neuroblastoma development are undetermined.

Methods: In this study, we used a lentiviral-based approach to established miR-125b-overexpressed neuroblastoma cell SK-N-DZ. We focused on the effects of miR-125b in proliferation, invasion and colony formation ability of neuroblastoma. The putative targets of miR-125b were predicted using bioinformatic approaches. The signaling pathways affected by the target of miR-125b, Lin28b, were analyzed by microarray. The expression of Lin28b and N-myc in neuroblastoma patients was assayed by qRT-PCR.

Results: Over-expression of miR-125b changed the cell morphology to neuron-like cells and inhibited proliferation by arresting cell cycle at G1 to S transition. MiR-125b also reduced neuroblastoma cell invasion and colony formation ability. In this study, we found Lin28b was the downstream target of miR-125b by bioinformatic methods. Overexpression of miR-125b suppressed the expression of Lin28b in both RNA and protein levels. Suppression of Lin28b by specific shRNA recapitulated the tumor suppressive effects of miR-125b and induced differentiation. Microarray analysis showed that knockdown of Lin28b significantly down-regulated the expression of N-myc and enhanced the expression of CDKN1A (p21). Notably, we examined the expression of Lin28b in primary neuroblastoma patients and showed that were positively correlated to N-myc expression. Highly expression of Lin28b in neuroblastoma of non-infant patients was marginally associated with poor prognosis.

Conclusions: These findings suggested that miR-125b exerted tumor-suppressive effects by suppressing Lin28b in neuroblastoma. Targeted repression of Lin28b might be a potential therapeutic strategy for neuroblastoma.

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POB030

Identification of prognosis-related miRNA expression profiles in neuroblastoma

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Background: Neuroblastoma (NB) is known to exhibit wide ranges of clinical behavior. We have been constructing the genomic and genetic profiles based risk classification system for NB. Recent studies indicated that miRNA expression levels are strongly correlated with patient prognosis of NB. To further improve our system by adding the miRNA classifier, we conducted miRNA expression profiling of favorable (F) and unfavorable (UF) NBs and searched for the prognosis-related miRNAs in our sample set.

Methods: 48 primary samples (19 stage 1 or 2; 16 stage 3 or 4 without MYCN amplification; 13 stage 3 or 4 with MYCN amplification) were analyzed by miRNA microarray (Agilent G4470A). Statistical analysis was conducted by using the information of patient outcome at 3 years after diagnosis.

Results: Among the 470 miRNAs analyzed, 79 miRNAs showed strong correlation to the prognosis ($p<0.05$): 17 and 62 miRNAs showed higher expressions in UF and F phenotypes, respectively. In the former miRNA group, 5 were located in 1q whose genomic copy number is frequently increased in UF type. On the other hand, the latter includes 5, 6 and 9 miRNAs in 1p, 19q13 and 14q32, respectively, which are frequently decreased in copy numbers in UF type. In addition, miR-542-5p, whose expression has been reported as a prognostic marker by other groups, was also in the latter group in our dataset ($p<0.001$, 3.3-fold lower in UF). Interestingly, miR-149 ($p=0.003$, 4.0-fold lower in UF) was one of the top-ranked members in the latter, and it has been reported that it has pro-apoptotic function by repressing Akt1 and E2F1 expressions in a neuroblastoma cell line.

Conclusions: The result suggested that miRNA expression profile exhibited strong correlation with NB prognosis, and combination with mRNA and genomic markers could improve the prediction system for NB prognosis.

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POB031

Genome-wide profiles reveal candidate miRNAs potentially involved in the regulation of ALK expression in neuroblastoma

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Background: We previously reported that ALK expression is significantly up-regulated in advanced/metastatic neuroblastoma (NB). Growing evidences have indicated that the deregulation of miRNAs contributes to the tumorigenesis and miRNAs are now recognized as a crucial family of molecules that target the transcriptome and regulate protein expression. Here, we hypothesized that miRNAs might be involved in the regulation of ALK expression in NB. Hence, we selected 16 NB samples among 48 of which we determined ALK expression, dividing them into two groups, showing either high or low ALK mRNA levels and we performed a genome-wide profiling to identify differentially expressed miRNAs.

Methods: Genome-wide profiling was performed on Human microRNA Microarray Release 14 (Agilent Technologies) containing 866 human miRNA probes. Slides were scanned and images extracted using Feature Extraction. Tab-delimited text files were analyzed in R using the limma package of Bioconductor. Probes with less than 50% of detected spots across all arrays were removed from the analysis. Data were log2-transformed and normalized for between-array comparison using quantile normalization. Differential expression analysis of miRNAs was performed using Student's t-test.

Results: The expression dataset considered for the quantitative analysis contained 531 miRNAs and 16 NB samples. We found out 27 differentially expressed miRNAs (raw p-value<0.05) between the two groups of NBs. Four out of 27 miRNAs showed a p-value<0.01(miR-218, miR-551b, miR-29c* and miR-130b*). Interestingly, two miRNAs from our list (miR-29c and miR-29b), which resulted down-regulated in high ALK expressing tumors, have already been described to be also down-regulated in ALK driven anaplastic large cell lymphomas.

Conclusions: In conclusion, we have identified 27 candidate miRNAs, which are differentially expressed between high and low ALK expressing NB samples and that may play a role in the pathogenesis of ALK driven NBs. These miRNAs may potentially affect ALK expression and regulate its activity in NB cells.

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POB032

miR-18a inhibits differentiation of MYCN-amplified neuroblastoma by deregulation of estrogen and NGF signaling

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Neuroblastoma is the most common tumor of the sympathetic nervous system and results in the highest number of cancer–related deaths in infants. One of the few prediction markers for poor outcome is amplification of MYCN. The MYCN amplified tumors frequently
co-expresses the TrkB neurotrophin receptor, while expression of the nerve growth factor (NGF) receptors TrkA and p75NTR negatively correlates with MYCN amplification and are associated with differentiated tumors with good prognosis. In an effort to delineate the molecular consequences in MYCN-driven neuroblastoma cells we have recently identified a role for the MYCN-regulated miRNA cluster miR17-92 in maintaining an undifferentiated phenotype. We demonstrated that MYCN overexpression as well as miR-18a upregulation downregulate the nuclear hormone receptor estrogen receptor alpha (ERα), a transcription factor involved in neuronal differentiation and development of the sympathetic nervous system. We further showed that ERα expression correlated with favorable disease outcome and an inverse relationship with MYCN expression. In addition, ectopic ERα expression led to neuronal differentiation of neuroblastoma cells in vitro. ERα has been shown to co-localize with NGF and its receptors both in adult and in the developing brain and cross-talk between estrogen and NGF signaling has been suggested to be required for neurite formation. We have found that activation of estrogen signaling either through ectopic ERα expression or by inhibiting miR-18a expression led to induction of NGF receptors. In addition, co-activation of estrogen and NGF signaling in neuroblastoma cells resulted in a prominent neuronal differentiation phenotype and expression of neuronal markers. Our results propose a novel pathway of how MYCN can maintain an undifferentiated phenotype in neuroblastoma through the activation of miR-17~92, which targets ERα leading to disruption of NGF signaling and inhibition of differentiation

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POB033

An unbiased high-throughput microRNA library screen identifies microRNA-interactomes of key neuroblastoma genes

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Background: MicroRNAs are small, non-coding RNA molecules that negatively regulate the expression of protein coding genes at the post-transcriptional level, most often through interaction with the 3'UTR. Although deregulation of miRNA function has been widely implicated in cancer and neuroblastoma pathogenesis, knowledge about which miRNAs interact with protein coding tumor suppressor and oncogenes is scarce, making it difficult to appreciate the true complexity of miRNA-cancer gene interactions. To address this issue we performed an unbiased high-throughput miRNA library screen to identify miRNA regulators of genes implicated in the pathogenesis of neuroblastoma and other cancers.

Methods: Interactions of 470 individual miRNAs with selected cancer genes (including neuroblastoma genes MYCN, MYC, ALK, PHOX2B and TP53) were explored with 3'UTR reporter gene assays and RT-qPCR upon miRNA overexpression and silencing, capturing expression changes on both mRNA and protein level.

Results: The majority of known miRNA-neuroblastoma gene interactions could be confirmed and various novel interactions could be identified. Noticeable, extensive regulation by multiple miRNAs is observed for MYCN and MYC, whereas ALK seems largely free of miRNA regulation. We also observed that the coordinated action of different miRNAs of the interactome can lead to more pronounced downregulation of neuroblastoma genes, which makes a case for the therapeutic use of miRNA mimic pools.

Conclusions: The screening effort presented here significantly contributes to the mapping of the complex interplay between miRNAs and protein coding genes involved in neuroblastoma pathogenesis. The resulting interactome catalogue helps prioritizing miRNAs for therapeutic use and better understanding of neuroblastoma disease pathways.

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POB034
Micro RNA-21 regulates the cisplatin resistance in human neuroblastoma cells
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Neuroblastoma is the most common malignancy affecting infants and young children. Current treatment regimens include surgery removal, radiotherapy and chemotherapy with combined use of anti-neoplastics. Cisplatin, a DNA damaging drug, is one of the most common chosen therapeutic agents. The effectiveness of treatment, however, remains unsatisfied for the incidence of drug resistance during treatment. MicroRNAs (miRNAs) are small RNAs with 19-23 nucleotides in length and function as a master regulator in cellular functions and homeostasis by binding to their target mRNAs, which is followed by RNA degradation or translation inhibition. Increasing evidences have implicated the significance of miRNA in tumorigenesis as well as tumor suppression. In this research, we aimed to investigate the role of miRNA in cisplatin resistance of neuroblastoma cells by comparing the expression of certain MYCN-related or neuroblastoma-associated miRNAs between parental and cisplatin-resistant cells. As revealed by miRNA quantification, in cisplatin-resistant neuroblastoma cells there was a significant increase in the expression of two tumorigenesis miRNAs, miR-21 and miR-143. However, only the antagomir against miR-21 but not miR-143 was able to convert resistant cells into sensitive ones. Also, ectopic expression of miR-21 in parental cells resulted in decreased sensitivity to cisplatin treatment. Furthermore, over-expression of miR-21 markedly hastened the proliferation rate of neuroblastoma cells, which would contribute to the reduced response toward cisplatin treatment. By analyzing the expression of PTEN, a proliferation-related miR-21 target, we found significantly lower level of PTEN mRNA in the resistant cells. In conclusion, these results demonstrated that miR-21 functioned as a regulator of drug resistance in neuroblastoma cells by modulating proliferation rate through inhibition on the expression level of PTEN. This provides a novel insight as using miR-21-specific inhibitors for the treatment of drug-resistant neuroblastoma cells.

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POB035
p53 expression and functional status in a TH-MYCN transgenic murine neuroblastoma cell line
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Background: The TH-MYCN transgenic murine model of neuroblastoma is an established system which recapitulates many features of human neuroblastoma, and is used for both molecular and preclinical studies. In neuroblastoma, p53 mutations are rare at diagnosis but inactivation of the p53 pathway is observed in relapsed tumours (Carr-Wilkinson et al, Clin Cancer Res, 2010). Consistent with this, introduction of TH-MYCN into a p53 insufficient background enhances MYCN-driven transformation, with concomitant decreased apoptosis and increased chemotherapy resistance (Chesler, et al, Neoplasia, 2008).

Methods: The expression and functional status of p53 were determined in the TH-MYCN mouse neuroblastoma 3402A cell line (Xue et al, Eur J Cancer, 2007). p53 mutational status was determined using automated gene sequencing of exons 5-8. The p53 functional response to DNA damage was assessed following 4Gy ionising radiation by flow cytometry for a G1 cell cycle arrest and by Western blotting for induction of p53 and p53 target genes MDM2 and p21WAF1.

Results: High basal levels of p53 were found in the 3402A cell line. Using gene sequencing, no mutations were detected in exons 5-8 of the p53 gene. However, after irradiation induced DNA damage, cells failed to G1 cell cycle arrest and there was no induction of p53, MDM2 or p21WAF1.

Conclusions and Future Work: The data shows that p53 is stabilised and non-functional in the 3402A cell line suggesting that if p53 is inactivated by mutation in this cell line, the mutation is outside of exons 5-8, or that p53 is inactivated by an alternative mechanism. Further experiments are in progress to extend these observations to a panel of TH-MYCN transgenic murine neuroblastoma cell lines and to determine their response to treatment with the potent MDM2/p53 antagonist, Nutlin. Murine TH-MYCN systems with inactivated p53 are valuable models for the study of chemotherapy resistant relapsed neuroblastoma.

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POB036
Characterization of tumor development, growth characteristics and spectrum of genetic aberrations in the TH-MYCN mouse model of neuroblastoma
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Background: The TH-MYCN transgenic mouse model provides a powerful tool to study neuroblastoma tumorigenesis and MYCN-dependent growth in vivo. We further characterized this model by sequencing the MYCN insert, studying tumor development and growth, and analysing genetic aberrations in the acquired tumors.

Methods: By primer walking, we sequenced the MYCN insert in the plasmid that was used to create the TH-MYCN model, and genomic TH-MYCN mouse DNA. We also performed copy number analysis of Affymetrix® Mouse Diversity Genotyping Arrays from 10 tumors.

Results: We provide the nucleotide sequences of the MYCN insert which confirm that it corresponds to the amino acid coding region of human MYCN cDNA. Following a total of 395 animals revealed no difference in perinatal survival of the three genotypes, respectively. However, some transgenic animals spontaneously died at an early age. In our hands 44% of the hemizygous mice developed palpable tumors between six to 19 weeks of age, median 9.1. All but one homozygous mouse developed a palpable tumor within four to eight weeks of age, median 5.4. Time from a palpable tumor to sacrifice, the "treatment window", for hemizygous mice was 14.6 days, independent of age at tumor development. In contrast the treatment window for homozygous mice was 5.2 days and early palpable tumors showed significantly longer treatment windows. Hemizygous mice developing palpable tumors as early as homozygous mice had a longer treatment window than homozygous mice, 15.4 versus 5.2 days, respectively. Array analyses revealed that seven out of 10 analysed tumors had a flat profile with no detectable segmental or numerical aberrations. Three tumor samples had one or more numerical aberrations. Of these, one had an event that corresponds to gain of human chromosome 17, no events corresponded to deletion of human 1p or 11q.

Conclusions: Our data emphasize that genotyping is essential to evaluate any therapeutic intervention since hemizygous and homozygous mice overlap in the time to tumor development but have different treatment windows. In addition, contrasting previous studies our data show that TH-MYCN tumors have very few genetic aberrations. This suggests that the oncogenic effect of the MYCN transgene is sufficient for significant early tumor development in this model.

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POB037
Identifying genes underlying bone marrow metastasis in neuroblastoma
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Background: Bone marrow is the most frequent site for neuroblastoma metastasis and the most common site of relapse. T his study aimed to identify genes contributing to the efficient colonization of the bone marrow by neuroblastoma cells.

Methods: Bioluminescent SK-N-BE(2) human neuroblastoma cells were injected into the tail vein of immunodeficient mice, with metastases arising in the adrenal medulla, liver and bone marrow. Tumor cell populations were then isolated from bone marrow and engrafted into secondary recipients to test for increased tropism to this site. Cell populations with varying ability to grow in bone marrow were then compared using whole genome expression analyses.

Results: Ten SK-N-BE(2)-derived cell populations were isolated from bone marrow. On engraftment into secondary recipients, each line efficiently colonized bone marrow, although their growth rates were variable. Surprisingly, an even greater variability for their growth in the liver was observed, and several lines were highly selective for bone marrow growth over liver growth. CXCR4 expression was substantially higher (at mRNA level) in the bone marrow selective lines, consistent with its established role in homing to bone marrow, and confirming the validity of the approach. Further gene expression analyses revealed a number of candidate genes that were associated with altered bone marrow and liver growth, including PAK2, SAMD11 and SCGN.

Conclusions: In vivo selection methods can be used to develop neuroblastoma cell populations with altered propensity to colonize bone marrow and liver. This approach may allow the identification of candidate genes underlying colonization and growth at each site. Candidate genes identified using theses methods will require further validation in both cell culture and animal models.

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POB038

The development of metastatic mouse models of neuroblastoma to bone and brain to identify the molecular mechanisms governing metastasis.

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Background: Metastasis is a key reason for the lack of success in treating children with neuroblastoma. Over 50% of patients present with metastatic lesions in the bone, brain, liver and lymph nodes, with a high fatality rate for patients that relapse with those metastases. Consequently, a better understanding of the molecular processes defining the complexity and multi-step nature of tumour cell dissemination is required for the effective treatment. Thus, we developed novel metastatic mouse models of neuroblastoma to bone and brain through in vivo selection to enhance metastatic capability and organ-specific tropism.

Methods: SK-N-AS, derived from a bone marrow metastasis, and IMR-32, a MYCN-amplified line derived from an abdominal mass, were tagged for in vivo monitoring using a triple reporter (TR) encoding TK, GFP and firefly luciferase, which allows for nuclear imaging, FACs analysis, and whole body bioluminescence. Reporter expression was confirmed by double sorting for GFP and testing for luciferase activity. Cells were introduced into the blood stream of NOD/SCID mice through intra-cardiac injection, and monitored bi-weekly using bioluminescence.

Results: SK-N-ASTR cells metastasized to the adrenal gland (87%, 13/15 animals) and bone (mandible, hindlimbs, 67%, 10/15 animals), whereas IMR-32TR cells metastasized to bone (100%, 10/10 animals) and brain (40%, 4/10 animals). Metastatic lesions were confirmed by ex-vivo bioluminescence, 3D bioluminescence, MRI or micro-CT. Cells from specific metastatic sites of individual mice were isolated, expanded in culture, and GFP sorted. These metastatic sub-populations were re-injected into a second cohort of mice. Metastases were established and are being analyzed for enhanced metastatic capability and organ-specific tropism. These sub-populations will be characterized to identify the molecular mechanisms governing organ-specific metastasis.

Conclusions: We have developed novel metastatic models of neuroblastoma that represent experimental systems for the identification of novel signaling pathways mediating organ-specific metastasis as well as drug discovery and validation.

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POB039

Molecular-Genetic Detection Of Minimal Disease In Neuroblastoma.

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Minimal disease (MD) was analyzed in neuroblastoma patients by quantitative PCR using TH, Phox2B as biomarker genes and GUS and B2M as control genes. Peripheral blood (PB) (n=32) and bone marrow (BM) samples (n=154, including those from left (n=48), right (n=49) os ilium or from sternum (n=2)) were analyzed. Samples from healthy donors and patients with other solid tumors were used as a control.

Paired comparison of TH expression from different BM locations showed discrepancy between MD level ($r=0.71$, $p<0.05$), that could be important when one of two samples gives negative result. It can reflect absence of residual tumor cells in negative sample, minute quantity of such cells in background of normal BM cells or weak TH activity in CD34+ cells of restoring BM. PB and BM samples from the same patient also showed variations in TH level ($r=0.66$, $p<0.05$). Analysis of MD in individual patients during therapy revealed that in some cases level of TH expression reflected general trends of tumor burden changes (correlated with relapse development), while in other patients expression varied depending on therapeutic interventions (surgery, chemotherapy) and could not reliably predict relapse especially in non-hematopoietic compartment. Expression of TH, PHOX2B as well as control genes (GUS and B2M) was compared in NB samples. It was found that GUS and B2M expressions were highly correlated ($r=0.99$, $p<0.05$, $n=63$), while TH and Phox2b had lower level of correlation ($r=0.74$, $p<0.05$, $n=63$).

We can conclude that TH, PHOX2B are sensitive and rather specific markers for detection of neuroblastoma cells. Their expression level can vary depending on therapeutic interventions. Level of biomarkers' expression reflects general trends of tumor burden change, but it can not reliably predict relapse, especially in non-hematopoietic tissues and can give false-negative results depending on location analyzed.

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POB040

MINA expression in neuroblastoma correlates with unfavorable patient outcome

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Background: The MYC-induced nuclear antigen (MINA) is overexpressed in many cancer types, including HCC and lung cancer. MINA is induced by cMYC and has been shown to exert a strong effect on proliferation of these tumor cells. Like many demethylases and methyl-histone interacting proteins, the MINA protein harbors a JmjC domain, implicating MINA as a possible regulator of chromatin structural modulation and transcription. We here aimed to analyze the role of MINA expression in neuroblastoma (NB).

Methods: MINA expression was analyzed in 88 primary NB using Affymetrix microarrays, and confirmed using RT-qPCR in an independent NB cohort of 351 NB. MINA expression was analyzed by RT-qPCR and western blotting in a NB cell line panel as well as the tamoxifen-inducible SHEP-NMYC-ER cell model. Transient (siRNA) and stable (shRNA) MINA knockdown was carried out in the NB cell lines Kelly and IMR32, and cell viability, proliferation and apoptosis was subsequently assessed.

Results: High MINA expression in primary NBs was significantly inversely correlated with event-free and overall survival in patients ($p < 0.001$) and strongly positively correlated with MYCN amplification in the tumors ($p < 0.001$). These results were confirmed in an independent cohort of 351 NB using RT-qPCR. High expression levels of MINA were confirmed in a panel of 4 amplified and 4 nonamplified NB cell lines using RT-qPCR and western blotting. MINA expression was also strongly induced by MYCN activation in SHEP-NMYC-ER cells. Targeting MINA expression by siRNA and shRNA significantly decreased cell viability and proliferation of NB cells and induced massive apoptosis.

Conclusion: MINA is a new target gene regulated by MYCN in NB cells, and MINA expression in primary NBs has prognostic value for NB patients. It has potential as a drug target circumventing the need to target MYCN directly in MYCN-dependent tumors, and warrants further functional investigation.

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POB041

The role of NEDD9 in neuroblastoma cell migration

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Background: Neuroblastoma is the most common extracranial solid tumour in children and despite advances in treatment only 40% of neuroblastoma patients are currently treated successfully. The very poor survival rate for patients with Stage IV disease is due to the extensive dissemination of the tumours throughout the body, preventing successful therapy. The neuroblastomas are grouped together with melanoma skin cancers and glioma brain tumours as neuro-ectodermal tumours. Given that the focal adhesion protein NEDD9/Cas-L/HEF1 has been shown to promote migration and metastasis in melanoma and glioma, we therefore questioned the role of NEDD9 in neuroblastoma cell migration.

Methods and Results: Analysis of publicly available data revealed a statistically significant, inverse correlation between NEDD9 expression and survival in neuroblastomas lacking MYCN amplification. By time-lapse imaging and confocal reflection microscopy, we have examined a panel of neuroblastoma cell lines that lack MYCN amplification and find that the cells with high level NEDD9 expression migrate in 3-D collagen gels in an elongated manner at a high speed. This is accompanied by obvious attachment to collagen fibres at the leading edge of the cells. As the targeting of NEDD9 to focal adhesions is critically associated with its function, we have begun to analyse how NEDD9 is targeted to focal adhesions. By immuno-fluorescence and fluorescence recovery after photobleaching (FRAP) analysis, our data reveal that NEDD9 localization to focal adhesions requires Focal Adhesion Kinase (FAK), while the rate of NEDD9 molecular exchange at focal adhesions is regulated by activity of Src family kinases.

Conclusions: Collectively, our data suggest that NEDD9 may be a key regulator of migration in invasive neuroblastoma. Significantly, we find that NEDD9 targeting to focal adhesions is dependent on FAK and Src, two kinases that are currently being targeted in novel anti-cancer therapies.

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POB042

A constitutively active TrkB receptor is sufficient to confer a highly aggressive and transformed phenotype in a rat neural crest derived cell line

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Background: Neuroblastoma arises from sympathoadrenal progenitors of the neural crest and expression of the neurotrophin receptor TrkB and its ligand, brain-derived neurotrophic factor (BDNF) is predictive of a poor prognosis and a more aggressive form of disease. Although activated TrkB signaling enhances the malignant phenotype of established neuroblastoma cell lines, whether TrkB signaling is sufficient to transform normal neural crest derived precursors has not been investigated.

Methods: We removed the two immunoglobulin-like ligand binding regions from the extracellular domain of the full length rat TrkB receptor (termed ΔIgTrkB) and stably transfected a rat neural crest cell line, NCM-1. Properties of the ΔIgTrkB expressing cell line were compared to those of the parent NCM-1 line (WT NCM-1).

Results: ΔIgTrkB is expressed at the cell surface and is constitutively active as shown by a 6-fold activation of phosphorylated Erk1/2 over basal levels ($p < 0.0001$). In PC12 cells, ΔIgTrkB promoted a 6-fold increase in process outgrowth ($p < 0.0001$), demonstrating the receptor is biologically active. In stably transfected ΔIgTrkB NCM-1 cells, significantly increased proliferation was observed, leading to a 2.5-fold increase in calcein AM fluorescence over WT NCM-1 cells after 4 days ($p < 0.0001$), together with a significant increase in protein expression of phospho-histone H3 ($p < 0.05$), a protein expressed preferentially in dividing cells. ΔIgTrkB expression also led to a 25-fold increase in anchorage independent cell growth in soft agar ($p < 0.001$), a 4-fold decrease in detachment-induced apoptosis ($p < 0.01$), and a 2.5-fold increase in matrigel invasion ($p < 0.05$). Furthermore, expression of ΔIgTrkB led to upregulation of transcripts encoding oncogenes including Cyclin D1 (436-fold, $p < 0.0001$), Twist1 (38-fold, $p < 0.0001$), and Hgf (29-fold, $p < 0.0001$), as well as downregulation of tumor suppressors such as Pten (-1.7-fold, $p < 0.001$) and Rb1 (-1.8-fold, $p < 0.001$).

Conclusions: These results indicate activated TrkB signaling is sufficient to promote the formation of a highly malignant phenotype in neural crest derived cells.

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POB043

Regulation of the wnt inhibitor DKK-1 in neuroblastoma

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Introduction: DKK1 is a gene frequently downregulated in many forms of cancer. It has been shown to be a marker of good prognosis in neuroblastoma, and to inhibit proliferation when overexpressed. DKK1 is known to act as an inhibitor of the canonical WNT pathway. Recent studies have demonstrated it to be inversely correlated with MYCN, both in expression analyses of tumor samples, and experimental studies. However, the nature of this regulatory relationship has not been identified. MYCN acts by inhibiting other genes by functioning as a transcription factor, it also acts by upregulating miRNAs and recruiting DNA methyltransferases and histone acetylators. Chromatine immunoprecipitation studies have revealed that it does not bind directly to the DKK1 promoter. We pursued to investigate the elusive regulatory relationship between MYCN and DKK1.

Methods: Neuroblastoma cells were incubated with 5-aza-2′-deoxycytidine(5-aza) and Trichostatin A to demethylate promoters and deacetylate histones respectively. The expression of DKK1 and the GAPDH/SDHA mRNA were investigated using qRT-PCR. Promoter methylation was investigated using methylation specific PCR(MSP).

Results: We found a close relation between MYCN knockdown and DKK1, as previously described. 5-aza treatment restored DKK1 expression in MYCN amplified neuroblastoma cell lines. We have further investigated the effect of MYCN overexpression and repression on the methylation status of the DKK1 promoter.

Conclusion: Our results indicate that DKK1 is methylated in the promoter region in MYCN amplified tumors.

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POB044

Individual caspase-10 isoforms play distinct and opposing roles in the initiation of death receptor-mediated tumour cell apoptosis.

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Background: The cysteine protease caspase-8 is an essential executioner of the death receptor apoptotic pathway. The physiological function of its homologue caspase-10 remains poorly understood, and the ability of caspase-10 to substitute for caspase-8 in the death receptor apoptotic pathway is still controversial. Both caspases-8 and –10 are often co-silenced in childhood tumour. Here we analysed the particular contribution of caspase-10 isoforms to death receptor-mediated apoptosis in neuroblastoma cells characterized by their resistance to death receptor signalling.

Methods: 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: 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. Overexpression experiments in various caspase-8 expressing tumour cells also demonstrated that caspase-10A and caspase-10D isoforms strongly increased TRAIL and FasL sensitivity, whereas caspase-10B or caspase-10G had no effect or were weakly anti-apoptotic. Further investigations revealed that the unique C-terminal end of caspase-10B was responsible for its degradation by the ubiquitin-proteasome pathway and for its lack of pro-apoptotic activity compared to caspase-10A and caspase-10D.

Conclusions: 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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POB045

CHD5, A Neuroblastoma (NB) Tumor Suppressor Gene (TSG), Also Regulates Spermiogenesis in Mice

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Background: We reported that CHD5 functions as a NB TSG, and recent studies showed that it also plays a cancer-suppressing role in many other tumors. CHD5 is a chromatin-remodeling protein, is expressed robustly in the brain and testis, and is localized in the nucleus.

Methods: We created a Chd5 knockout (KO) mouse model to study the role of CHD5 in normal development and cancer predisposition. We recorded the body weight, and we assessed both behavior and fertility. Tissues were fixed and studied with H&E staining, immunohistochemistry, or EM. Real-time RT-PCR and Western blots were used to evaluate gene expression in mice.

Results: Chd5 KO mice showed normal physical development, and to date they have not developed neural tumors. However, they have deregulated spermatogenesis, characterized by immature sloughing of elongated spermatids, spermiation failure and disorganization of the spermatogenic cycle. This results in the inappropriate placement and juxtaposition of germ cell types within the epithelium. Sperm that did enter the epididymis displayed abnormal morphology, with an irregularly shaped sperm heads. These sperm also stained positive for acidic aniline and retained histone 3 in the chromatin. This indicates a failure of cytoplasm removal and lack of proper chromatin condensation during spermiogenesis, possibly due to a failure of histone/protamine exchange. Electron microscopy showed that Chd5 KO mouse seminiferous tubules have extensive nuclear deformation, with an irregularly shaped heads of the elongated spermatid. However, the mRNA expression levels of other genes controlling spermatogenesis (e.g., GOPC and Prm2) were not affected.

Conclusions: Chd5 deletion may lead to the failure of histone/protamine exchange, which leads to deformation of the condensing spermatid nucleus leading to a failure of normal spermiation. These data show that CHD5 is a critical regulator of chromatin modification/organization, which may provide insight into its role as a TSG in NB.

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POB046

CHD5 Nucleosome Remodeling and Deacetylation (NuRD) Complex in Neuroblastoma (NB) Cell Lines

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Background: CHD5 maps to chromosome 1p36.31 and acts a tumor suppressor gene in NBs and other cancers. It is preferentially expressed in neural tissues, whereas expression is consistently low or absent in NB cell lines. CHD5 shares significant homology with CHD3 and CHD4, which are core subunits of a NuRD complex. We performed studies to determine if CHD5 forms a similar chromatin-remodeling complex.

Method/approach: The NB line NLF was stably transfected with CHD5 cDNA in the sense or antisense orientation, and stable clones were isolated. The NB line NBLS expresses endogenous CHD5. Immunofluorescence microscopy was used to determine subcellular localization of CHD5 protein. CHD5 NuRD components were detected after immunodepletion of CHD4 followed by using GST-FOG1 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 and associated proteins in the complex.

Results: Immunofluorescence demonstrated nuclear localization of CHD5 protein. Therefore, we examined nuclear extracts from either CHD5-transfected NLF cells, or NBLS cells, to determine if CHD5 forms a NuRD complex similar to CHD4. V5/His-tagged CHD5 was immunoprecipitated from nuclear extracts with either V5 or CHD5 antibody, or pulled down with GST-FOG1 after CHD4 depletion. CHD5 associated with all known NuRD components, including MTA1/2, GATAD2A/B, HDAC1/2, RBBP4/7 and MBD2/3. In addition, we also pulled down cytoplasmic dynein heavy chain 1 (DYNC1H1), as determined by Western blotting and LC/MS, as an associated protein of CHD5-NuRD complex.

Conclusion: Our data suggest that CHD5 forms a NuRD complex very similar to CHD4. The CHD5-NuRD complex in association with DYNC1H1 presumably plays an important role in chromatin remodeling and tumor suppression. This may occur by transcriptional repression and/or activation of genes and factors important in regulating neural-specific expression and/or differentiation.

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POB047

Identification and characterization of FOXP1 as a candidate tumor suppressor gene in neuroblastoma

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Background: To investigate the mechanisms of spontaneous regression in neuroblastoma (NB), genes differentially expressed between favorable and unfavorable NB were identified, including the putative tumor suppressor gene forkhead box P1 (FOXP1). FOXP1 is aberrantly expressed in a variety of carcinomas and belongs to a diverse family of winged-helix transcription factors that have diverse roles in cellular proliferation, differentiation and neoplastic transformation. Together with the fact that FOXP1 maps to chromosome 3p14, a tumor suppressor locus frequently deleted in aggressive NB, these findings indicate that FOXP1 might be a tumor suppressor candidate in NB.

Methods: Microarray expression profiles of 476 NB specimens were generated and genes differentially expressed between favorable and unfavorable NB were identified. FOXP1 expression was correlated to clinical markers and patient outcome. To determine whether promoter hypermethylation is involved in silencing of FoxP1 expression, promoter methylation analysis of primary NB (high FOXP1 expression, n=23; low FOXP1 expression, n=24) was performed. Furthermore, FOXP1 was re-expressed in three NB cell lines to study the effect of FOXP1 on growth characteristics of NB cells.

Results: Low expression of FOXP1 is associated with markers of unfavorable prognosis like stage 4, age >18 months and MYCN amplification ($p<0.001$ each). Moreover, low FOXP1 expression is correlated with unfavorable gene expression-based classification and adverse patient outcome ($p<0.001$ each). Low FOXP1 protein levels are found in NB cell lines and in samples from patients with poor outcome. Methylation analysis revealed no evidence of epigenetic FOXP1 silencing. Re-expression of FOXP1 in NB cell lines results in reduced cell viability, diminished clonogenic growth and impaired migration as well as increased apoptosis, thus supporting the hypothesis of FOXP1 as a tumor suppressor gene in NB.

Conclusions: Collectively, our results suggest that loss of FOXP1 function is involved in NB pathogenesis and contributes to tumor progression and unfavorable patient outcome.

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POB048

Functional analysis of a novel 1p tumor suppressor DMAP1

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Background: In neuroblastoma (NB), 1p LOH highly correlates with MYCN amplification, indicating that important tumor suppressor genes (TSGs) which relate MYCN amplification may be located at 1p. We previously reported the p53 functional inactivation in NB and its relation to MYCN amplification (Oncogene 2008; Eur J of Cancer, 2010). Together, there could be important TSGs at 1p which are driven by MYCN and regulate p53 in NB.

DNA methyltransferase 1-associated protein 1 (DMAP1) is coded on 1p34.1 and its physical and functional interaction with MYCN were reported. These observations prompted us to study DMAP1 as a candidate of 1p TSG which regulates p53 in NB.

Methods: Gene knockdown and over-expression were performed by lentiviral systems. DMAP1 expression was studied by qPCR and immune-histochemical (IH) analysis.

Results and Discussion: Low expression of DMAP1 was significantly correlated with unfavorable prognosis. IH analysis also indicated low expression of DMAP1 was related to unfavorable pathology. 1p Loss appeared to be one of the mechanism of DMAP1 reduction. Bisulfite sequencing indicated low methylation status of the DMAP1 promoter region in NB; pathological gene mutations were not detected by WAVE analysis and direct sequencing. These results suggest that there may be the other epigenetic mechanisms to suppress DMAP1.

MYCN-induced DMAP1 up-regulation and ATM/p53 activation were detected in both MYCN-single NB cells and human fibroblasts, suggesting the existence of MYCN-dependent DMAP1/ATM/p53 pathway activation. Over-expression and knockdown of DMAP1 in NB showed: (i) DMAP1 upregulates p53 pathway through ATM activation; (ii) DMAP1 suppresses cell cycle progression and increases apoptosis in a p53-dependent manner.

Together, we identified the MYCN-related ATM/p53 pathway activation by DMAP1 and its suppression in NB tumors. This may be one of the important mechanisms of p53 functional inactivation in MYCN-amplified NBs.

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POB049

TRIM16 is an E3 ubiquitin ligase which directly binds and activates Caspases 2 and 3 leading to neuroblastoma cell apoptosis

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Background: Tripartite motif 16 (TRIM16) is a member of the diverse TRIM family of proteins. TRIM16 acts as a tumour suppressor, affecting neuritic differentiation, cell migration and replication through direct interactions with cytoplasmic vimentin and nuclear E2F1 proteins in neuroblastoma cells. Many TRIM proteins function in ubiquitination through a RING protein domain.

Methods: We used human neuroblastoma cancer cell lines, molecular modelling, co-immunoprecipation, TUNEL assays, immunofluorescence studies and ubiquitination assays, to examine the hypothesis that TRIM16 can heterodimerize with other TRIM proteins leading to E3 ubiquitin ligase activity, and, apoptosis of cancer cells.

Results: Here we show that TRIM16 can homodimerize and heterodimerizes with other TRIM family members; TRIM24, Promyelocytic leukaemia (PML) protein and Midline-1 (MID1). Although TRIM16 has no RING domain, three-dimensional modelling of TRIM16 suggested that it's B-box domains could adopt RING-like folds, and thus E3 ubiquitin ligase activity. Consistent with this hypothesis, we demonstrated that TRIM16, although devoid of a classical RING domain, has auto-polyubiquitination activity via its B-Box domain and E3 ubiquitin ligase activity in vivo and in vitro. TRIM16 protein overexpression in neuroblastoma cells induced apoptosis via directly binding to caspase 3. TRIM16 co-localised with caspases 2 and 3 in the cytoplasm of cancer cells and regulated the levels of pro-caspases 2 & 3. Neuroblastoma cells treated with caspase 2- and 3-specific inhibitors, lost the capacity for TRIM16-induced apoptosis.

Conclusion: TRIM16 is a novel E3 ubiquitin ligase, which interacts with other important TRIM family members and plays a role in the apoptosis death pathway in neuroblastoma cells.

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POB050
HoxC9 activates the intrinsic pathway of apoptosis and is associated with spontaneous regression in neuroblastoma
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Background: The embryonic nature of neuroblastoma (NB) suggests the involvement of key developmental regulator genes in its pathogenesis. Homeobox (HOX) genes constitute an important family of developmental regulators which play fundamental roles in morphogenesis and cell differentiation during embryogenesis. This study aimed at elucidating the clinical impact of HOX genes in NB in general and the role of HoxC9 in NB spontaneous regression and differentiation in particular.

Methods: Microarray gene expression profiles from 651 NB specimens were generated and expression levels of HOX Class I genes were correlated with prognostic markers. A HOX gene expression based classifier was established using PAM and the association of classification results with clinical covariates was determined. HoxC9 was re-expressed in 3 NB cell lines to investigate the molecular function of HoxC9 in cell differentiation and apoptosis.

Results: Expression of the majority of the 39 HOX genes correlated significantly with clinical covariates in NB. A HOX gene expression-based classifier was able to predict NB patient outcome with a predictive accuracy of 76%. Among all HOX genes, HOXC9 expression was most prominently associated with favorable prognostic markers, and was particularly elevated in spontaneously regressing tumors. Re-expression of HoxC9 leads to a significant reduction in NB cell viability. We found that HoxC9 induced apoptotic cell death through activating the intrinsic caspase cascade by increasing the Bax:Bcl-2 ratio and subsequent release of mitochondrial cytochrome c in the cytosol. In IMR-32 cells, neuronal differentiation was observed upon HoxC9 up-regulation combined with a down-regulation of the RE1-silencing transcription factor REST, a master negative regulator of neurogenesis.

Conclusions: Our data suggest that HOX genes have a prognostic impact in NB, and indicate a critical role of HoxC9 in NB spontaneous regression and differentiation.

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POB051
Modeling the G1-S Transition in Neuroblastoma
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Background: MYCN–amplification in neuroblastoma is predictive for poor clinical outcome. MYCN–amplified cells proliferate strongly and exhibit impaired cell cycle arrest. Unlike in other tumor entities, the aberrant cell cycle arrest is rarely attributed to genetic mutations (e.g., of p53). It appears to be due to an imbalance in the expression and function of p53, MDM2 and CDKs, which is influenced by deregulated MYCN.

Method: To rationalize how deregulated MYCN is related to aberrant G1-S transition and escape of DNA damage–induced cell cycle arrest we utilized a systems-biology approach. Cell lines that allow targeted overexpression or knock down of MYCN were used to describe MYCN-dependent perturbations of the p53-MDM2 module and the E2F1 mediated G1-S transition. We established a mathematical model for the MYCN dependence of the restriction point control by means of mass action kinetics. The interactions of main regulatory proteins (G1–CDKs, pRb, E2F1, SKP2, p27, p53, MDM2, p21) and transcriptional activation were modeled.

Results: Due to the activating feedback loop of E2F1 via SKP2 this model shows bistability. The bifurcation diagram can now be used as an output of the model to analyze the restriction point behavior under change of protein concentrations. In cells with moderate MYCN level the p53 induced p21 response after DNA damage seems to be deregulated, however leaving the cell cycle arrest functional. Whereas for cells with relatively high MYCN level and an enhanced CDK4 signal (a MYCN target) the bistable region vanishes completely and the model stays in an activated state even under DNA damage.

Conclusion: Although in cells with moderate MYCN level both p53 and MDM2 are overexpressed, MDM2 appears to be dominant for the p21 response upon DNA-damage. For MYCN high cells points to reintroduce bistability for elevated CDK4 level were identified.

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POB052
NLRR2 inhibits tumor growth by activating ER stress signals and its down-regulation is associated with poor prognosis in neurobalstoma
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Background: The novel human family genes encoding Neuronal Leucine Rich Repeat (NLRR) proteins were identified as prognostic markers from screening of our primary neuroblastoma (NB) cDNA libraries. Among this family, NLRR1 and NLRR3 were associated with regulation of cellular proliferation and differentiation, respectively. However, the function and clinical significance of NLRR2 in NB remain elusive.

Methods: We evaluated the expression of NLRR2 in 123 NBs by quantitative real time PCR. Nude mice were used for tumor xenograft study. Endoplasmic reticulum (ER) stress and neuronal differentiation were induced by Tunicamycin (TM) and Retinoic acid (RA) treatments, respectively.

Results: The high levels of expression of NLRR2 mRNA were significantly associated with good prognosis (p=0.000). In in vitro experiments, NLRR2 suppressed cell growth and reduced colony formation ability in both NB and non-NB cells. Of interest, the TM treatment induced the ER stress-related gene, ATF4, in the NLF NB cells stably overexpressing with NLRR2 as compared with the control cells. The inhibition of NLRR2-mediated cell growth was cancelled when expression of ATF4 was knocked down, suggesting that NLRR2 might function through the induction of ATF4. We then transplanted with the NLRR2 stably expressing tumor cells into nude mice, and found that the tumor formation was significantly inhibited ($p<0.01$). Endogenous expression of ATF4 was positively correlated with the expression of NLRR2 in primary NBs ($n=123$, $p<0.002$). More interestingly, the high expression levels of ATF4 were significantly associated with good prognosis of NB ($p<0.01$).We further found that NLRR2 is an inducible gene upon treatment with RA. JNK downstream protein c-JUN was found to be recruited onto NLRR2 promoter. Thus, NLRR2 may function in induction of both cell death and differentiation in NB.

Conclusion: Our data suggest that NLRR2 might contribute to aggressiveness of NB by induction of differentiation and/or the ER-stress-induced cell death.

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POB053
Possible role of Delta-like 1 homolog in the chemoresistant behavior of Neuroblastoma.
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Introduction: Neuroblastoma (NB) is the most frequent extracranial pediatric solid tumor and a particularly heterogeneous and devastating disease. As in other cancers, the development of chemoresistance represents a major obstacle to effective treatment of high grade NB. DLK1, a member of the Notch/delta/serrate family has been linked to NB differentiation and progression.

Methods: To address the mechanisms underlying the chemoresistant phenotype in NB, we compared the gene expression profiles of doxorubicin-resistant cells (LAN-1-R) and parental sensitive LAN-1 cells.

Results: DLK1 was identified as a moderately, but significantly, overexpressed gene in the resistant variants. Its high level of expression in neuroendocrine tumors like NB, suggests a possible involvement in the disease development. We confirmed the increase in DLK1 expression at mRNA and protein level in LAN-1-R vs LAN-1 cells, as well as higher amounts of released DLK1 in LAN-1-R cells culture fluid. Overexpression of DLK1 in LAN-1 cells highly influenced their proliferative behavior without modifying their drug sensitivity. In contrast, silencing of DLK1 in LAN-1-R cells by lentiviral-mediated micro-adapted shRNA only resulted in slight drug sensitivity to four chemotherapeutic drugs, suggesting a possible but not crucial role of DLK1 in the drug resistant phenotype of these cells. When the endogenous DLK1 expression was inhibited in other well-characterized cell lines such as LAN-1, IMR32 and SKN-Be2c, a lower proliferation rate and/or increased resistance were observed which was compatible with acquisition of a more differentiated phenotype. In contrast no effect of DLK1 inhibition in the LAN-1 cells was observed.

Conclusion: Our data which fully support a recent report implicating DLK1 in enhanced tumorigenic and undifferentiated characteristics of NB cells, further indicate a complex and cell type-dependent role for DLK1 in multi-drug resistant phenotype. These observations which associate DLK1 to multiple mechanisms leading to the particularly malignant behavior of NB deserve further investigation.

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POB054

p19INK4D inhibits neuroblastoma cell growth, and its low expression is associated with poor neuroblastoma outcome

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Background: Loss of G1-S-phase checkpoint control, resulting from deregulated cyclinD-CDK4-RB pathway, critically contributes to neuroblastoma (NB) malignancy. Mechanisms leading to aberrant CDK4 kinase activity in NB are only partially understood. In this study, we assessed the role of the CDK4 inhibitor p19INK4D in NB biology.

Methods: p19INK4D mRNA expression in primary NBs was determined using oligo microarray data of 251 patients. Tetracycline-inducible ectopic p19INK4D expression was used to explore the proteins' impact on growth and phenotype of NB cells. All-trans retinoic acid (ATRA) treatment and ectopic expression of TrkA in combination with nerve growth factor (NGF) treatment were used to test p19INK4D response during NB differentiation.

Results: Low p19INK4D expression was significantly associated with advanced disease stage and poor event-free and overall survival in a set of 251 primary NBs. Ectopic expression of p19INK4D significantly decreased viability and clonogenicity of SH-EP, SH-SY5Y and IMR5-75 cells and significantly reduced the capacity of IMR5-75 to grow anchorage-independent. G1/0 cell cycle fraction was significantly increased in p19INK4D-induced SH-EP, SH-SY5Y and IMR5-75 cells. On the molecular level, ectopic p19INK4D expression activated the RB pathway in SH-EP and SH-SY5Y cells, evidenced by a decrease in CDK4-specific pRB phosphorylation and decreased mRNA and protein expression of E2F target genes. Intriguingly, ectopic p19INK4D expression was accompanied by induction of differentiation and senescence in SH-SY5Y and SH-EP cells, respectively. Further, p19INK4D expression was activated upon NGF-TrkA- and ATRA-mediated differentiation of SH-SY5Y and Be-(2)C cells, respectively.

Conclusions: The present study indicates that low p19INK4D expression provides a selective advantage in malignant NB. This is in line with an extensive oncosuppressive effect of p19INK4D in NB cells, comprising growth suppression and induction of differentiation or senescence. Collectively, this study identifies p19INK4D as a crucial component of NB growth control and differentiation.

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POB055

Contribution Of Ataxia Telangiectasia Mutated (ATM) Loss Of Function To Neuroblastoma Progression.

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Background: Loss of chromosome 11q22-23 is frequent in neuroblastoma, where it correlates with tumor progression and poor prognosis. The molecular basis of this phenomenon remains to be defined. 11q22-23 contains ATM, the gene mutated in ataxia-telangiectasia, a cancer syndrome characterized by marked sensitivity to ionizing radiations and cancer predisposition. ATM encodes a homonymous serine/threonine kinase playing a key role in the cellular response to DNA double strand breaks, through the activation of a phosphorylation cascade that activates cell cycle checkpoints, DNA repair, apoptosis or senescence. We wanted to investigate the possibility that diminished expression of ATM, as it results from the loss of chromosome 11q, increases the aggressiveness of neuroblastoma.

Methods: We generated human neuroblastoma cells with stable silencing of ATM by transfection of short hairpin (sh)RNA vectors specifically targeting ATM mRNA. The cells were tested for their capacity to grow in soft agar, to form spheres under stem cells and cancer stem cell culture conditions (i.e. when grown in suspension in serum-free medium), and to form tumors in nude mice.

Results: Stable inhibition of ATM expression in human neuroblastoma cells by several ATM shRNAs resulted in an increased capacity to grow as multicellular colonies both in the soft agar assay and under stem cell and cancer stem cells culture conditions. In addition, when injected subcutaneously into nude mice, human neuroblastoma cells with stable ATM silencing formed considerably bigger tumors than control cells. ATM silencing had no effect on other human cell types not previously reported to have loss of ATM as a potential tumor promoting event.

Conclusions: Our findings represent the first experimental evidence that ATM loss of function contributes to neuroblastoma progression. To identify potential mediators of the ATM tumor suppressor function in neuroblastoma, we are currently studying the ATM-mediated transcriptional responses in human neuroblastoma cells.

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POB056

Mismatch repair protein expression and gene promoter methylation in paired neuroblastomas pre and post-chemotherapy

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Background and Aims: The development of chemoresistance presents a significant obstacle to the successful treatment of high-risk neuroblastoma. Defects in mismatch repair have been identified in many cancers and correlate with resistance to alkylating agents. The aim of this study was to determine whether defects within the mismatch repair (MMR) pathway develop in neuroblastomas during treatment and to explore the underlying mechanisms.

Methods: Protein expression and promoter methylation status of the mismatch repair genes MLH1, MSH2, and MGMT were determined in 37 paired neuroblastomas pre- and post-induction chemotherapy (6 intermediate risk and 31 high risk) by immunohistochemistry and methylation-specific polymerase chain reaction respectively. Immunohistochemical expression was scored using a combined index (CI) (Labelling index (% of positively immunostained cells) x Intensity).

Results: The median CI for MLH1 and MSH2 expression was significantly lower in neuroblastomas studied post-chemotherapy compared with pre-chemotherapy ones ($p = 0.026$ and 0.001 respectively, Wilcoxon test), but this was not related to MLH1 or MSH2 promoter hypermethylation. Using the median CI as a cut off, low MLH1 expression post-chemotherapy was associated with improved overall and progression free survival ($p < 0.01$ log rank, overall survival). There was no relationship between MLH1 or MSH2 expression and INPC differentiation. In contrast there was no significant change in MGMT expression during treatment, no relationship with survival, but a high CI was found in INPC undifferentiated tumours post- chemotherapy ($p=0.031$ Chi-square).

Conclusions: The reduced expression of MLH1 and MSH2 in neuroblastomas following chemotherapy was not due to promoter hypermethylation. The association between high MGMT expression and undifferentiated tumours may relate to MGMT's oncogenic functions. These studies suggest that acquired defects of mismatch repair do not influence survival in neuroblastoma in the same way they do in other tumours and may help to explain the sensitivity of relapsed neuroblastomas to temozolamide.

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POB057

Role Of Carcinoembryonic Antigen-Related Cell Adhesion Molecule 1 (CEACAM1) In The DNA Damage Response Of Human Neuroblastoma Cells.

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Background: Inactivation of p53 is infrequent in primary neuroblastoma. However, it often occurs in post- chemotherapy or radiotherapy relapses. Since p53 is likely to play a key role in the response of primary neuroblastoma to radiotherapy and chemotherapy, we planned to study it in human neuroblastoma cells by focusing on carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), a known tumor suppressor that we recently identified as a new target of the ataxia-telangiectasia mutated (ATM)/p53 pathway (Oncogenesis, in press).

Methods: CEACAM1 regulation by ATM/p53 is being studied by quantitative real-time PCR, Western Blotting and p53 chromatin immunoprecipitation in several human cell types with known p53 status, including human neuroblastoma cells. Human tumor specimens are being studied by CEACAM1 immunohistochemistry. In human neuroblastoma cells, the role of CEACAM1 in the response to radiotherapy and chemotherapy will be studied by stable silencing of CEACAM1, as well as by inducible CEACAM1 overexpression.

Results: In human mammary and colon epithelial cells, CEACAM1 is upregulated by ATM/p53 starting from the lowest doses of DNA double strand break inducers used. In these cells, induction of CEACAM1 is required for the establishment of cellular senescence in response to DNA damage. CEACAM1 is also induced by ATM/p53 in several human neuroblastoma cell lines. We are currently studying the role of CEACAM1 in the DNA damage response of human neuroblastoma cells.

Conclusions: CEACAM1 is a known tumor suppressor gene that we discovered to be regulated by ATM/p53 in many different human cell types including neuroblastoma cells. In human mammary and colon epithelial cells, CEACAM1 is required for the establishment of cellular senescence in response to DNA damage. Our current experiments will tell us if CEACAM1 plays a role in the ATM/p53 mediated cellular response of neuroblastoma cells to radiotherapy and chemotherapy, and if CEACAM1 dysregulation or mutation occur in relapses.

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POB058

FOXO3a Is A Critical Target of PI3K/AKT Pathway Activity In Neuroblastoma

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Background: Recently, the PI3K pathway has come to light as a pathway of strong prognostic importance in neuroblastoma. Despite recent advances, it is still unclear what aspects of PI3K activity are vital in neuroblastoma. FOXO3a is a transcription factor phosphorylated and inactivated by AKT signaling which has many evolutionarily-conserved roles within the pathway. In other systems it has been identified as a pivotal player in the PI3K/AKT pathway with defined roles in apoptosis, autophagy, cell cycle arrest and resistance to oxidative stress.

Methods: Here we use inducible over-expression and inducible shRNA-mediated knockdown of FOXO3a to delineate the role of this factor in neuroblastoma cells within the context of PI3K/AKT pathway inhibition accomplished using the small molecule inhibitors PI-103 and MK-2206. RNA microarray profiling was also employed to study the downstream effects on gene expression.

Results: Neuroblastomas have the highest expression of FOXO3a relative to all other tumors and normal tissues and this expression is prognostically significant. We demonstrate that knockdown of FOXO3a rescues SKNBE(2c) cells from the cell death induced by PI-103 treatment and that over-expression in SY5Y cells greatly sensitizes them to PI-103 as well as the AKT inhibitor MK-2206. Across a cell line panel, we find that increased MK-2206 sensitivity correlates with increased protein levels of FOXO3a. RNA microarray profiling of SY5Y reveals PI3K and FOXO3a gene expression signatures which are highly prognostic in neuroblastoma tumors.

Conclusions: FOXO3a is a vital executioner of cell death downstream of PI3K/AKT signaling in neuroblastoma cells and is abundantly expressed in virtually all neuroblastoma tumors. Therefore, PI3K signaling may be vital in high-stage neuroblastoma to inactivate FOXO3a and allow tumors to escape an otherwise tumor suppressive program. FOXO3a expression, phosphorylation and activity may represent useful biomarkers to monitor the efficacy of PI3K/AKT inhibitors in neuroblastoma therapy.

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POB059

Understanding the biological role of caspase-8 in neuroblastoma

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Background: Expression of caspase-8 (casp8), a mediator of extrinsic apoptosis, is suppressed in many but not all neuroblastoma tumors. Data from our group and others suggests that decreased casp8 may facilitate neuroblastoma tumorigenesis and/or metastasis. Paradoxically, expression of casp8 increases cell attachment and migration in vitro and enhances tumor growth in SCID mice. One explanation for the ability of casp8 to have multiple and sometimes opposing functions is post-translational modifications which may decrease casp8 activity and shifts the role of casp8 towards survival. This hypothesis is supported by data demonstrating that Src phosphorylation of casp8 decreases its activity and induces Erk signaling. Ataxia telangiectasia mutated kinase (ATM), an essential mediator in the DNA double strand break repair mechanism, is activated by chemotherapeutic treatments, radiation and cellular stress. Casp8 has one consensus phosphorylation site that has been reported to be phosphorylated in a genome-wide screen for ATM substrates. Here we test whether casp8 is an ATM substrate.

Methods: We developed a novel ultrasound guided method to establish orthotopic NB para-adrenal tumors. Analysis of the growth of tumors which lack casp8 or express casp-8 with and without mutation in the ATM phosphorylation site was performed weekly using ultrasound and luciferase imaging. Casp8 activity was also assessed.

Results: ATM can phosphorylate casp8 on Ser219. This phosphorylation suppresses casp8 enzymatic activity in vitro and increases tumor establishment and growth in vivo in the orthotopic xenograft model.

Conclusion: These data indicate that casp8 enhances orthotopic xenograft tumor establishment which may explain why casp8 expression is maintained in some NB tumors. Furthermore, they suggest that exposure to DNA damaging agents suppresses the apoptotic function of casp8 via ATM-mediated phosphorylation, thereby might allow cell survival and metastasis. This outlines a possible mechanism by which tumor cells may avoid cell death when exposed to chemotherapeutic agents.

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POB060

Frizzled receptor 6 (Fzd6) is a new marker for neuroblastoma stem cells.

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Background: The expression of surface molecules typical of stem cells is thought to be a key feature of tumor initiating cells. In neuroblastoma, stem cell surface markers such as CD133 or CD34 have been detected, but none of them is associated with the neural crest, the embryonal tissue from which neuroblastoma originate. Wnt signalling is an important component of vertebrate development, required for specification of the neural crest. In this study we investigated the role of the Wnt receptor Frizzled 6 (Fzd6) in Neuroblastoma.

Materials and Methods: Mouse neuroblastoma cells (R6-2) were labelled with an anti-Fzd6 antibody, separated by FACS sorting and subjected to neurosphere assay. Sorted cells were also plated in invasion matrigel chambers to assess the invasive potential. Gene expression characterisation was performed with quantitative real time-PCR. Fzd6 positive and negative human neuroblastoma cells, were injected subcutaneously and orthotopically into NOD-SCID mice.

Results: Expression of Fzd6 is associated with poor survival in Neuroblastoma patients and marks rare, HIF1/2 α-positive cells in tumour hypoxic areas. Fzd6-positive cells formed neurospheres more efficiently than the negative subpopulation, were resistant to doxorubinic treatment, were more invasive and expressed high levels of mesenchymal markers such as Twist1 and Notch1. Fzd6 is required for the signalling and expression of genes of the non-canonical Wnt pathway and the spheres forming activity. When transplanted into SCID mice, Fzd6 positive cells grew more aggressively than their negative counterparts.

Conclusions: In this study we show that Fzd6 predicts poor survival in neuroblastoma patients, marking rare cells with stem cell-like features. We propose that Fzd6 is a new marker and signalling receptor for neuroblastoma stem cells. Pharmacological inhibition of Fzd6 positive tumour cells could improve the treatment of recurrent and aggressive forms of neuroblastoma and possibly of other cancers expressing this marker.

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POB061

BMI1 regulates neuroblastoma apoptotic cell death and differentiation

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Background: BMI1 is a polycomb protein and its overexpression correlates with tumorigenesis and aggressiveness. Importantly, BMI1 expression in neuroblastoma (NB) also related to patients' prognosis. In this report, to confirm BMI1 as a new target of NB therapy, we studied the effects of BMI1 reduction on NB cell death and differentiation.

Methods: Using shRNA and siRNA, we determined the effect of reducing BMI1 levels on NB in vitro and in vivo.

Results: shRNA-mediated BMI1 knockdown induced: 1. significant differentiation with neurite extension and NF68/GAP43 induction and inhibition of sphere formation in 5 NB cell lines, suggesting that BMI1 has a role in un-differentiation status of NB cells; 2. apoptotic NB cell death in a p53-dependent, with induction of pro-apoptotic PUMA and NOXA (3 cell lines), and a p53-independent manners (2 cell lines). Importantly, reduction of BMI1 in fibroblasts did not induce proliferation failure and apoptosis. All-trans retinoic acid (ATRA) treatment and BMI1 knockdown synergistically enhanced apoptotic cell death in vitro, although ATRA administration could not induce apoptosis in the most of NB cell lines. As a therapeutic model experiment, BMI1 knockdown by shRNA significantly suppressed xenograft tumor formation of NB cells in nude mice. Surprisingly, treatment with combination of BMI1 KD and ATRA resulted in nearly complete suppression of tumor growth. Moreover, we observed a strong antitumor effect of BMI1 siRNA mixed with atelocollagen in the NB xenograft tumors. Pathological analysis indicated significant apoptotic cell death was induced by the BMI1 siRNA treatment in the xenografts. As molecular mechanisms of BMI1-KD related cell death, the significance of both of p53 and p73 was confirmed by knockdown experiments; the p53/p73 activation was further studied by comprehensive chip analysis.

Conclusion: BMI1 KD and combination of ATRA with it appear to be expected therapies for the advanced and refractory NBs.

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POB062

Reprogramming of human neuroblastoma cells using iPSC technology

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Background: Our global genomic and RNA expression profile provide a novel stratifications of neuroblastoma (NB). These molecular signatures are strongly correlated with patient prognosis, suggesting a subtype specific course for progression. Recently, induced pluripotent cancer cells (iPCCs) already established in several cancers and are being used to investigate the stemness of cancer cells. Here we aimed to generate iPCCs from NB and elucidate the mechanistic insight behind development of deadly disease in infants.

Methods: Sendai virus (SeV) vector-system was employed to reprogramme SH-SY5Y I-type NB cells. 244K human array CGH (aCGH) microarray was carried out to examine gene expression profile in iPCCs. Embryonic stem (ES) cell-related genes expression was examined by immunocytochemistry and quantitative real time RT-PCR (qRT-PCR). Apoptotic cells were detected using TUNEL assay after treating with chemotherapeutic reagents.

Results: SH-SY5Y intermediate (I)-type cells maintains cancer stem cell (CSC)-like properties. Upon treatment with retinoic acid (RA) they easily differentiate to neuroblastic (N) and substrate adherent(S) types. Since the vector is derived from Sendai virus which is an RNA virus, the transgenes do not integrate into host genome. Forty days after infection, we observed iPS-like colonies, and alkaline phosphatase(AP) staining confirm iPCCs formation. Array CGH data reflect the same genomic aberrations in iPCCs as those of their parental cells, suggesting intact genomic status were maintained during the reprogramming process. Immunocytochemistry indicate that iPCCs express ES cell surface markers such as SSEA4, TRA-1-60 and TRA1-81. Quantitative RT-PCR data revealed that, endogenous reprogramming factor genes including NANOG, OCT4, SOX2, KLF4 and hTERT were highly expressed in iPCCs. Finally, iPCCs shows more resistant phenotype against CDDP treatment as compared with their parental cells.

Conclusions: We generated and characterized iPCCs from human NB cells, which may provide a new opportunity of the diseases modeling, patient-specific drug screening and personalized cell-based-therapies of NB.

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POB063

Identification of tumoral glial precursor cells in neuroblastoma

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Background: Neuroblastic tumors (NBT) are composed by neuroblasts and Schwannian stroma. The ratio of both cell types varies and a correlation has been established between the proportion of the Schwannian-like stroma and the outcome of the patient. The aim of the present study was to explore in NBT the presence of cells that express simultaneously neuronal (GD2) and glial (S100A6) cell lineage markers, bipotential cells that could embody a potential NBT precursor or initiating cell.

Methods: 37 NBT (18 NB, 2 GNB and 2 GN at diagnosis and 15 NB post-treatment) specimens and 8 metastatic bone marrow biopsies were analyzed from patients managed at HSJD. Nine tumors were used for FACS analysis. To recapitulate in vitro the differentiation process involved in the development of GD2+/S100A6+ neuroblasts in vivo, we induced neuronal differentiation with ATRA in 2 I- and 1N-type cell lines.

Results: GD2 and S100A6 double immunostaining was performed in 18 non-treated samples. Concomitant expression was observed in a subpopulation of neuroblasts (<25% of the total) in 16 (89%) of the 18 samples. The majority of the GD2+/S100A6+ neuroblasts within the tumor were found isolated and surrounded by GD2+/S100A6_ neuroblasts. The GD2+/S100A6+ neuroblastic subpopulation was found to be enriched in low risk NB, distributed around the perivascular niche. Metastatic BM specimens also showed GD2+/S100A6+ cells. By FACS analysis the isolated subclones (GD2+/S100A6_; GD2+/S100A6+ and GD2_/S100A6+) displayed a similar level of NMYC amplification suggesting the existence of a common tumor precursor cell. During in vitro treatment with ATRA, few GD2+ neuroblasts acquired positive nuclear S100A6 staining. The GD2+/S100A6+ round neuroblasts changed their morphology to flat and enlarged cytoplasms, increasing their adherence, all distinctive features of S-cell phenotype.

Conclusions: We conclude that GD2+/S100A6+ neuroblasts represent a tumoral glial precursor subpopulation in NBT.

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POB064

Histone deacetylase inhibitors reduce the self renewal capacity of neuroblastoma tumor initiating cells

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Background: Several reports indicate the existence of tumor initiating cells in neuroblastoma. We aimed at isolating representative cells from neuroblastomas to investigate the influence of agents used for differentiation therapy on this cell population.

Methods: Primary tumor sphere cultures were established from 18 neuroblastoma biopsies obtained for diagnostic intervention following informed consent. Genetic profiling was based on M-FISH karyotyping and MYCN in situ hybridization. Functional studies included neurosphere initiation capacity and proliferation assays, subcutaneous tumor implantation in mice, and western blot and qRT-PCR for MYCN and stem cell marker expression analyses.

Results: Cells isolated responded to mitogens promoting the propagation of normal neural stem cells. Neuroblastoma cells derived from low risk tumors lost their self-renewal capacity in the neurosphere assay in the 1st or 2nd round of passaging. In contrast, cells derived from medium or high risk neuroblastomas were expandable for at least 5 passages. We next analyzed the proliferation of cells derived from medium or high risk neuroblastomas and found that MYCN amplified cells proliferated distinctly faster than those with MYCN single copy status, indicating that MYCN amplification promotes the proliferation of cells growing under neurosphere culture conditions. For further functional analysis, we selected the NB#8 cells due to their strong self-renewal capacity in vitro and tumorigenicity in mice. HDAC-inhibitors reduced their neurosphere initiation capacity up to 10-fold and proliferation up to 2.5-fold in a dose-dependent fashion. On a molecular level, HDAC-inhibitors suppressed MYCN and stem cell marker expression.

Conclusion: Our data support the concept of tumor initiating cells in neuroblastoma and show that differentiating agents like HDAC-inhibitors impact stem cell specific properties such as self-renewal, indicating a possible clinical application of HDAC-inhibitors in the treatment of high risk neuroblastoma through targeting of the tumor initiating cell compartment.

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POB065

MYCN or ALKF1174L are sufficient to drive neuroblastoma development from neural crest progenitor cells

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Background: Few recurrent genetic changes contributing to neuroblastoma (NB) formation, such as amplification of the MYCN oncogene and activating mutations of the ALK oncogene, have been identified to date. NB is presumed to arise from the neural crest, but models directly demonstrating its development from neural crest progenitor cells are still missing. Here we present direct evidence that neural crest progenitor cells give rise to NB using a multipotent murine neural crest progenitor cell line, JoMa1.

Methods: The JoMa1 cell line was maintained in an undifferentiated state by a tamoxifen-activated c-Myc transgene (c-Myc-ERT). Either MYCN, the oncogenic F1174L ALK variant identified in primary NBs, TrkA or GFP were ectopically expressed in JoMa1 cells. Subsequently, growth independence of c-Myc-ERT transgene was monitored in vitro, and tumorigenicity of transfected cells was evaluated in nude mice.

Results: JoMa1 cells expressing MYCN or ALKF1174L were able to grow independently of c-Myc-ERT activity in vitro and formed tumors in nude mice, as opposed to parental JoMa1 cells and JoMa1 cells expressing TrkA or GFP. Tumors resembled human NBs in morphology, and expressed the NB markers, TH, DBH, Phox2b, DC56 and synaptophysin. In addition, neurosecretory vesicles and synaptic structures were visible in electron micrographs of the tumors. Tumorigenicity was enhanced upon serial transplantation of tumor-derived cells as subcutaneous grafts in nude mice. MYCN-driven tumor cells remained susceptible to the MYC-inhibitor, NBT-272, indicating that cell growth depended on functional MYCN.

Conclusion: Our findings support neural crest progenitor cells as the precursor cells of NB, and indicate that NBs arise as their malignant progeny. The Joma1 model system is a well validated and rapid tool to investigate genes suspected of contributing to neuroblastomagenesis in a mouse model or implement screening strategies to identify such genes. The former strategy has already been successfully applied for FoxR1, Lin28b and miR-17-92.

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POB066

From human embryonic stem cells to sympathetic neurons: A model for understanding neuroblastoma pathogenesis

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Background and Aims: Neuroblastoma is an embryonal tumour originating from neural crest cells which give rise to the sympathetic nervous system (SNS). Our aim is to establish a model of normal human SNS development by differentiating human embryonic stem cells (hESC) to sympathetic neurons to further our understanding of the pathogenesis of neuroblastoma.

Methods: H9 and NCL-14 hESC cell lines were differentiated to sympathetic neurons using the stromal derived inducing activity of PA6 mouse feeder cell co-culture, and high concentrations of BMP4 followed by N2 supplemented growth media.

Results: Following 7 days of co-culture of hESC with PA6 cells, markers of neural crest, autonomic progenitors and sympathetic neurons were present on differentiated cells. Expression of neural crest specifier genes by RT-PCR including, Snail, Slug, Sox-9, and the noradrenergic marker, dHAND increased following 7 and 14 days of PA6 co-culture, subsequently decreasing by day 21. Following 21 days of co-culture extensive neuronal differentiation was observed by immunofluorescence with high expression of the neuronal markers NCAM, MASH-1, Phox2b, peripherin and tyrosine hydroxylase. The addition of BMP4 10ng/ml at days 5-9 of co-culture led to an increase in peripherin expression by RT-PCR. FACS sorting for the NC stem cell marker p75, led to enrichment for cells expressing p75, dopamine beta-hydroxylase, peripherin and tyrosine hydroxylase. Dual immunofluorescence for peripherin and tyrosine hydroxylase identified sympathetic neurons. Optimal cell culture conditions favouring the expansion and survival of sympathetic neurons include the use of Poly 'L' Ornithine/Laminin plates in the presence of N2 neuronal supplement and the addition of nerve growth factor (NGF) 10ng/ml from day 7 of differentiation.

Conclusions: We have derived sympathetic neurons from two hESC lines. This system provides a model of normal SNS development which can be used to understand more about neural crest development and neuroblastoma pathogenesis.

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POB067

Epigenetic Regulation of Differentially Expressed microRNAs in Neuroblastoma Cancer Stem Cell-like Population

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Background: Our laboratory has recently defined a novel subpopulation of highly tumorigenic neuroblastoma cells based on their surface expression of CD114 (G-CSFR). These cells are phenotypically similar to induced pluripotent stem cells (iPSCs) (see abstract by Patterson et.al). Based on data demonstrating that bivalent H3K27me3 and H3K4me3 histone modifications characterize the de-differentiated pluripotent state of embryonic stem cells (ESCs) and iPSCs, we hypothesized that similar epigenetic changes would be found in the cancer stem cell (CSC)-like neuroblastoma subpopulation. Here we focused on microRNAs up-regulated in ESCs and iPSCs that were also found elevated in the CSC-like neuroblastoma subpopulation.

Methods: Genome-wide histone maps for pluripotent and differentiated cell lines (data obtained from the Epigenome Atlas, http://www.genboree.org/epigenomeatlas) were analyzed using the microRNA loci associated with our novel stem-cell like population. CHIP-qPCR for these marks were performed on different cell lines using 10,000 and 1x107 cells as starting material.

Results: Principle component analysis (PCA) confirmed that histone modifications at these microRNA loci clearly differentiate pluripotent populations (ESCs and iPSCs) from more differentiated cell lines. Next, we performed chromatin-immunoprecipitation-qPCR and validated the 'bivalent' H3K27me3 and H3K4me3 modifications in the H9 ESC and Tz16 iPSC cell lines in contrast to differentiated neuroblastoma cells. We are further evaluating the stem cell specific histone modifications at the MCM7 (hosts miR-25-93) and MIRGH1 (hosts mir-17-92a) and other loci of microRNAs up-regulated in neuroblastoma CSC-like sub-population using the FACS sorted CD114+ and CD114- cells.

Conclusions: Pluripotent cell populations have distinct patterns of histone modifications and altered microRNA expression compared to differentiated cells. Our data suggest active changes in H3K27me3 and H3K4me3 histone marks alter microRNA expression in neuroblastoma and may help to further distinguish a defined CSC-like subpopulation from differentiated progeny. Ongoing evaluation of these modifications and the mechanisms regulating microRNAs in neuroblastoma are a focus of our laboratory.

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POB068

Interest of Orthotopic injections in NOD/SCID/Il2rg Null (NSG) mice to study cancer stem cells neuroblastoma

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Background/Aims: Neuroblastoma (NB) is the most common and deadly extracranial solid tumor of childhood. The concept of Cancer Stem Cells (CSCs) appears pertinent to the metastases, chemoresistance and unfavorable prognosis commonly observed in NB. Expanding evidence highlights the role of CD133 as a reliable marker of CSCs in various human tumors such as medulloblastoma. The recently described NOD/SCID/interleukin-2 receptor γ (Il2rg) null (NSG) mice have been shown to be one of the most immunodeficient hosts since these mice are deficient in both T and B lymphocytes, lack NK cells and display impaired dendritic cell functions. With about half of all primary NB occurring in the adrenal medulla, orthotopic transplantation of NB cells in the adrenal gland of NSG mice is the most relevant xenotransplant for NB research. The aim of this study was to verify the stemness property of cells expressing CD133 using orthotopic injections in NOD/SCID/Il2rg Null (NSG) mice.

Methods: Firstly, two cells lines were sorted for CD133 by FACS (CD133high and CD133low). Secondly, NB cells were transfected with shRNA plasmids (for CD133 silencing) or CD133 plasmids (for CD133 overexpression). Five to six NSG mice were injected ortothopically with either 500 CD133high, CD133low, CD133 silenced or CD133 overexpressing cells. .

Results: At necropsy, large tumors were observed in adrenal glands of the NSG mice injected orthotopically with 500 CD133high sorted cells. The concomitant histological and immunohistochemical analysis confirmed the presence of primary tumors and frequent metastases to the lung (4/6), liver (3/6) and bone marrow (3/6). In mice injected with 500 CD133 overexpressing cells, 2/5 presented a large primitive tumor in the adrenal gland without metastasis. No primary tumor was found in mice injected with CD133low sorted or CD133 silenced cells.

Conclusion: The orthotopic injection of cells in NSG mice demonstrated the stemness property of cells expressing CD133.

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POB069

EFNA2 implication in stemness properties of neuroblastoma

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Background/Aim: Neuroblastoma (NB) is a frequent paediatric tumour. Despite aggressive treatments survival of children with advanced and metastatic NB is poor (only 40% to 66%)and are associated to chemoresistance , . Interestingly, cancer stem cells (CSCs) are a distinct subpopulation of cancer cells that possess characteristics normally associated with stem cells as well as being chemoresistant. Our precedent studies demonstrated that cells expressing CD133 possessed stemness properties. The aim of this study is to determine which genes or proteins contribute to the stemness property of CSCs in NB.

Methods: Using six cells lines, we sorted by FACS two populations CD133high and CD133low. After DNA extraction, a differential genotyping analysis was realized with Affymetrix SNP 6.0 arrays on CD133low and CD133high populations. Among the abnormalities found after this analysis, the gene EFNA2 seemed to be the most interesting. Also, we transfected NB cell lines with shRNA plasmids (for CD133 silencing) or CD133 plasmids (for CD133 overexpression). To determine the gene expression, Western blots (WB) were performed on sorted and transfected cells for CD133 as well as 6 NB tumors positive or not for CD133. Using a blocking antibody, we evaluated the influence of EFNA2 on neurospheres formation.

Results: In the majority of NB cell lines, the differential genotyping analysis identified a gain in two common regions (16p13.3 and 19p13.3) containing the EFNA2 gene in the CD133high population. EFNA2 protein expression was correlated to CD133 expression in tumors and in sorted or tranfected cells. Moreover, the increased concentration of anti-EFNA2 antibody in the culture medium decreased the size of neurospheres formed.

Conclusion: EFNA2 presence in cells expressing CD133 highlights its importance in the development of CSCs and appears to be an attractive target in the development of new therapeutic strategies for NB targeting CSCs.

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POB070

Isolation and verification of new neuroblastoma tumor-initiating cell lines

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We previously identified neuroblastoma (NB) tumor-initiating cells (TICs) from bone marrow (BM) metastases of high-risk patients that displayed a mixed phenotype of neural and hematopoietic origin. Subsequent analysis of three of these lines, NB12, NB88R2, NB122R, revealed that expression of B-cell markers CD20, CD22, and CD45 resulted from contaminating Epstein Barr Virus (EBV)-transformed lymphocytes likely of patient origin, and probable fusion of NB and EBV-B-cells. While early passages of lines contained a mixture of NB cells and lymphocytes, NB cells were almost entirely absent from later passages. We recommend that these lines not be used for NB studies (see Pahlman, Kaplan letters to Editor, Can Res 72:821-824).

We have established new sphere-forming lines in serum-free conditions from tumors, BM, and brain metastases that are devoid of EBV by multiple methods including qPCR. NB100, NB153, NB249, NB272, and NB273 express NB markers such as GD2, NB84, and nestin, and do not express CD20, CD22, and CD45. They highly express CD74, the receptor for the cytokine MIF that is upregulated in B-cell malignancies and some carcinomas, and a therapeutic CD74 antibody is in Phase I/II clinical trials for multiple myeloma. NB249 and NB272 form NB tumors in an orthotopic model with as few as 500 cells, although they must be passaged as xenografts in mice. We have repeated in vitro assays of a number of targets from our previous drug screens using NB153, a tumor-derived line, and found that DECA-14 and the PLK1 inhibitor BI2536 were cytotoxic at low nanomolar concentrations. Furthermore, the third generation PLK1 inhibitor BI6727, in Phase II trials for adult cancer, potently suppressed NB249 tumorigenesis in vivo. We suggest that our highly tumorigenic EBV-free NB lines are new models for NB target discovery and validation, and that CD74 remains a viable therapeutic target for NB.

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POB071

Factors associated with recurrence and length of survival following relapse in patients with neuroblastoma: a pilot study

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Background: Despite advances in therapy for neuroblastoma, survival from high risk disease is poor. This UK based study aimed to investigate epidemiological, clinical and biological factors associated with recurrence and length of survival following relapse in neuroblastoma to compare with a recent international study.

Methods: All cases of relapsed neuroblastoma diagnosed from 1990-2010 were identified from two specialist tumour registries. Kaplan-Meier survival analyses were used to calculate the overall survival (OS) time from diagnosis and post relapse overall survival (PROS). Log rank tests and Cox regression analyses were used to investigate survival.

Results: 82 cases of relapsed neuroblastoma were identified. The median age at diagnosis of neuroblastoma was 2.9 years (range 0-19), 72 cases had stage 4 disease at diagnosis and 8 had stage 3 (2 unknown). MYCN status was known for 61 (74%) cases, 22 (36%) were MYCN amplified and 39 (64%) were non-MYCN amplified. 1p status was known for 30 (37%) cases, 18 (60%) had 1p deleted tumours, 12 (40%) had no 1p abnormality. Median OS was 23 months and median PROS was 4 months. MYCN amplified disease was associated with worse OS 15 months (95% CI 10.0-28.5) vs 28 months for non-amplified cases (95% CI 24.5-39.4) (P=0.0006) and worse PROS, 2 months (95% CI 1.05-4.40) vs 9 months for non-amplified cases (95% CI 4.23-12.74) (P<0.0001). Cox univariate analysis showed PROS was worse for MYCN amplified cases (P<0.0001), stage 4 disease (P<0.0001), 1p deleted cases (P=0.01) and time from diagnosis to first relapse (P=0.006). Patients who relapsed less than 6 months from diagnosis had the highest risk of death. Stage 4 disease and MYCN amplified disease were significant in multivariate analyses.

Conclusion: This preliminary analysis from the UK confirms that relapsed neuroblastoma cases have worse survival if MYCN amplified and stage 4, but also if 1p deleted. We are currently obtaining more cases from two other centres for a further analysis.

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POB072

Long-term vascular access at chemotherapy in children with oncology: optimisation, prevention and treatment of aftereffects

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From July 2010 till February 2012 95 children and adolescents at the age of 6 months to 17 years with localized and metastatic tumors, 10 with neuroblastoma, underwent implantation of subcutaneous venous ports for carrying out polychemotherapy and supporting therapy. The children underwent the implantation with local anesthesia together with intravenous sedation of Propofoly. The adolescents underwent the procedure with local anesthesia. Ultrasonic marking of deep jugularis venous was applied in advance. All the implantations were done in the cath lab. Postoperative complications were not registered. Taurolock was used as a solution for locking the ports between the courses of chemotherapy.From the moment of implantation all the systems are successfully functioning. The thrombosis was registered in 6 cases and was successfully treated by injection Urocinaza to the port-system. Catheter-related bloodstream infections are not registered.Usage of the implanted subcutaneous venous ports and Taurolock as a solution for locking the ports gave opportunity to avoid numerous central venous punctions before each course of chemotherapy and complication caused by their presence, such as pneumatothorax, lung's trauma and catheter-related bloodstream infections, that helped to increase patients' life quality.

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POB074

CDK4 inhibition increases chemosensitivity of MYCN-amplified neuroblastoma cells

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Background: Understanding mechanisms of resistance to drug-induced DNA damage response is essential to develop new therapeutic strategies for relapsed neuroblastomas. We hypothesize that functional restoration of pRB through CDK4 inhibition will sensitize MYCN-amplified cells to doxorubicin-induced DNA damage.

Methods: The DNA damage response phenotype after doxorubicin treatment was characterized using flow cytometry in fourteen neuroblastoma cell lines with distinct chromosomal aberrations: amplified MYCN, CDK4/MDM2 or CCND1, mutant TP53 or deleted p14ARF/p16INK4A. The role of MYCN on G1-S transition after doxorubicin treatment was determined using SH-EP-MYCN (TET21N) expressing an inducible MYCN transgene and IMR5/75-C2 cells expressing an inducible shRNA targeting MYCN. To determine if CDK4 or CDK2 inhibition could functionally restore pRB, we used siRNAs/shRNAs, small molecule inhibitors and conditional expression of p16INK4A or p19INK4D and measured G1-S arrest, viability and apoptosis after doxorubicin treatment.

Results: Amplified MYCN in cooperation with additional chromosomal aberrations affecting the p53 and/or pRB pathway abolished G1/S checkpoints. This was associated with G2/M cell enrichment and apoptosis resistance in neuroblastoma cells after doxorubicin treatment. Despite p53-p21 induction after doxorubicin treatment, MYCN-amplified cells failed to arrest in G1-S, which was due to a p21 shift away from CDK2 into highly abundant CDK4-containing complexes. As result, both kinases remained highly active after doxorubicin treatment, leading to pRB hyperphosphorylation and increased SKP2 expression. Inhibition of either CDK4 or CDK2 restored G1-S arrest in MYCN-amplified cells, but only CDK4 inhibition sensitized for doxorubicin-induced cell death. p19INK4D and p16INK4A induction both restored G1-S arrest, but only p19INK4D sensitized for doxorubicin-induced cell death.

Conclusion: High CDK4 activity - as a consequence of amplified MYCN and/or chromosomal aberrations directly affecting the CDK4/CCND1 complex, significantly contributes to impaired G1-S arrest and apoptosis resistance following doxorubicin treatment. CDK4-specific inhibitors may potentiate DNA-damaging drugs currently used in treatment regimes for high-risk neuroblastomas.

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POB075

Radiosensitization of neuroblastoma cells after DNA-PK inhibition with NU7026

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Background: Effectivity of radiotherapy in neuroblastoma might be ablated by restoration of the radiotherapy-induced DNA damage by naturally occuring intracellular DNA repair pathways. Ionization radiation (IR) destroys cancer cells by inducing lethal double strand breaks (DSBs) in DNA. Since these DSBs are primarily repaired by DNA-dependent protein kinase (DNA-PK)-mediated non-homologous end-joining (NHEJ), we evaluated the radiosensitizing effects of DNA-PK inhibition in neuroblastoma cells.

Methods: Affymetrix microarray analysis of a tumour panel of human neuroblastoma samples, derived from primary tumours of untreated patients, was performed to study gene expression profiles. Gene levels in neuroblastoma were compared with various other tumour types and normal tissue. The radiosensitizing effect of DNA-PK inhibition was studied in the human neuroblastoma cell line NGP. NGP cells were treated with the DNA-PK inhibitor NU7026, 1 hour prior to exposure to ionization radiation.

Results: Gene expression of PRKDC, encoding for the catalytic subunit of DNA-PK, was significantly higher in neuroblastoma tumours as compared with normal tissues and the observed elevated PRKDC expression was associated with a more advanced tumour stage and worse prognosis. Prior inhibition of DNA-PK with NU7026 resulted in sensitization of NGP cells to IR. The degree of sensitization was higher for lower quantities of IR. Synergistic effect was most advantageous when treating cells with 10 μM NU7026 and subsequently 0.5 Gy IR, and became more pronounced at later time points after irradiation.

Conclusions: Concomitant treatment with a DNA-PK inhibitor might be an attractive approach to radiosensitize neuroblastoma and prevent radioresistance. Future animal studies are needed to confirm these results and give more insight into the most optimal in vivo treatment schedule.

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POB076

The antifungal drug ciclopirox olamine as a potential therapeutic agent for the treatment of neuroblastoma

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Background: Off-patent drugs with previously unrecognized anticancer activity could be rapidly repurposed for this new indication. Though originally developed as antimicotic agent, ciclopirox olamine (CPX), a well-known iron chelator, has recently been demonstrated to be an effective anti-tumor agent for the treatment of leukemia and myeloma cells in vitro. Our preclinical results raise the possibility that this drug could be used as a clinically relevant therapeutic for neuroblastomas.

Methods: Cytotoxic effects of CPX and other chemically different molecules with known iron chelation activity on NB cells were evaluated by the AlamarBlue assay. The intracellular iron chelation ability of the compounds was investigated by FACS. Microarray gene expression analysis was performed on polysomal RNA isolated from control cells and cells treated with CPX. The identified hits were confirmed by quantitative RT-PCR. Protein levels were assessed by Western Blotting. The synergism of CPX and 17AAG (an HSP inhibitor) was determined by combination index analysis.

Results: Among the iron chelators tested, CPX was shown to be one of the most effective in NB cells. CPX decreased growth of NB cell lines, MNA and non-MNA, with IG50 values of about 1-10 μM that appear pharmacologically achievable. Iron chelation was shown to be functionally important for its cytotoxicity. Microarray analysis on the polysomal samples from CPX-treated vs untreated CHP134 cells demonstrated downregulation of individual members of the human heat-shock protein (HSP) family. The downregulation was validated by quantitative RT-PCR and subsequently confirmed at the protein level. However, the effect was only partially mediated by the iron-chelation ability of CPX. In addition, consistent with its effect on HSPs level, CPX sensitizes cells to 17AAG. Future studies delving into the possible mechanism are warranted.

Conclusions: HSPs contribute to tumorigenesis through their well-documented antiapoptotic activity. Our data point out the suppression of HSPs as a potential mechanism of CPX-mediated cell-death in neuroblastoma.

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POB077

A screening for natural products identifies isorhamnetin as a synergistic compound with 13-cis retinoic acid in neuroblastoma

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Background: High-risk cases of neuroblastoma have poor survival rates, and novel therapies are needed. Well-chosen drug combination strategy can provide new opportunities. 13-cis-retinoic acid (13-CRA) is a differentiating agent used in most current neuroblastoma treatment regimens, while naturally occurring polyphenols have been shown to exert anti-proliferative effects in a wide variety of cancer cell lines. The purpose of this study was to identify natural products potentiating the effect of 13-CRA against neuroblastoma cells, and investigate the molecular mechanisms behind.

Method: A library of about 160 natural compounds was screened for viability of CHP134 cells challenged with a sublethal dose of 13-CRA for 48h. Drug combination experiments of 13-CRA and isorhamnetin, identified as a hit from the initial screening, were carried out, by exposing the cells for 24 and 48h to medium alone, single drugs alone or to different concentrations of the combination of the two drugs, and finally assessing cell viability. The synergism of 13-CRA and isorhamnetin was determined by combination index (CI) analysis. Changes in cell proliferation and cell death dynamics were investigated by flow cytometry and fluorescence microscopy. Microarray gene expression analysis was performed on polysomal RNA isolated form control cells and treated cells for 24 h with a single concentration of 13-CRA and isorhamnetin alone and with their combination.

Results: The in vitro screening of a natural compound library in combination with 13-CRA identified a flavonoid, isorhamnetin, as a potential enhancer of 13-CRA activity on neuroblastoma cells. Median effect analysis of drug combination revealed that 13-CRA and isorhamnetin exerted synergistic inhibition of the growth of neuroblastoma cells both at 24h and 48h treatment (CI<1 across a broad range of doses, corresponding to 20-80% loss of viability). Treatment of 13-CRA plus isorhamnetin induced enhanced apoptosis and led to coherent perturbations of gene expression which allowed to advance hypotheses on the molecular mechanism of this pharmacological synergism.

Conclusions: The combination of 13-CRA with isorhamnetin is more effective, at therapeutically relevant concentrations, than the sum of each single compound alone, in inhibiting neuroblastoma cell growth in vitro, suggesting potential clinically therapeutic capabilities

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POB078

MR and Hemodynamic Response Imaging (HRI) for monitoring the efficacy of a novel anti-angiogenesis drug combination on a Neuroblastoma murine model

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Background: Highly vascularized neuroblastoma (NB) is correlated with aggressive disease. Despite recent advances, outcome for high risk patients remains low and new therapies are urgently needed. TL-118 is a novel drug combination optimized for its anti-angiogeneis effect (a low-dose cytotoxic agent, a Histamine type2-receptor antagonist, COX1/2-inhibitor and an NF-kB inhibitor). Gemcitabine (Gem) is a chemotherapy used for pancreatic cancer therapy. TL and TL-Gem combination are currently in phase-ιι clinical trials, showing promising results. In this study we utilized an fMRI method combined with hypercapnia and hyperoxia (HRI) in order to evaluate the efficacy of TL-treatment on NB tumors in vivo.

Methods: 10^6 SK-N-BE cells were injected to the adrenal gland of NOD/SCID mice (n=25) to generate an orthotopic model. TL and Gem were daily administered by i.p injections starting on day of tumor detection (19± 2.24) by MRI. Tumor progression and response to treatment were followed bi-weekly by MRI. HRI was performed to assess the vascular changes within tumors. Finally, tumors were evaluated by histology.

Results: TL-118 (n=10) successfully decelerated NB growth In-vivo. Furthermore, the TL-Gem combination (n=5)prolonged the dormant phase, slowed tumor progression and significantly prolonged survival. Moreover, the TL-Gem combination significantly increased apoptosis and inhibited cell proliferation. HRI results demonstrated heterogeneousness response within the tumor, indicating for higher vessel density and maturation status; confirmed by immune-histochemical staining for smooth-muscle actin(α-sma) and CD31 (endothelial marker).

Conclusions: These results suggest that TL-118 and TL-Gem combination harbors a potential clinical benefit for NB-patients. Additionally, HRI proved to be sensitive and beneficial for monitoring tumor response to anti-angiogenic therapy.

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POB079

Genetic and Epigenetic Determinants of Differentiation Potential

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Understanding the molecular mechanisms regulating differentiation programs and cell fate decisions has broad implications for human disease. Specifically, controlling the process of differentiation represents a potential mechanism for treating cancers, as terminal differentiation induces a stable growth arrest. For example, treating neuroblastomas with cis-retinoic acid to promote differentiation has proven an effective strategy. In addition, the degree of differentiation noted in these tumors at diagnosis correlates strongly with prognosis, with more differentiated tumors associated with significantly superior outcomes. Overall, however, the development of novel agents directed towards tumor differentiation is a relatively understudied strategy.

To this end, we have examined how differentiation programs are executed by two different but structurally homologous bHLH proteins, NeuroD2 and MyoD. Comparison of genome-wide binding and expression profiles of NeuroD2-mediated neuronal differentiation in P19 cells and MyoD-mediated muscle differentiation in fibroblasts demonstrates a set of factor-specific binding sites and gene targets that is determined by binding site sequence. NeuroD2 and MyoD binding is limited, however, to E-boxes located within an accessible chromatin context, although additional factors serve to modulate binding within these areas of open chromatin. This indicates that the epigenetic state of a cell can significantly modulate its response to differentiation factors. In support of this, we observed that when expressed in an alternate cell type, NeuroD2 and MyoD can activate genes while failing to induce differentiation, and that this is due to an absence of binding near specific neurogenic or myogenic genes, likely due to their inaccessibility. Thus, while differentiation programs are genetically encoded by unique E-box sequences, chromatin accessibility appears to play a critical role in defining lineage potential by determining binding site availability for each factor. This suggests that modification of chromatin accessibility may represent a rational method for inducing differentiation in resistant cells.

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POB080

The neuroblastoma and ganglion components of nodular ganglioneuroblastoma share the same genetic changes, distinct from Schwann cells

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Background: Nodular ganglioneuroblastoma (nGNB), which represents <10% neuroblastoma cases, is characterized by a macroscopic nodule of stroma-poor neuroblastoma within a ganglioneuromatous component. These two components have been considered separate clones, one of them potentially more aggressive, to account for the poor prognosis in nGNB; however, there are few genetic data to support this concept. The aim of our study was to identify unique genetic changes in this rare subtype, and to investigate the clonal origin of its cellular components (neuroblasts, ganglion cells and Schwann cells).

Methods: Paraffin-embedded tumour samples from eight cases of nGNB were analyzed by SNP array and in situ hybridization (ISH). DNA was extracted separately for neuroblastoma and ganglioneuromatous areas using histology as a guide.

Results: By ISH, no cases were scored as MYCN amplified (>10 gene copies/nucleus); however, MYCN gain (4-10 gene copies/nucleus) was detected in 7/8 neuroblastoma samples, and was always associated with increased copies of chromosome 2. In ganglioneuromatous regions, gains were also detected in ganglion cells but were absent in Schwann cells. The SNP array studies identified chromosome losses (11q and 14q) and gains (12, 13q, 17q and 18q) in the neuroblastoma component whereas the ganglioneuromatous component showed far fewer genetic alterations or was normal. No changes unique to nGNB were detected. By ISH, ganglion cells in the ganglioneuromatous component showed the same alterations detected in the immature neuroblasts, whereas those changes were never detected in Schwann cells.

Conclusions: MYCN gain, but not amplification, is a frequent event in nGNB. Neuroblasts and ganglion cells in ganglioneuromatous components are genetically related, suggesting they are not likely to originate from separate clones. In contrast, the Schwann cells likely have a different origin and may be reactive. Our results suggest that the poor prognosis of nGNB cannot be explained by separate clones with distinct genetic signatures.

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POB081

MLL is a strong candidate driver gene for high risk MYCN non amplified neuroblastomas: a multi-dimensional high-resolution genomic data mining approach

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Background: Only a few driving events are known to be implicated in neuroblastoma including MYCN amplification and ALK activation but in most of the high risk MYCN non amplified tumors convincing driver gene has been identified thus far. To investigate this further, we used the CONEXIC algorithm that combines both gene copy number and gene expression data [Akavia et al., Cell, 2010].

Methods: DNA copy number, mRNA and miRNA expression data were available for 209 neuroblastoma tumor samples. CONEXIC was applied with focus on the two high risk neuroblastoma genomic subgroups (subtype 2A: 11q deletion and 17q gain) and subtype 2B (MYCN oncogene amplification and 17q gain).

Results: A list of 145 candidate driver genes was identified using this integrated data mining approach. These genes were subsequently prioritized using whole genome methylation and mutation data, chromosomal location and literature mining. As expected, MYCN emerged as a key driver for the subtype 2B group. Interestingly, for the subtype 2A group that is often marked by 11q deletions, the MLL gene was identified as a candidate driver. MLL belongs to a family of histone H3K4 trimethylases. Recent studies implicated MLL2 and MLL3 loss of function in medulloblastoma in keeping with previous copy number alterations of these loci in a subset of tumors. Amongst the possible mechanisms through which MLL genes might act in embryonic cancers is a putative role in aberrant proliferation of precursor cells.

Discussion: This study represents the first extensive integrated data mining effort in order to identify new candidate drivers for neuroblastoma. We propose MLL as a key putative driver gene for subgroup 2A neuroblastoma. Given the fact that few mutations are found in embryonal tumors, this approach may be critical in prioritizing rare mutated genes for further functional analyses and mouse modeling.

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POB082

Mapping the neuroblastoma epigenome: perspectives for improved prognostic biomarkers

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Background: Neuroblastoma (NB) is a childhood tumor originating from sympathetic nervous system cells. Although recently new insights into genes involved in NB have emerged, the molecular basis of neuroblastoma development and progression still remains poorly understood. Current risk assessment schemes unfortunately result in a significant proportion of patient misclassifications, leading to under - or overtreatment. Clearly, a more objective and accurate classifier is needed for improved outcome prediction. Next to genomic changes, epigenetic alterations have been described as well. Most of these methylation markers are found using 'candidate gene' approaches and the methylation frequencies are usually very low.

Methods: In order to find novel methylation markers that can be used for improved prognosis, we applied a whole-genome methylation screen. This technique relies on capturing with the MBD2 protein, containing a methyl-binding domain, with a very high affinity towards methylated genomic regions. In an initial phase, MBD2-seq was performed on 8 NB cell lines (for which mRNA profiles before and after treatment with the demethylating agent DAC were also available).

Results: An integrated analysis (MBD2-sequencing, re-expression analysis, analysis of public expression data) led to the selection of 43 candidate biomarkers, which were validated using real-time methylation-specific PCR in 89 primary NB patients in three prognostic groups. More than ten novel biomarkers in NB were found to be related with classical NB risk factors and survival. MBD-sequencing data on 45 primary NB tumors of the same three prognostic groups are underway, allowing us to make a genome-wide map of the epigenome of NB. An integrated data-analysis across the genomic (exon-arrays and array CGH) and epigenomic information, will lead to the identification of highly sensitive and specific biomarkers for improved risk classification and treatment.

Conclusions: Mapping the complete neuroblastoma methylome gives insight in NB biology and opens perspectives for improved prognosis.

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POB083

Promoter methylation analysis identifies prognostic methylation biomarkers in neuroblastoma

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Background: Due to the heterogenic biology of neuroblastoma (NB), current risk assessment schemes sometimes lead to misclassification of the patient with respect to therapy protocols. To reduce unnecessary under- or overtreatment and to improve the power of predictive outcome, additional tumor-specific prognostic markers are required. As several genes have already been shown to be silenced by hypermethylation of their promoter region in NB, this study aimed at identifying tumor DNA-methylation markers with prognostic power in order to improve NB patient stratification.

Methods: To identify genes silenced by promoter methylation we applied two genome-wide methylation screening methodologies on 8 NB cell lines: a re-expression analysis using 5-aza-2'-deoxycytidine (DAC) and massively parallel sequencing after capturing with a methyl-CpG-binding domain (MBD). To select for prognostic markers, 212 genes upregulated after DAC-treatment were subsequently screened using methylation-specific PCR (MSP) on primary tumors. After integrated data analysis, 43 unique candidate biomarkers were further analyzed with MSP on 89 primary tumors, representing low- and high-risk surviving and deceased patients.

Results: We identified 12 novel DNA-methylation markers (COL6A3, miR-1225, miR-3177, PCDHA6, PLXNC1, ANKRD43, ADRB2, APOE, miR-671, QPCT, KCND2 and PRPH), methylated in >50% of the primary tumors. Promoter methylation of DPP4, HIST1H3C and GNAS was found to be associated with overall and event-free survival, and the methylation status of TGFBI, TNFRSF10D, KRT19, TNFRSF10A, RARRES1, FAS, PRPH, CNR1, QPCT, HIST1H3C, ACSS3, GNAS and GRB10 with at least one of the classical risk factors, such as age, stage and MYCN status.

Conclusions: This study, being the most extensive methylation study in NB performed thus far, has led to the discovery of several novel prognostic DNA-methylation markers, and is of great value for the development of a DNA-methylation-based prognostic classifier. In addition, methylation in microRNA (miRNA) promoter regions could be demonstrated in primary NB tumor samples for the first time.

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POB084

A map of genomic copy number alterations in neuroblastoma based on annotation-guided breakpoint detection

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Background: Previous microarray studies of neuroblastoma chromosomal copy number have identified two distinct types of alterations: numerical and segmental (Janoueix-Lerosey, et al. 2009, Schleiermacher, et al. 2010). Tumors with numerical alterations exhibit gain or loss of entire chromosomes and are associated with good clinical outcome. In contrast, segmental alterations arising from chromosomal translocations usually indicate poor outcome. Thus, to construct an accurate model for predicting patient prognosis based on copy number profile data, we must first precisely characterize the locations of breakpoints and copy number alterations.

Methods: We analyzed array CGH data for 598 neuroblastomas. Due to variations in microarray technologies and tumor cell content across samples, existing copy number analysis algorithms produced visually inaccurate segmentations. So we designed new GUI software that allows a biologist to inspect a copy number profile and define regions of the genome that clearly contain breakpoints, gains, or losses (Hocking, et al. 2012). Then, we used the region annotation data to train segmentation models that provide highly accurate estimation of breakpoint locations and copy number alterations for each tumor genome.

Results: Figure: http://cbio.ensmp.fr/~thocking/neuroblastoma/ANR-figure.html We present a map of breakpoints and copy number alterations found in our database of 598 neuroblastomas. We detected frequent breakpoints in several previously characterized regions on chromosome arms 2p, 3p, 11q, and 17q. We characterize several additional genomic regions that frequently contain breakpoints and copy number alterations.

Conclusions: Our new software allows a biologist to visually train segmentation models, leading to very accurate breakpoint detection in the neuroblastoma copy number profiles we examined. The detected breakpoints and copy number alterations provide a map of genomic changes in neuroblastoma that we will use for predicting patient prognosis.

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POB085

TRANSLATOME PROFILING OF NEUROBLASTOMA CELL LINES REVEALS EXTENSIVE TRANSLATIONAL DEREGULATION OF HISTONE GENES

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Background: Sequence-dependent translational regulation of gene expression is exerted by modulation of mRNA translation rates by means of RBPs and ncRNAs. In the last years, it has gained increased acceptance as a powerful determinant of cell phenotypes, but its role in cancer development and progression is still largely not addressed. MYCN-amplified neuroblastoma is the most aggressive form of the disease. We analyze, for the first time, 13 neuroblastoma cell lines at both the transcriptome and translatome level in order to infer which translational mechanisms both may be altered in this disease.

Methods: We performed gene expression microarray analysis of 13 MYCN-amplified neuroblastoma cell lines, either on total and polysomal mRNA fractions. Resulting profiles were clustered in order to understand, on a genome-wide scale, the degree of relatedness of neuroblastoma transcriptome and translatome. mRNAs with a divergent behavior in the transcriptome and the translatome were then identified by various methods and obtained lists underwent ontological analysis to detect gene class enrichment. Next, the observed interesting genes groups were mined for known and likely post-transcriptional regulatory interactions by means of our internally developed AURA database (aura.science.unitn.it), in order to prioritize potential mechanisms producing these alterations in neuroblastoma.

Results: Total and polysomal gene expression profiles are significantly different: indeed, a number of cell line polysomal mRNA profiles group with each other instead that with their corresponding total mRNA profile. Fraction of mRNA with divergent behavior vary depending on the method used to score them, and being almost 12% with the RankProd method. Ontological enrichment highlighted a group of 64 translationally enhanced histone genes, which can potentially be regulated by various factors including the RBPs AGO1, SLBP and miRNAs of the mir-29 family.

Conclusions: We present here the first translatome profiles of cancer cells: the results of the comparison between these and the conventional, matching transcriptome profiles highlights that derangement of translational control of specific genes could play a significant influence on neuroblastoma onset and progression.

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POB086

M2 macrophages express CD1d and are selectively targeted by NKT cells in neuroblastoma

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CD1d-reactive Va24-invariant Natural Killer T cells (NKTs) play an important role in tumor immunity. However, the mechanism by which NKTs mediate antitumor responses against CD1d-negative tumors has remained enigmatic. Recent findings by us and others suggest that instead of attacking tumor cells directly, NKTs may target CD1d-positive tumor-supportive myelomonocytic cells. We have demonstrated that NKTs co-localize with tumor-associated macrophages (TAMs) in primary human neuroblastoma (NB). The phenotypic analysis of TAMs in primary tumors has revealed the existence of both M1- and M2-polarized subsets. However, only the numbers of CD163-positive M2 macrophages correlated with poor clinical outcome. Importantly, we found that CD1d is selectively expressed on M2, but not on M1 macrophages, both in TAMs isolated from primary NB tissues, and in in vitro polarized macrophages. NKTs mediated CD1d-dependent in vitro cytotoxicity against M2 macrophages or polarized them to an M1 phenotype via soluble mediators. The latter was in part dependent on NKT-derived GM-CSF and IFNγ. Moreover, adoptive transfer of ex-vivo expanded human NKTs resulted in a significant reduction of M2 TAMs in NB xenografts in humanized NOD/SCID/IL2Rγ(null) mice that correlated with anti-metastatic activity. Thus, targeting of tumor-supportive M2 TAMs by NKTs reveals a novel mechanism of tumor immunity that can be exploited for the development of effective immunotherapy of neuroblastoma and other tumors.

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POB087

The promoter methylation may lessen the increased pro-apoptotic impact of RASSF1A in triploid neuroblastoma found by mass screening

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Background: Although mass screening (MS) for neuroblastoma has been discontinued, the tumors found by MS give us opportunities to study their unique biology.

Methods: We examined promoter methylation of RASSF1A in 207 patients with neuroblastoma using conventional methylation specific PCR (cMSP) and quantitative MSP (qMSP); 123 infants found by MS (group A) and 84 children >12 months diagnosed clinically (group B). Tumors were classified into 2 types (diploid, 2n and triploid, 3n) by flowcytometry and/or FISH on chromosome 1. One and 30 patients in groups A and B, respectively, died of the disease.

Results: While 3n tumors were more frequently methylated than 2n tumors in group A (P=0.02), such correlation was not found in group B based on cMSP. Among group A tumors, while 3n tumors were more frequently methylated in the early stages, 2n tumors were more frequently methylated in the metastatic stages. In contrast, among group B tumors, the methylation occurred in both 2n and 3n tumors at the metastatic stages. Thus, the stage distributions of the methylated tumor were different between 2n and 3n tumors in group A, but not in group B. qMSP analysis showed that the methylation percentage was higher in 2n tumors of group B than 3n tumors of group A (P<0.01).

Conclusions: Previous studies reported that RASSF1A methylation was found in neuroblastomas at the advanced stages, and associated with poor outcome. However, in the present study of 2n and 3n tumors found by MS, RASSF1A methylation was frequently found in early stage tumors of 3n type and not associated with unfavorable outcome. Furthermore, 3n tumors were more frequently methylated than 2n tumors. The methylation in infant 2n and childhood 2n and 3n tumors represses its tumor suppressor activity, but that in infant 3n tumors could lessen the increased pro-apoptotic impact of triplicated RASSF1A.

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POB088

Inactivation of hSgo1 shows synthetic phenotype to MYCN amplification.

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Background/Aims: Unfavorable neuroblatoma is known to be characterized by MYCN amplification. Targeting a gene that is synthetic phenotype to MYCN amplification should kill only cancer cells and spare normal cells. Therefore such gene(s) provides a clue for the development of MYCN related cancer-specific cytotoxic agents.

Methods and Results: To search for genes that show synthetic effects with MYCN amplification, we compared the gene expressions using microarray between the normal ganglion and tumor of MYCN transgenic mice. We found hSgo1, which is essential for accurate chromosome segregation, was highly expressed in tumor. Additionally, MYCN overexpression in MYCN single copy cells induced hSgo1 expression. hSgo1 knockdown led to almost complete suppression of proliferation, G2/M delay and senescence associated phenotype in MYCN amplified cells, whereas hSgo1 knockdown did not affect in single copy cells. Supporting these in vitro data, patients bearing tumors with amplified MYCN could benefit from decreased hSgo1.

Conclusions: MYCN regulates hSgo1 expression. Downregulation of hSgo1 shows synthetic phenotype to MYCN amplification in human neuroblastoma cells, suggesting that hSgo1 could be a potent candidate of molecular target for neuroblastoma therapy. Our data also revealed that hSgo1 plays a critical role in interphase in addition to M phase.

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POB089

PES1 is a MYCN-related driver gene of neuroblastoma and shows modified histone binding

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Background: In neuroblastomas, some genes, including MYCN, are known as "driver genes" which confer a selective growth advantage to the cells. Although histone modifications have been linked to the multistep of carcinogenesis, few reports have elucidated the relationship between the development of neuroblastoma and histone modification.

Methods: Human neuroblastoma mRNA array data (GSE3960) were analyzed to find MYCN-related genes. To assess the relevance to the clinical features, we utilized Kaplan–Meier analysis of the human neuroblastoma prognosis data (AMC neuroblastoma profiling). mRNA array data of MYCN transgenic mouse were also referred to narrow candidate genes down.

Results: PES1, originally known as a pre-rRNA processing protein, was selected as a candidate of MYCN-related driver gene of neuroblastoma. Its mRNA and protein expressions were correlated with MYCN in neuroblastoma cell lines. Based on published human prognosis data, PES1 high patients showed worse prognosis than PES1 low patients (p=0.01). PES1 knockdown using shRNA caused growth inhibition in neuroblastoma cell lines. These results were in line with the gene selection strategies. Because PES1 contains a BRCA1 C-terminal interaction (BRCT) domain, phospho-protein binding domain, we performed LC-MS/MS to find binding partners of PES1. Histones were recognized as partners and the binding was confirmed by immunoblotting. Of particularly interest, heterochromatin forming histone modifications, such as H3K9me2 or H3K9me, was found to bind PES1, on the other hand, modifications related to euchromatin were not. These results suggested that PES1 specifically binds heterochromatin forming nucleosome and this association could be the key of the oncogenic role of PES1.

Conclusion: PES1 were considered to be a MYCN-related driver gene of neuroblastoma. Its expression was correlated with MYCN expression and had a significant impact on human prognosis data. Binding with heterochromatin forming histones suggested that its growth poromoting role was through epigenetic histone modification.

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POB090

Exploiting synthetic lethality for the Identification of therapeutic targets in MYCN-amplified neuroblastoma.

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Background: Amplification of the MYCN protooncogene is a common feature of advanced neuroblastoma and is associated with a poor outcome of the disease. It has been shown that high MYCN expression can sensitise cells to apoptosis. We thus speculated that neuroblastoma cells with amplification of MYCN should express high levels of antiapoptotic genes whose targeting could represent a potent strategy of eliminating MYCN-amplified cancer cells. The aim of this study was to identify synthetic lethal partners of MYCN, which can be used as therapeutic targets to selectively kill aggressive neuroblastoma cells, while sparing non-cancerous ones.

Methods: To identify the desirable targets, we performed a negative shRNA screening utilizing pGIPZ-shRNAmir whole genome library in isogenic neuroblastoma cell lines with or without MYCN over-expression.

Results: Out of 20,000 genes targeted, around 188 were identified as synthetic lethal partners of MYCN in our model system. We selected Adenosylhomocysteinase (AHCY), the Bloom syndrome, RecQ helicase-like (BLM) and Protein Kinase, membrane associated tyrosine/threonine 1 (PKMYT1) genes based on the screening results and validation experiments. We found that the selected genes are co-expressed with MYCN in neuroblastoma tumours and cell lines and are directly regulated by MYCN. Knockdown of AHCY and BLM by lentiviral shRNA caused increased apoptosis in MYCN-amplified neuroblastoma cell lines. Inhibition of S-adenosylhomocysteine hydrolase (SAHH), the protein encoded by the AHCY gene, and PKMYT1 by the compounds 3-deazaadenosine and PD 166285, respectively, led to proliferation arrest of MYCN negative and death of MYCN-amplified neuroblastoma cell lines. BLM knockdown and PD 166285 treatment sensitized neuroblastoma cells to DNA damage, but caused p53-induced apoptosis exclusively in MYCN-amplified cells.

Conclusions: Our findings suggest that pharmacological inhibition of AHCY, PKMYT1 and BLM could be an effective approach in the treatment of aggressive MYCN-amplified neuroblastomas.

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POB091

MXI1 INHIBITS N-MYC-MEDIATED PROLIFERATION AND INDUCES NEUROBLASTOMA CELL APOPTOSIS

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Background: Neuroblastoma is the most common extracranial malignancy of childhood. Myc family proteins regulate cell proliferation in response to mitogenic stimulation, and N-Myc plays a role in the pathogenesis of a subset of high-risk neuroblastoma tumors. MYCN-amplified neuroblastoma tumors are associated with a poor prognosis. Mxi1, a MAD family transcriptional regulatory protein, counteracts Myc by repressing transcription of Myc target genes. We hypothesize that Mxi1 antagonizes N-Myc activity in neuroblastoma by inhibiting cell proliferation and viability.

Design/Methods: We constitutively expressed Mxi1 in non-MYCN-amplified SHEP neuroblastoma cells, and also SHEP cells stably transfected to express high levels of MYCN (SHEP/MYCN). We also created neuroblastoma cell lines (Non-MYCN-amplified SH-SY5Y and MYCN-amplified IMR32) that inducibly express Mxi1 upon exposure to doxycycline. Cell proliferation and survival were quantified using BrdU and MTT assays, respectively. Apoptosis was measured by propidium iodide staining and caspase-3, caspase-8, and caspase-9 immunohistochemistry.

Results: In non-MYCN-amplified SHEP cells, Mxi1 overexpression independently inhibits cell proliferation and induces cell apoptosis. In the context of N-Myc overexpression in SHEP/MYCN or IMR32 cells, Mxi1 inhibits N-Myc-dependent cell proliferation and blocks N-Myc-dependent apoptosis. In the absence of N-Myc expression, Mxi1 induces cell apoptosis via the caspase 8 pathway. In contrast, in SHEP/MYCN or IMR32 cells that undergo N-Myc-dependent apoptosis upon exposure to low serum, the caspase 9 pathway is active.

Conclusion: Overexpression of Mxi1 in MYCN-expressing neuroblastoma cell lines leads to inhibition of both cell proliferation and low serum-induced apoptosis. Furthermore, Mxi1 expression results in decreased cell proliferation and induction of apoptosis independent of N-Myc. The use of distinct apoptotic pathways by N-Myc (caspase 9) and Mxi1 (caspase 8) highlights the complexity of cell death regulation in neuroblastoma. The interaction between Mxi1 and N-Myc in neuroblastoma should be considered in the development of novel targeted therapies to improve outcomes in children with neuroblastoma.

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POB092

Neuroblastoma cell lines express embryonic neural crest stem cells genes

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Background: We hypothesized that pathways characteristic of embryonic neural crest stem cells could play important roles in neuroblastoma cells. The aim of our study was to identify genes that were differentially regulated in neural crest stem cells and determine their expression in neuroblastoma.

Methods: Dorsal root ganglia (DRG) from rat embryos (E14.5) were flow sorted for the neural crest stem cell markers CD49d (integrin alpha) and CD271 (p75NGFR). Affymetrix microarray (Rat 230 2.0) was used to compare RNA expression profiles of CD49d+/271+ high and CD49d-/CD271- low populations (Partek v 6.5, ingenuity pathway analyses). Results were compared to microarray data obtained by comparing rat facial SKPs (neural crest precursors from the dermis) to sciatic nerve Schwann cells. Differentially expressed genes were validated by qRT-PCR in the DRG model. Expression of selected genes was determined using RNA from a panel of neuroblastoma cell lines and normal adrenal tissue.

Results: 796 genes were differentially expressed in stem vs. control cells in the rat DRG model. 143 of these genes were also differentially expressed in the SKPs/Schwann cell model. Proteins encoded by these genes have been implicated in signalling pathways involved in cardiac development, apoptosis, tumour initiation and metastasis. Genes that were upregulated in primary neural crest stem cells and neuroblastoma cell lines (vs. adrenal control) included EDNRA, RGS5 and IGF2R. Genes with lower expression in these cells included KCNJ8, LOX, NALCN, GDF10, RASGRF1, and iroquoise-1.

Conclusions: A neural crest stem cell gene expression profile signature has been identified using two different models of rat neural crest stem cells. A subset of these genes were also differentially expressed in neuroblastoma. Further studies will determine whether these genes are involved in NB proliferation, apoptosis, or invasion using neuroblastoma cell lines and tumour-initiating cell models.

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POB093

NLRR3 negatively regulated by MYCN induces neuronal differentiation through proteolytic processing by ECEL1

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Background: Neuronal leucine-rich repeat 3 (NLRR3) is a family member of orphan receptors with unknown function. We previously reported that high-level NLRR3 expression was significantly associated with favorable outcome in NB, and its expression was negatively regulated by MYCN in association with Miz-1. To find the functional roles of NLRR3, we here report that NLRR3 receives proteolytic processing and induces NB differentiation.

Methods: Suppression of NLRR3 expression by siRNA and overexpression of NLRR3 in NB cells were used for analyses of proliferation and differentiation. Immunohistochemistry, immunocytochemistry and western blotting were used to examine the expression and cellular localization of NLRR3 in NB cells and primary tumors.

Results: Overexpression of NLRR3 induced a neuronal differentiation in NB cells, whereas NLRR3 knockdown by siRNA significantly reduced the differentiation. Intriguingly, in retinoic acid-treated NB cells, NLRR3 expression was increased along with differentiation. Furthermore, ectopic-expression of NLRR3 repressed proliferation and reduced the colony numbers. Immunohistochemical study using anti-NLRR3 C-terminal antibody showed that NLRR3 was strongly expressed in favorable NB tumor cells, especially in the cell nuclei. Immunocytochemical study also showed the nuclear localization of NLRR3 protein in NB cells. Additionally, western-blot analysis showed multiple short fragments of overexpressed NLRR3. Based on these results, we hypothesized that NLRR3 is cleaved by the proteolytic enzyme and that its intracellular fragments translocate into nucleus to regulate differentiation process. We have found that NLRR3 interact with endothelin-converting enzyme like 1 (ECEL1), which is a new member of putative zinc-binding metalloendopeptidase with unknown substrate. Upon binding, NLRR3 cleaved at extracellular region by ECEL1. Interestingly, ECEL1 has been found to be expressed at significantly high levels in favorable NBs ($p<0.05$).

Conclusion: Our data suggest that NLRR3 is one of the important regulators to induce differentiation of NB, and its negative regulation by MYCN may define the clinical behavior of NB.

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POB094

RUNX3, whose gene is mapped to chromosome 1p36, facilitates protein degradation of MYCN in neuroblastoma

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Background: Neuroblastoma is a common pediatric solid tumor of neural crest origin. Among the prognostic indicators of neuroblastoma, the deletion at chromosome 1p36 and MYCN amplification are strongly associated with advanced stages, rapid tumor progression, and poor outcome. RUNX3, mapped to chromosome 1p36.2, encodes a Runt-related transcription factor. Originally, RUNX3 was identified as a candidate tumor suppressor gene in solid tumors of diverse origins, such as gastric, lung, and colon cancer. Previously, we reported that the expression level of RUNX3 is significantly down-regulated in advanced stages of primary neuroblastomas.

Methods: We hypothesized that high expression of RUNX3 could overcome the oncogenic property of MYCN in neuroblastoma. To prove this, we employed cell biological and biochemical assays, such as colony formation, immuno-precipitation, and immunofluorescence microscopy.

Results: We found that higher expression level of RUNX3 is closely correlated with better prognosis in neuroblastoma patients in which MYCN is highly expressed. RUNX3 inhibited cellular growth and migration of neuroblastoma cell lines, confirming the tumor suppressive capability of RUNX3. In MYCN-amplified neuroblastoma cells, the expression level of MYCN was significantly decreased at protein level by overexpression of RUNX3, while mRNA expression of MYCN was not affected. RUNX3 protein physically interacted with MYCN both in vitro and in vivo. As well, RUNX3 and MYCN colocalized in the nucleus. Interestingly, overexpression of RUNX3 induced poly-ubiquitination of MYCN. Consistent with this, addition of a proteosomal inhibitor MG132 inhibited degradation of MYCN, indicating that RUNX3 destabilizes MYCN through the proteosome.

Conclusions: Interaction between RUNX3 and MYCN facilitates protein degradation of MYCN via ubiquitin-proteosome pathway, suggesting a novel molecular mechanism of RUNX3 to suppress MYCN-mediated tumorigenesis. We propose that clinical treatment targeting RUNX3 may provide with an important clue to develop a novel therapeutic strategy in neuroblastoma.

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POB095

ARPP19 negatively regulates MYCN stability and enhances differentiation of neuroblastoma both in vivo and in vitro

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Background: ARPP19 is a member of highly conserved family of cyclic-AMP regulated phosphoproteins. It is involved in the regulation of neuronal differentiation upon NGF treatment. In neuroblastoma (NB), MYCN was reported to be a key factor to regulate differentiation but the role of ARPP19 remains elusive. We here hypothesized that ARPP19 might regulate NB differentiation by regulating MYCN.

Methods: We have employed retinoic acid (RA) mediated SK-N-BE cell differentiation to assay the role of ARPP19 and MYCN. We performed real-time PCR to check ARPP19 expression in the primary NBs. For tumor xenograft study in nude mice, we used TGW clones stably expressing ARPP19.

Results: Overexpression of ARPP19 promoted SK-N-BE cell differentiation upon RA treatment. We found that endogenous ARPP19 was increased in protein level but not at mRNA level upon RA mediated differentiation of NB cells, suggesting that ARPP19 is an inducible gene and regulates differentiation. The MYCN protein degradation was accelerated in the SK-N-BE clones expressing ARPP19 suggesting that ARPP19 enhances differentiation by suppressing MYCN protein. Consistently ARPP19 overexpression increased phosphorylation of MYCN at Thr58 position which is necessary for MYCN degradation. ARPP19 expressing SK-N-BE and TGW cells were characterized with the reduction of cell growth and colony formation ability. Moreover, the in vivo tumor growth was significantly reduced in mice bearing the TGW cells stably overexpressing with ARPP19 ($p<0.001$). Interestingly, the tumors derived from ARPP19 expressing clone were characterized by less angiogenesis. In 123 primary NB samples, Kaplan Meier survival curves have shown that high expression of ARPP19 is significantly associated with favorable outcomes ($p = 0.0095$). More interestingly, high expression of ARPP19 was significantly associated with good prognosis ($p=0.041$) among the aggressive NBs with MYCN amplification.

Conclusion: Present evidences suggested that ARPP19 may play an important role in NB pathogenesis by promoting differentiation and limiting tumor growth.

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POB096

NCYM, a MYCN antisense gene product, induces OCT4 promotes cell proliferation in neuroblastoma

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Background: MYCN amplification is frequently observed in unfavorable neuroblastoma (NB). MYCN maintains embryonic stem (ES) cell pluripotency, suggesting that MYCN plays an important role in tumorigenesis via maintenance of pluripotency. NCYM is a natural antisense transcript of MYCN. However, the functional role of NCYM remains unclear. We hypothesized that, like MYCN, NCYM might be critical for regulation of stem-like states of the cell. Here, we found that NCYM directly regulates OCT4.

Methods: mRNA was obtained from primary NBs. The mRNA expression was examined by quantitative RT-PCR. Transcriptional activation was investigated by chromatin immunoprecipitation analysis. Cell proliferation was evaluated by WST assay.

Results: The mRNA expression levels of NCYM, MYCN and ES-related genes were evaluated in primary samples. NCYM and OCT4 were significantly correlated. Overexpression of NCYM induced expression of ES-related genes (OCT4, SOX2, NANOG and LIN28) in SK-N-AS cells. Therefore, we focused on the regulation of OCT4 by NCYM. Knockdown of NCYM inhibited the expression of OCT4 in SK-N-BE cells. NCYM bound to the OCT4 promoter region and enhanced euchromatic histone modifications. We then investigated regulation of NCYM by OCT4. Overexpression of OCT4 induced the expression of NCYM. Knockdown of OCT4 suppressed the expression of NCYM. Finally, we evaluated a biological function of NCYM-OCT4 pathway. During cell differentiation with treatment of ATRA, the expression of NCYM and OCT4 were repressed. The mRNA expression patterns of NCYM and OCT4 were similar to each other. Moreover, overexpression of NCYM or OCT4 promoted cell proliferation in SK-N-AS and SK-N-BE cells.

Conclusions: Our results suggest that there is mutual regulatory mechanism between NCYM and OCT4 in NB cells, whose pathway may be important for promotion of the cell proliferation.

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POB097

The MYCN target gene AHCY drives methylation reactions and is thus involved in tumourigenesis

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Background: MYCN target genes are numerous and mostly unknown. We found AHCY to be directly regulated by MYCN and suspect function in tumourigenesis. AHCY codes for S-adenosylhomocysteine hydrolase (SAHH), which catalyses the hydrolysis of S-adenosylhomocysteine (SAH) to adenosine and homocysteine. High SAHH increases the conversion from S-adenosylmethionine (SAM) to SAH releasing CH3-groups for methylation reactions. SAM is essential for DNA and H3K27 methylation and thus silencing of genes.

Methods: To define neuroblastoma-associated target genes of MYCN, we carried out chromatin immunoprecipitation (ChIP) of MYCN in neuroblastoma cell lines, and correlated this data with genome-wide gene expression. Differential regulation of MYCN target genes were defined by the epigenetic marker H3K4me3 (activation), H3K36me3 (elongation) and H3K27me3 (silencing) at gene promoters using ChIP. Gene expression profiles of genes bound by MYCN and by distinct epigenetic marks were analysed in primary tumours (n=478). Using an all-trans-retinoic acid (ATRA)-induced neuroblastoma differentiation model, MYCN-regulated genes were further analysed. After revealing AHCY as an important MYCN target, we preceded with knockdown and inhibitor studies.

Results: In all tumours, genes are aberrantly silenced or activated due to changes in DNA methylation or histone modifications. We found a direct link between MYCN activation and methylation potential in neuroblastoma. AHCY expression is activated upon MYCN induction in Tet21N cells and correlates with MYCN expression in primary tumours. Also, high AHCY expression predicts poor outcome. We found AHCY to be bound by MYCN, clearly activated and elongated. AHCY expression is decreased upon differentiation with ATRA. Knockdown of AHCY with siRNA was synthetic lethal with amplified MYCN. Knockdown or SAHH inhibition presumably leads to changes in epigenetic markers and expression due to the inhibitory effect of SAH in the system.

Conclusions: Our data suggests that MYCN oncogenic function is partly mediated by SAHH, which might be involved in silencing tumour suppressor genes.

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POB098

Disrupting the N-Myc/Aurora-A complex as an approach to control N-Myc levels in childhood neuroblastoma

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MYCN encodes a transcription factor of the Myc family (N-Myc) that regulates multiple genes involved in cell growth, metabolism and cell cycle progression. Amplification of MYCN is a driver mutation in a subset of human neuroendocrine tumors including neuroblastoma and predicts poor prognosis. No small molecule inhibitors that target N-Myc are currently available for therapy.

To identify potential molecular targets for the therapy of MYCN-amplified neuroblastoma we have previously used an RNA-interference screen. One of the identified genes encodes the mitotic kinase Aurora-A. In neuroblastoma cells, N-Myc forms a complex with Aurora-A, which stabilizes N-Myc and protects it from proteasomal degradation.

Stabilization of N-Myc is a kinase independent function of the Aurora-A protein. Nevertheless, we now show that two available inhibitors of Aurora-A (MLN8054 and MLN8237), which induce an unusually distorted conformation of the kinase domain, disrupts the Aurora-A/N-Myc complex and promote degradation of N-Myc via the Fbw7 ubiquitin ligase. Furthermore, degradation of N-Myc contributes to efficient suppression of neuroblastoma cell proliferation by MLN8054. In a transgenic mouse model of MYCN-driven neuroblastoma, inhibition of Aurora-A suppresses N-Myc transcriptional and oncogenic functions and induces both mitotic arrest and differentiation of neuroblastoma cells. Our data show that Aurora-A is an accessible target that allows the manipulation of N-Myc stability for tumor therapy.

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POB099

The JARID1C histone demethylase is upregulated in aggressive neuroblastomas independent of MYCN amplification

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Background: Defining reliable risk factors for progression and fatal outcome in advanced stage neuroblastoma (NB) patients with tumors not harboring a MYCN amplification remains challenging. We here used mRNA expression data to identify novel genes, potentially involved in NB pathogenesis, that were associated with poor outcome independent of MYCN amplification.

Methods: Microarrays including all human exons were used to generate mRNA expression profiles from 113 primary NBs. Correlation analyses were used to identify genes associated with patient outcome independent of known risk factors. Candidate target gene expression was assessed in the SHEP, NB69, IMR5 and IMR32 cell lines using immunohistochemistry and western blotting. Candidate target gene expression was downregulated using siRNA in SHEP and IMR5 cells, and expression confirmed using RT-qPCR and western blotting. Subsequently, viability and cell cycle analyses were performed to monitor the cellular response upon target gene knockdown. In addition, the methylation status of H3K4 was assessed by western blotting, since the target gene JARID1C demethylates both trimethyl and dimethyl H3K4.

Results: Analysis of mRNA levels in primary NBs revealed that expression of the JARID1C histone demethylase was significantly elevated in relapse tumors independent of MYCN amplification status. The JARID1C protein was detectable in all NB cell lines analyzed, but subcellular localization was cell-line specific. Analyses of H3K4 methylation status in SHEP and IMR5 cells following siRNA-mediated JARID1C knockdown confirmed inhibition of JARID1C function. JARID1C knockdown significantly decreased cell viability, induced morphological changes, and resulted in an increase in the fraction of apoptotic cells, which was also confirmed by flow cytometry.

Conclusions: We identified JARID1C as a novel and independent marker of aggressive NB. Since siRNA-mediated knockdown of JARID1C in NB cell lines significantly decreased cell viability and increased the number of apoptotic cells, it is likely that JARID1C expression could be linked to NB tumor maintenance.

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POB100

Exploitation of the chick embryonic microenvironment to reprogram MYCN-amplified neuroblastoma cells to a benign phenotype, lacking detectable MYCN expression

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Background: Neuroblastoma exhibits an unusually high propensity for spontaneous regression. This phenomenon occurs most frequently in the youngest patients, under 18 months of age, perhaps because developmental cues still present prompt belated differentiation of the tumour cells. This led to the working hypothesis that factors from an appropriate embryonic environment may be capable of activating the correct molecular switches to encourage the differentiation or reprogramming of tumour cells to a benign phenotype.

Methods: EGFP-labelled MYCN-amplified Kelly cells were injected into the extra-embryonic vitelline veins of embryonic day (E) 3 and E6 chick embryos. Spatial and temporal responses of injected cells were then analysed at E10 and E14.

Results: Kelly cells injected at E3 respond to neural crest migratory cues and integrate into neural crest-derived tissues: some neural, such as the sympathetic ganglia and enteric nervous system, although never the adrenal gland; and others non-neural, such as the heart, meninges, jaw region and tail. Kelly cells injected at E6 do not show such targeting, integrating into tissues such as the liver, kidney and meninges. The cells respond to their respective microenvironments, and in sympathetic ganglia some cells differentiate, show reduced cell division, and crucially such cells have undetectable MYCN expression by E10. In non-neural locations, cells form more rapidly dividing clumps and continue to express MYCN. The down–regulation of MYCN is dependent on continuous and direct interaction with the sympathetic ganglion environment.

Conclusions: The Kelly cells' morphology, behaviour and gene expression are altered by the sympathetic ganglia microenvironment. Taking these key observations, we speculate that the Kelly cells' amplicon may likely contain the required DNA regulatory sequences to enable MYCN expression to be altered in response to the embryonic environment. These findings show that transcriptional control of MYCN expression in neuroblastoma tumours represents a suitable therapeutic target.

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POB101

CHD5 Promoter Regulation by MYCN in Human Neuroblastoma (NB)

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Background: CHD5 is located on 1p36.31 in the region that is consistently deleted in unfavorable NBs. CHD5 forms a NuRD-type chromatin remodeling complex, but the genes that it regulates is unknown. CHD5 expression is high in brain and in favorable NBs, but it is very low in unfavorable NBs and most NB cell lines. Previously, we found CHD5 promoter methylation in NB lines with 1p deletion and MYCN amplification. We performed CHD5 promoter analysis to identify factors that regulate CHD5 expression.

Method/Approach: Luciferase assay: Luciferase plasmid vectors with different portions of the CHD5 promoter were transfected into NB lines (NBLS, SKNSH, NLF). After 24 hr incubation, luciferase activity was detected with a fluorescence reader. ChIP assay: Nuclear proteins were prepared from NB cell lines by crosslinking and sonication. Immunoprecipitation was performed with MYCN antibodies and control IgG. After purification of bound DNA, Q-PCR was performed with primers designed around the E-boxes on the CHD5 promoter, as well as positive and negative control primers.

Results: High luciferase activity was found with sequences from -78 bp up to -358 bp upstream of CHD5 exon1, but with partial down-regulation from -140 bp to – 313 bp. Activity was also down-regulated from -600 kb up to -1 kb in all three NB lines. Previously, we showed DNA methylation from -400 bp to -800 bp in NLF and IMR5 lines, but not in SKNSH or SKNFI. There are five E-boxes located between -600 bp and -900 bp, and one at -214 bp. ChIP analysis showed MYCN binding to the upstream E-box region around -800 bp.

Conclusion: The CHD5 promoter has a negative regulatory domain from -600 bp to -1 kb, and we found MYCN binding to this multi E-box region. This suggests that MYCN may play a role in direct regulation of CHD5 expression.

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POB102

MYCN up regulation activates the folate metabolism and sensitizes cells for thymidylate synthase inhibitors

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Background: MYCN amplification (MNA) leading to MYCN up regulation has a substantial effect on the gene regulation network in neuroblastoma (NB). A meta-analysis of gene expression studies published during the last decade, has shown that genes of the one carbon pool by folate pathway are higher expressed in MNA compared to non-MNA tumors and cell lines. We hypothesised that the folate metabolism could be one of the pathways under direct transcriptional control of MYCN and an essential link in the MYCN oncogenic network.

Methods: MYCN-DNA interactions and histone modifications in four neuroblastoma cell lines were analysed using ChIP-seq. Functional analyses were performed after RNAi and during treatment with the thymidylate synthase inhibitors (TSI) raltitrexed and pemetrexed in four MNA and four non-MNA NB cell lines. Expression changes of folate genes due to the perturbations were monitored via qPCR.

Results: The ChIP-seq experiments showed that ten of the eighteen genes of the folate pathway (ATIC, AMT, GART, MTR, TYMS, MTHFS, MTHFD1, MTHFD1L, MTHFD2, and MTHFD2L) are direct transcriptional targets of MYCN and histone modifications related with gene induction were found in the majority of these genes. Inhibiting the gene expression of the folate genes (e.g. MTHFD2) resulted in a sharp reduction of viability and a moderate increase in apoptosis. Subsequent, cell cultures with a concentration series of raltitrexed and pemetrexed showed that MNA cell lines are approximately 10 to 100 times more sensitive compared to non-MNA cell lines.

Conclusions: MYCN overexpression up regulates the one carbon pool by folate in NB and makes MNA cells more susceptible for treatment with the anti-folates raltitrexed and pemetrexed in comparison with non-MNA cells which could be a new opportunity in the treatment of high stage MNA NB tumors.

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POB103

Mutation of the N-terminal T58 phosphorylation site of N-Myc stabilizes N-Myc protein expression and enhances its oncogenic potential

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Background: Deregulated expression of the MYC family of oncogenes occurs in many cancers. Mutations of phospho-residues within the N-terminal conserved phospho-degron domain (CPD) alter MYC stability and are found clinically in Burkitt's lymphoma. Increased phosphorylation of the CPD phospho-residues of N-Myc has also been associated with tumour progression in a medulloblastoma model. Amplification of MYCN occurs in approximately 25% of neuroblastoma (NB) patients and is associated with poor survival; however, the mechanisms underlying MYCN-mediated NB progression remain poorly understood. Using a panel of isogenic NB cells expressing either wild-type (WT) or CPD mutated N-Myc protein, we characterised the functional consequences of manipulating N-Myc stability through alteration of these residues.

Methods/Results: We engineered a panel of SHEP NB cell lines to express murine constructs encoding N-MycWT, N-MycS62A, N-MycT58A or N-MycS62A/T58A (Kenney, A.M. et al.2003). We show that N-MycWT and CPD mutant cell lines display a significant increase in cell growth (MTS and Flow Cytometry) over parental SHEP cells. Furthermore, we show that SHEP/T58A cells exhibit a defect in apoptosis (by Caspase activity and Annexin V assays) that is rescued upon ectopic expression of the pro-apoptotic BIM protein. SHEP CPD mutants also show increased oncogenicity in clonogenic assays and in vivo where N-MycT58A, N-MycS62A/T58A xenografts demonstrate superior ability to drive tumourigenesis compared to N-MycWT. Conversely, the SHEP CPD mutant cell lines show a defect in DNA damage repair conferring heightened sensitivity to both radiation and DNA damage inducing chemotherapeutics.

Conclusions: NB cell lines expressing CPD-stabilised N-Myc proteins show a significant increase in oncogenesis in both cellular and in vivo assays. Additionally, N-Myc CPD mutants show enhanced sensitivity to DNA damage inducing agents - potentially uncovering a synthetically lethal relationship between N-Myc stability and the DNA damage response. This approach has important implications for the discovery of MYC-targeted therapeutics in early-phase clinical trials.

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POB104

Integration of genomic and proteomic data identifies MYCN-regulated genes, proteins and interaction networks in neuroblastoma cells

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Background: The most common marker prognostic of poor outcome in children with neuroblastoma is amplification of the MYCN oncogene, yet the biological programs by which MYCN effects its aggressive phenotype are largely unknown. To identify biological pathways affected by MYCN amplification, we performed a comparative global analysis of genes and proteins in Tet 21/N neuroblastoma cells.

Methods: MYCN expression in Tet21/N cells is regulated by tetracycline. Gene arrays were performed on MYCN-high and MYCN-low Tet21/N cells and SH-EP parental cells using Affymetrix GeneChip® Human Exon 1.0 ST. For proteomic analyses, the cells were stable isotope-labeled for relative quantitation, enriched for phosphoproteins to enhance detection of signaling proteins, and analyzed by GeLC-MS/MS analysis. Integrative pathway analysis was performed on the genomic and proteomic datasets using DAVE and GeneGo.

Results: Integrating genomic and proteomic data in MYCN-high and MYCN-low expressing cells identified 88 proteins and 300 genes that change in abundance. We compared these results with previous studies and found both novel and previously identified genes and proteins. Integrating proteomic and genomic data identified over 50 gene products that are present in both analyses and are altered in abundance in response to modulating MYCN expression. The majority of these have discordant abundance changes, with protein levels more frequently altered with no change in gene expression. We have validated changes in protein levels using Western blots, including NPM1 and MATR3. We present interaction networks and pathways that correlate with MYCN expression, most notably those involved in cell adhesion and cytoskeletal remodeling.

Conclusions: We demonstrated a significant discordance between gene and protein expression, underscoring the need for integrative genomic and proteomic analysis to describe complex systems. We have identified previously reported and novel genes and proteins and networks regulated by MYCN expression that may provide new insights into the biology of MYCN-amplified tumors.

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POB105

Identification of synthetic lethal genes to MYCN-amplification

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Background: Despite the identification of MYCN amplification as an adverse prognostic marker in neuroblastoma, no drugs that target MYCN have yet been developed, in part due to the inherent difficulty in designing therapeutic molecules that can target transcription factors. An alternative approach is to identify genes that are essential for the growth of MYCN-amplified neuroblastoma.

Methods: We have screened for MYCN synthetic lethal genes using a highly efficient microRNA-short-hairpin RNA (miRNA-shRNA) library that contains more than 200,000 shRNAs targeting almost all predicted genes in the human genome. Each shRNA transcript in the library is linked to a unique 60 nt DNA barcode. If a shRNA leads to cellular death, the barcode linking to this shRNA will diminish in the cell population. The level of each shRNA was measured both by hybridizing genomic PCR products to microarrays that contain complementary sequence to the barcodes and by high-throughput sequencing of the DNA barcodes.

Results: We have performed an shRNA screen utilizing the SHEP-21N cell line, which expresses MYCN under a tetracycline controlled promoter. 396 significantly ($P<0.01$) depleted or enriched genes by both best shRNA and integrated gene level were identified. Gene set enrichment analysis was performed utilizing the Pathway Commons data set. 25 potential synthetic lethal genes have been examined by secondary screening utilizing Dharmacon SMARTpool siRNAs. Potential synthetic lethal genes include ATF5, eIF3M, WDR12, MAP2K2, and genes in the Shh pathway.

Conclusions: By the use of a genome wide RNAi screen, we identified potential synthetic lethal genes to MYCN amplification. After further validation, this may provide novel targets for treatment of neuroblastoma.

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POB106

Distinct roles for the CXCL12 receptors, CXCR4 and CXCR7 in human neuroblastoma

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Background: The chemokine CXCL12, and its receptors CXCR4 and CXCR7 have been involved in progression and dissemination of various cancers. In neuroblastoma (NB), CXCR4 expression is associated to undifferentiated tumours and poor prognosis, while the role of CXCR7, the newly identified second CXCL12 receptor, has not yet been elucidated. As functional interactions between CXCR4 and CXCR7 were recently proposed in different tumour systems, we explored not only the individual role of CXCR7 in NB biology, but also its impact on CXCR4/CXCL12-mediated signaling.

Methods: CXCR7 expression was screened in a large panel of NB tissues and cell lines by using tissue-microarray technology and RT-PCR analyses, respectively. Individual CXCR7, CXCR4, or both receptors were ectopically expressed in NB cell lines, and resulting cell migration and clonogenic properties were explored in vitro. Proliferative and invasive capacities of transduced NB cells were further evaluated upon nude mouse sub-cutaneous (s.c) or orthotopic tumour cell implantations.

Results: CXCR7 expression was essentially associated to the more differentiated neural tumour cells in differentiated tissues, while almost undetectable in undifferentiated tumours. Moreover, CXCR7 expression was found in a minority of NB cell lines, but could be further induced upon neuronal differentiation of NB cells in vitro. In contrast to CXCR4, CXCR7 strongly reduced in vitro colony formation, and impaired CXCR4/CXCL12-mediated chemotaxis. CXCR7 also drastically reduced in vivo growth in s.c conditions, and affected CXCR4-mediated orthotopic tumour take in a CXCL12-producing environment. No effect of CXCR7 was observed on tumour cell dissemination.

Conclusions: Our data suggest different roles for CXCR7 and CXCR4 in NB. While CXCR4 favors NB growth, CXCR7 elicits anti-tumorigenic properties and may be associated with NB differentiation. Importantly, CXCR7 may act as a negative modulator of CXCR4 signaling, further opening new research perspectives for the role of the global CXCR7/CXCR4/CXCL12 axis in NB.

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POB107

High AXL promotes migration in non-MYCN amplified neuroblastoma cell lines

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Background: Neuroblastomas (NBL) are highly metastatic(50% of patients). In general, in NBL MYCN amplification (NMA) is the most important prognostic factor. However NMA is infrequent and 70% of NBL are non-NMA, without a known driving oncogenic event. We identified among 17 NBL cell lines four non-NMA NBL cell lines with a distinct gene expression profile (HU133plus2.0 arrays, Affymetrix) and high expression of the AXL gene. AXL is a tyrosine kinase receptor, associated with metastatic spread of several cancers. We hypothesized that AXL functions as an oncogene in non-NMA neuroblastomas and expect AXL silencing to diminish malignant properties of high AXL expressing cell lines.

Methods and Results: High expression of AXL was detected in G-IM-EN, SK-N-AS and two subclonal cell lines SH-EP-2 and SH-EP-21N. AXL was silenced (lentiviral AXL shRNA expression plasmids, pLKO.1_shAXL, Sigma-Aldrich) in GI-M-EN and SH-EP-2 with an infection efficiency of >90% and 80-85% AXL-mRNA knockdown. We examined the silencing effect on migration and invasion. A significant reduction of migration (74.7%) and invasion (99.8%) was achieved in GI-M-EN. In the intermediate AXL expressing cell line SH-EP-2 migration reduction was less (41.9%). Furthermore, GI-M-EN and SH-EP-2 were compared for cytoskeletal, morphological changes after silencing. Cells became rounded with small lamellipodia and filopodia and F-actin fluorescent staining showed induction of F-actin positive stress-fibers (17.3%), most prominent in high AXL expressing GI-M-EN. In SH-EP-2 the number of cells with stress fibers was increased (9.9%). Expression of ,matrix metalloproteinase 9 frequent down-stream target of EMT-like processes, was not changed upon silencing of AXL. Also, we observed no effects on cell proliferation, apoptosis or downstream pathways (PI3K-AKT, MAPK-ERK pathway).

Conclusion: The AXL gene is highly expressed in a subset of non-NMA NBL cell lines and seems involved in migratory and invasion properties. AXL is a possible mediator of NBL metastasis.

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POB108

Prokineticins promotes neuroblastoma progression by maintaining a de novo population of c-KIT expressing cells

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Background: c-KIT+ cells are endowed with stem-like features, and may contribute to neuroblastoma (NB) progression and heterogeneity. High expression of Prokineticin receptors (PK-R1 and R2) are associated with NB progression. In this study, we have defined the roles of Prokineticin signaling in the maintenance of c-KIT+ population and tumor progression of NB.

Methods: Expression of c-KIT in various NB cell lines, NB tumor initiating cells (TICs) and tumor tissues derived from these cells were analyzed using flow cytometric analysis and immunohistochemical staining (IHC). Quantitative RT-PCR and IHC were also performed to examine the expressions of PK-R1 and PK-R2 in different NB cell lines, TICs lines and tumors. Growth, proliferation, clonogenicity, as well as tumorigenicity of c-KIT+ cells and its counterpart were compared. The dynamic changes of c-KIT+ compartment in tumor bulk were monitored with in vitro and in vivo models.

Results: c-KIT+ cells were consistently detected not only in the primary NB samples, but also in tumors derived from c-KIT- NB cell lines and TICs, suggesting that c-KIT+ cells can be de novo generated and maintained during tumor formation. Intriguingly, subsequent isolation of c-KIT+ cells from NB cells indicated that they are highly migratory, drug resistant and endowed with high colony formation capacity and tumorigenicity. In addition, we found that c-KIT+ cells indeed are bidirectionally regulated in tumor bulk, while Prokineticin is implicated in the maintenance of this phenotypic distinct population via PK-R2. Knocking down PK-R2 could significantly reduce c-KIT+ population in tumor bulk.

Conclusions: In summary, c-KIT+ cells represent the aggressive entity of NB, and they may continually arise or disappear dependent of the tumor context to support the tumor growth and contribute to the tumor heterogeneity. Therapies against the Prokineticin/PK-R2 may have profound impacts on attacking this moving target and eradicating the tumor bulk.

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POB109

Role of ATP and myeloid-derived suppressor cells in neuroblastoma microenvironment

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Background: 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 of A/J mice. Bioluminescence imaging (BLI) was used to detect extracellular ATP in living animals.

MDSC were phenotypically characterized in the peripheral blood (PB), bone marrow, spleen and tumor from healthy and NB-bearing mice by flow cytometric analysis. MDSC Gr-1high and Gr-1low were isolated from the spleen of NB bearing mice and evaluated for expression of immunosuppressive molecules such as arginase-1, reactive oxygen species (ROS), nitric oxide and TGF-beta.

The functional expression of P2X7 receptors was evaluated in MDSC Gr-1high and Gr-1low by RT-PCR, immunofluorescence and spectrofluorimetric analysis.

Results: Extracellular ATP was specifically detected in the tumor microenvironment of NB bearing mice in amounts that increased as tumor porgresses. The percentage of CD11b+/Gr-1+ cells was found to be higher in the spleen and PB of NB bearing mice compared to healthy animals. In particular, granulocytic Gr-1high MDSC producing higher levels of ROS and arginase-1 were detected in the spleen of NB bearing mice. Both Gr-1high and Gr-1low from NB bearing mice expressed P2X7 receptor which was found to be functional as an ion channel.

Conclusions: Extracellular ATP and functional MDSCs were found in NB microenvironment where they likely promote NB progression.

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POB110
Characterization and proteomic analysis of neuroblastoma-derived exosomes

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Background: Exosomes are nanometer-sized membrane vescicles derived from the multivesicular bodies (MVBs) of the endocytic pathway and released by normal and neoplastic cells.

Methods: exosomes were isolated from the conditioned media of HTLA-230 human Neuroblastoma (NB) cells by ultrafiltration and ultracentrifugation. Size and morphology of the purified nanoparticles were studied by electron microscopy and dynamic light scattering (DLS). Proteomic profile of NB-derived exosomes was evaluated with a 2DLC separation and MS/MS analyses using the MudPIT strategy. Exosome-derived proteins were compared to the profiles of HTLA-230 membrane proteins and to the Exocarta database. Flow cytometry of NB-derived exosomes was carried out after vesicle adsorption onto latex beads.

Results: NB-derived exosomes exhibited the characteristic cup-shaped morphology and size distribution analysis showed a bell-shaped curve with a peak at 130 nm and a polydispersity factor of 0.1. Zeta Potential value was -32 mV, suggesting a good nanoparticle stability. Proteomic analysis revealed that nearly 70% of the proteins identified in NB derived exosomes are present on Exocarta, including well characterized protein markers such as tetraspanins, heat shock proteins, MVBs proteins and cytoskeleton related proteins. Moreover, several NB cell-related proteins were identified in HTLA-230-derived exosomes, including CD133, CD147, CD276 and Fibronectin. The presence of tetraspanins, CD133, CD147 and CD276 was confirmed by flow cytometry. Noteworthy, flow cytometric analysis showed that NB-derived exosomes highly expressed GD2 disialoganglioside, one of the most specific marker of NB disease. Moreover, we developed an ELISA protocol based on the presence for the first time of the GD2 molecule on exosomes.

Conclusion: this study shows that NB-derived exosomes express a discrete set of proteins involved in defense response, cell differentiation, cell proliferation and the regulation of other important biological processes, suggesting that NB-derived exosomes may play an important role in the modulation of tumor microenvironment and may provide potential diagnostic biomarkers.

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POB111
Cell survival signalling through PPAR delta in neuroblastoma

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Background: The balance between cell death and survival signalling determines resistance to retinoic acid (RA). After treatment, RA promotes phospholipase A2 (PLA2) mediated arachidonic acid (AA) release. AA is metabolised by lipoxygenases (LO) to form leukotrienes and eicosanoids. We hypothesise that LO metabolites promote cell survival through PPAR delta activation.

Methods: Small molecule inhibitors targeting enzymes that metabolise AA were used in combination with all-trans retinoic acid (ATRA). Combinations were tested on SH-SY5Y, NB-69 and NGP cells using cell viability XTT assays. SH-SY5Y subcutaneous xenografts were established and mice were treated daily with ATRA, celecoxib or a combination of the two drugs. The role of PPAR delta was investigated using PPAR response element luciferase reporter assays and PPAR delta siRNA. PPAR delta expression was measured by quantitative real time PCR.

Results: Celecoxib (COX2 and 5-LO inhibitor) and ATRA synergistically promote apoptosis in neuroblastoma cells in vitro and slow tumour growth in vivo. Inhibition of PLA2 (AACOCF3), 5-LO (MK886) and PPAR delta (GSK0660 and PPAR delta knockdown) sensitised cells to ATRA induced apoptosis. Both ATRA and the 5-LO metabolite 5-oxo-ETE activated endogenous PPAR transcriptional activity. Conversely, PPAR delta knockdown and 5-LO inhibition reduced transcriptional activity after ATRA treatment. This demonstrates that ATRA activates PPAR delta and 5-LO is necessary for this activation. Interestingly, expression of the inhibitory isoform, PPAR delta 2, increased significantly in cells treated with a combination of 5-LO inhibitors and ATRA. Finally, treatment with the PPAR delta agonist L165-041 or 5-oxo-ETE rescued cells from ATRA and celecoxib induced cell death.

Conclusions: AA released after RA treatment is metabolised by 5-LO to produce 5-oxo-ETE. 5-oxo-ETE activates PPAR delta and promotes cell survival. Targeting this pathway sensitises neuroblastoma cells to apoptosis in vitro and enhances the efficacy of RA in vivo.

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POB112
The JMJD2c histone demethylase is strongly expressed in neuroblastoma and maintains the undifferentiated state in vitro

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Background: Epigenetic changes in DNA and histone methylation are hallmarks of most cancers. Several histone demethylases have been identified, most of which catalyze the removal of methyl groups from histone H3 lysine residues, thereby influencing gene expression. The JMJD2c histone demethylase regulates H3K9me3 demethylation, and has been shown to work together with the epigenetically crucial LSD1 histone demethylase. A recent report from our own group implicates LSD1-targeted therapy as a new option against neuroblastoma. Here we analyzed the role of JMJD2c in neuroblastoma cell lines and its usefulness as a therapy target.

Methods: Cell viability, proliferation, death and differentiation were analyzed in the neuroblastoma cell lines, SHEP, LAN1, SK-N-AS and IMR5, after siRNA-mediated JMJD2c knockdown. In the same cell line panel, cell viability, proliferation and death were analyzed after inhibition of JMJD2c, LSD1 or both using novel small molecule inhibitors.

Results: JMJD2c knockdown significantly reduced cell viability and proliferation, and increased expression of neurotensin, a marker of neuronal differentiation. Similar results were obtained when LSD1 was inhibited using a novel small molecule inhibitor, whereas a small molecule inhibitor of JMJD2c had no effect on cell viability in vitro. Combinatorial treatment with both inhibitors, however, produced synergistic effects on both cell viability and induction of differentiated cell morphology.

Conclusions: High levels of JMJD2c expression in neuroblastoma cells are likely to contribute to the maintenance of an undifferentiated state in vitro. The combinatorial inhibition of JMJD2c and LSD1 produces a synergistic effect on cell viability and a stronger induction of cell differentiation. These results indicate that integrating agents targeting cooperating pools of epigenetic regulators into molecular targeted therapy approaches could be beneficial for the treatment of neuroblastomas.

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POB113
Cellular mechanisms regulating Anoikis Resistance in Neuroblastoma

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Background: More than half of neuroblastoma patients have metastatic disease at diagnosis, and the mechanisms by which neuroblastoma cells spread are not well understood. As a barrier to metastases, cells normally undergo apoptosis after detachment from the extracellular matrix (ECM), a process termed 'anoikis'. Resistance to anoikis enables cells to survive in an anchorage-independent manner after detachment from primary tumors and while traveling through blood vessels and lymphatics. Anoikis resistance is considered to be a critical step for tumors to metastasize, and thus represents an attractive tumor-specific therapeutic target.

Methods: To understand the regulation of anoikis resistance of neuroblastoma, we conducted a novel high-throughput screen to identify chemical compounds (Prestwick library) that sensitize neuroblastoma cells to anoikis. We also performed an Affymetrix GeneChip analysis to determine which genes are differentially regulated in neuroblastoma cells resistant to anoikis. Neuroblastoma cells were plated on standard tissue culture plates to promote adhesion and on hydrogel-coated ultralow binding plates for suspension conditions.

Results: Thirteen neuroblastoma cell lines were analyzed for growth in non-adherent conditions. MycN amplification strongly correlates with the ability of cells to grow in anchorage-independent conditions. Our initial high-throughput chemical screen did not identify unique compounds that differentially induced apoptosis in suspension but not adherent conditions. Future efforts will focus on optimization of this assay. Microarray analyses identified signaling pathways previously known to regulate anoikis resistance in other cell types such as components of the integrin, focal adhesion, extracellular matrix receptor interaction and membrane trafficking networks, as well as other signaling modules previously not implicated including members of the TGFbeta pathway, cell cycle, and Rho GTPase signaling.

Conclusion: The acquisition of anoikis resistance promotes survival and distant spread of cancer cells. Identification of the molecular mechanisms utilized by neuroblastoma cells to regulate anoikis resistance will lead to the development of innovative therapies.

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POB114

Calreticulin mediates nerve growth factor/TrkA-elicited neuronal differentiation

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Background: The nerve growth factor (NGF)/TrkA-signaling is necessary for neural development, and its abnormality has been tightly associated with the tumorigenesis of various cancers originated from the nervous system. The characterization of key molecules involved in the NGF/TrkA-mediated neuronal differentiation could pave the way for the development of novel therapeutic strategies against neural malignancy. We have previous demonstrated that calreticulin (CRT) is a favorable prognostic factor highly expressed in primary neuroblastomas (NBs) with a more differentiated histology. In the present study, we sought to determine whether CRT could take on an active in governing neuronal differentiation of NB.

Methods: The NGF-elicited neuronal differentiation of PC-12 cells were quantitatively examined by immunofluorescence confocal microscopy and Western blotting in response to either overexpression or down-regulation of CRT. The correlation between CRT expression and TrkA level in 68 historically confirmed NB tumors were also determined by Kaplan-Meier analysis.

Results: We found that the level of CRT was enhanced in NGF-stimulated differentiation of PC-12 cells through the extracellular signal-regulated kinase (ERK)-dependent mitogen-activated protein kinase (MAPK) pathway. A deficiency of CRT significantly decreased NGF-elicited neuronal differentiation. Furthermore, overexpression of CRT enhanced neuronal differentiation via simultaneously activating the ERK-dependent MAPK pathway. The Ca2+-regulating capacity of CRT was demonstrated to be indispensable for NGF-elicited neuronal differentiation. Intriguingly, the expression levels of CRT and NGF receptor TrkA were highly correlated in NBs with differentiated histology, and the coexistence of CRT and TrkA in NB tumors synergistically predicted a better 5-year survival rate.

Conclusions: Our present findings delineate a novel CRT-dependent regulation of NGF-induced neuronal differentiation. The intimate correlation between CRT and TrkA suggests a mechanistic synergy in favoring a better clinical outcome of NB.

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POB115

Identifying TrkA and TrkB specific pathways in neuroblastoma through phosphoproteomic analysis

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Background: Neuroblastoma is the most common solid tumor found in children and is difficult to treat given its genetic and clinical heterogeneity. The tyrosine kinase receptor, TrkB, is often co-expressed with the MYCN oncogene in high-risk tumors, whereas the TrkA receptor is most often found expressed in low-risk, non-MYCN amplified samples. There are differences in the gene expression profiles of TrkB- and TrkA-over-expressing cell lines, but they do not explain the phenotypic variation. We hypothesize that differences in protein translation and post-translational modifications have profound downstream effects on cellular signaling and disease phenotype.

Methods: We performed quantitative proteomics using stable isotope labeling, phosphopeptide enrichment, and tandem mass spectrometry on parental SY5Y neuroblastoma cells and cell lines stably transfected with either TrkA or TrkB. Receptors were activated with NGF or BDNF and activation was inhibited with CEP-701 (Lestaurtinib), a selective tyrosine kinase inhibitor, currently in clinical trials. Samples were separated by gel electrophoresis, digested with trypsin, and applied to the mass spectrometer for protein identification.

Results: We have performed quantitative phosphoproteomic analysis and compared protein expression levels and patterns in TrkA overexpressing, TrkB overexpressing, and parental SY5Y cells. The TrkA and TrkB receptors were activated with NGF and BDNF ligands, respectively, as evidenced by increased phosphorylation of ERK and AKT, with inhibition by CEP-701. Changes in protein abundance and pathway activation following both ligand binding and inhibition are being determined by quantitative phosphoproteomic analysis, and TrkA-specific and TrkB-specific differences will be presented.

Conclusions: As current genomic techniques may underestimate the differences in protein expression, proteomic profiling holds great promise for describing how post-translational modifications such as phosphorylation can affect tumor phenotype. Our work may reveal key elements of TRK signaling pathways important in neuroblastoma tumorigenesis, and may lead to the identification of novel targets for therapy development.

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POB116

Rab15 alternative splicing correlates with differentiation of neuroblastoma cells

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Background: Neuroblastoma is characterized by its tumor heterogeneity driven by the differentiation of tumor-initiating cells (TICs) that can be isolated as spheres. Although many diagnostic and/or prognostic biomarkers have been proposed, the response of high-risk neuroblastoma patients to current therapies is still unpredictable. Aberrant alternative splicing is intimately associated with an increasing number of cancers, and its use as a new diagnostic and/or prognostic biomarker has attracted considerable attention. Misregulation of Rab family small G proteins, key regulators of membrane traffic, results in an increasing number of cancers. Rab15 is originally isolated as a brain-specific Rab protein regulating the endocytic recycling pathway and is recently identified as a downstream target of neural bHLH transcription factor Atoh1 implicated in preventing medulloblastoma progression.

Methods: Neuroblastoma TICs were isolated as spheres grown in serum-free non-adherent culture. Neuronal differentiation of neuroblastoma cells was induced by all-trans-retinoic acid. Full-length Rab15 cDNA containing seven exons was amplified by RT-PCR using primers corresponding to a human counterpart of rat Rab15, and nine independent clones were sequenced. Rab15 isoform expression and balance were analyzed by real-time RT-PCR using the comparative CT method.

Results: In addition to a human counterpart of rat Rab15 designated as Rab15CN, alternative splicing of exon 4 generated three isoforms designated as Rab15AN1, Rab15AN2, and Rab15AN3. Although Rab15AN2 and Rab15AN3 contained premature termination codons, all isoforms showed tissue-specific distributions. Rab15CN and Rab15AN3 were predominantly expressed in brain, Rab15AN1 in testis, and Rab15AN2 in brain and testis. Rab15 isoform balance measured by the Rab15CN/Rab15AN1+AN2+AN3 ratio was regulated in a tissue-specific manner and was significantly decreased in spheres and increased during neuronal differentiation of neuroblastoma cells.

Conclusion: Rab15 isoform balance correlated with differentiation of neuroblastoma cells and may deserve the further evaluation as a new diagnostic and/or prognostic biomarker.

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POB117

Impact of neuroblastoma TrkB target Galectin-1 on immune effector cells

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Background: Galectin-1 (Gal-1) is a multifunctional protein that enhances tumor aggressiveness by inducing angiogenesis and contributing to the tumor immune escape. We have previously reported Gal-1 expression to be correlated with TrkB expression in neuroblastomas (NB), and to mediate TrkB-triggered aggressive properties of NB cells in vitro. Here, we aimed to assess the effect of Gal-1 on immune effector cells as a first step to decipher the role of NB-derived Gal-1 in the tumor-host interaction.

Methods: We first determined the immune phenotype of neuroblastic tumors and control tissue in tumor-bearing mice using the established TH-MYCN NB mouse model. Next, we ectopically co-expressed Gal-1 and GFP in CD4+ T cells from wildtype (wt) mice, allowing for FACS-based isolation of Gal-1-expressing CD4+ T helper cells including a subset of CD4+CD25+ regulatory T cells (Treg). We monitored the autocrine and paracrine effect of ectopic Gal-1 expression in CD25- T helper cells on T cell proliferation. In comparison, the additional effect of paracrine Gal-1 derived from Treg cells was assessed in co-culture experiments.

Results: NB tumor tissue from TH-MYCN transgenic mice showed higher lymphocyte infiltration even compared to lymphocyte-rich spleen tissue, with CD4+ T cells being most prominently elevated. We, therefore, focused our first analyses on the interaction of Gal-1 with CD4+ T cells. Overexpression of Gal-1 in transduced CD4+ T cells was confirmed by western blotting. Ectopic expression of Gal-1 in CD4+ T cells from wt mice resulted in autocrine- as well as paracrine-mediated inhibition of T cell proliferation. The paracrine inhibitory effect of Gal-1 on T cells was additionally increased when the molecule was ectopically expressed in co-cultured Treg cells.

Conclusions: Our data provide insights into Gal-1 mediated immune escape mechanisms of NB cells and add further evidence to the potential of Gal-1 as a promising new target for neuroblastoma therapy.

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POB118

A genome-scale shRNA screen identifies GSK3 as a critical regulator of p75NTR transcription in high risk neuroblastoma.

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Background: High expression levels of TRKA and P75NTR neurotrophin receptor genes sensitize neuroblastoma cells to NGF-mediated apoptosis and correlate with low risk neuroblastoma and favorable outcome. We have recently shown that in the most aggressive cases, particularly those characterized by amplified MYCN, both genes are transcriptionally silent. Although MYCN appears to contribute to repression of the two receptors, the precise mechanism(s) by which this happens has not been completely elucidated.

Methods: We used a shRNA screening strategy to identify genes involved in p75NTR silencing. SK-N-BE and L-AN-1 cell lines lacking p75NTR expression were infected with a human whole genome shRNA library (pLK0.1) Following expression, shRNAs were selected based upon strong re-expression of p75NTR in cells. p75NTR positive cells were selected by FACS, and shRNAs were amplified and sequenced. shRNA target genes were bioinformatically identified.

Results: Using this approach, we identified 31 genes whose functional knockdown leads to significant p75NTR re-expression. We have focused on one of these genes, GSK3β, since its downregulation caused the strongest re-expression of p75NTR. Data were validated by qRT-PCR, Western blotting and FACS analysis. The role of GSK3β on p75NTR biology is under investigation.

Conclusion: Our findings support a model in which the GSK3β pathway can exerts a negative control on p75NTR transcription in high risk neuroblastoma and highlight the possibility to use GSK3β inhibitors as potential therapeutic drugs to treat neuroblastoma with low p75NTR.

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POB119

PKA-mediated phosphorylation of EZH2 at serine 21 suppresses H3K27me3 and induces neuronal differentiation of neuroblastoma

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Background: Increased expression of EZH2, the polycomb repressive complex proteins 2(PRC2) histone methylase, represses transcription of genes with tumor suppressor or differentiation-inducing function in undifferentiated, high-risk neuroblastoma (NB). Mechanisms regulating EZH2 activity in NB cells are not well-studied. We've identified a novel post-translational modification to EZH2 mediated by Protein kinase A(PKA) that inhibits EZH2 activity.

Methods: Differentiation was induced using 5μM retinoic acid(RA) in KCNR and SY5Y cells. Immunoprecipitation(IP) and Western blotting assessed protein levels and binding interactions. Pharmacologic and siRNA strategies were used to target EZH2 and signaling pathways. EZH2 target genes(RARß and NTRK1) were assessed by Chromatin IP(ChIP). EZH2-wild-type(GFP-EZH2-WT) or mutant EZH2-S21A plasmids were transfected into NB for functional studies.

Results: At steady-state EZH2 binds, methylates H3K27me3 and represses transcription at RARß and NTRK1 genes. RA(6hrs) induces increases in phosphorylation of EZH2 ser21(P-EZH2ser21) which decreases EZH2 binding to histone H3, decreases nuclear EZH2 and increases cytosolic EZH2. This causes decreases in H3K27me3 and induction of RARß and NTRK1 mRNA. Pharmacologic or genetic inhibition studies indicate PKA but not AKT or MAPK binds to and phosphorylates P-EZH2ser21. High-risk NB patients have low levels of PRKACB mRNA, a catalytic subunit of PKA(R2 database, bonf.p=2.8e-7). Reconstitution of PRKACB in NB cells leads to increases in P-EZH2ser21. RA increases cAMP levels leading to increased P-EZH2ser21, decreased cell growth and induction of differentiation which is completely blocked by transfection of the phospho-mutant EZH2- S21A.

Conclusions: Lesions in PKA signaling may exist in NB. PKA-mediated P-EZH2ser21 is an early event in the RA mediated differentiation and reverses epigenetic silencing of key developmental genes such as RARß. These studies identify a novel mechanism by which cAMP and PKA, key mediators of extracellular signaling pathways function to regulate EZH2 activity. This indicates agents that raise intracellular cAMP levels may be therapeutically relevant to target EZH2 activity.

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POB120

DLL1 expression in Neuroblastoma correlates with angiogenic processes and modulates endothelial cell branching.

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As was previously shown in breast cancer models, tumor-endothelial cell interactions are of regulatory importance in the process of endothelial cell tube formation. Specifically, Notch ligands expressed on the tumor cells appeared to alter Notch signaling in the forming vessels. In the case of breast cancer, the ligand responsible for this effect was found to be JAG2, however upon analysis of available neuroblastoma expression arrays, no correlations between JAG2 expression and angiogenic processes were found. Instead, observations implied a similar role for the Notch ligand DLL1 in neuroblastomas. Treatment of cultured neuroblastoma cell lines with hypoxia did also result in an up-regulation of DLL1 expression. In addition, upon knockdown of DLL1 in neuroblastoma cells, co-culture experiments with endothelial cells displayed a reduction in both number and length of endothelial tubes formed. These results suggest that hypoxic up-regulation of DLL1 in neuroblastoma cells may mediate a similar cross-talk between tumour and endothelial cells as is seen with JAG2 in breast cancer.

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POB121

HOX gene and associated long noncoding RNA expression correlates with neuroblastoma cell line phenotype and response to 13-cis retinoic acid

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Background: Hox genes and associated long noncoding RNAs (lncRNAs) are master regulators of body and organ development. Dysregulation of their expression has been appreciated in multiple cancer types, including neuroblastoma. However, the effects of these gene products are dependent on their overall expression pattern, and comprehensive evaluation of that expression has not been previously performed.

Methods: 5 neuroblastoma cell lines (SK-N-AS, SK-N-SH, SHSY5Y, LAN5, and KANR) were grown under standard conditions in media supplemented with either 2 μM 13-cis retinoic acid (RA) or DMSO for 7 days. RNA was purified, processed, and hybridized to the Nimblegen 385K HOX tiling array. For each sample, robust multichip average normalized intensity values for previously defined peaks encoding HOX-coding-gene exons and HOX lncRNAs were determined relative to reference cDNA. Expression of selected genes and lncRNAs were validated by RT-qPCR using 8 cell lines (the original five, plus KANR, NBL-W-S, and LAI-5S).

Results: In NB cell lines treated with vehicle alone, HOXC genes were more highly expressed compared to HOXD or HOXB genes; HOXA genes were silenced in N-type cells but expressed basally in S-type cell lines. Upon treatment with RA, expression of HOXC and HOXD genes increased significantly in N-type cells and correlated with markers of neuronal differentiation. In S-type cell lines, expression of HOXA and HOXB genes directly correlated with lack of response to RA, specifically in inhibition of proliferation. Expression of lncRNAs tended to correlate with expression of adjacent HOX genes; over 100 lncRNAs were found to be expressed within the HOX clusters in NB cell lines.

Conclusions: Neuroblastoma cell lines show distinct patterns of HOX gene and lncRNA expression basally, correlating with N- or S-phenotype, and when induced to differentiate with RA. These changes provide insight into the response or resistance of patient disease to RA.

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POB122

RAB1A: a novel marker of in vitro invasion in neuroblastoma.

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Functional studies have determined that miR-542-5p inhibits neuroblastoma cell invasiveness, although the molecular mechanism has remained elusive. Microarray expression analysis of mRNA from cell lines ectopically over-expressing miR-542-5p contained no enrichment for 3'UTR target sites for this miRNA, leading us to hypothesize that miR-542-5p has a more significant impact on protein levels through translational inhibition than mRNA degradation. In order to elucidate the molecular mechanism of miR-542-5p action, we carried out proteomic analysis using mass spectrometry (LC-MS/MS) of neuroblastoma cell lines over-expressing miR-542-5p. 27,000 peptides were examined per sample, with 17 proteins changing significantly in MYCN amplified Kelly cells (6 down-regulated and 11 up-regulated) and 14 proteins changing significantly in MYCN single copy SKNAS cells (9 down-regulated and 5 up-regulated). The R2 database (http://r2.amc.nl) was used to assess any associations of these proteins with poor overall (OS), or event-free survival (EFS) in neuroblastoma. Among the 31 proteins differentially expressed, 26% were significantly associated with poor patient survival ($p < 0.05$). None of the 31 proteins had miR-542-5p seed sites in the 3' UTR of their mRNAs, indicating that the changes were indirect effects. Of particular interest was RAB1A, which increased in abundance in both miR-542-5p transfected cell lines. Decreased expression of RAB1A was associated with poor OS ($p=0.015$) and EFS ($p=0.00016$), consistent with its up-regulation in response to miR-542-5p over-expression in cell lines. siRNA mediated depletion of cellular RAB1A enhanced the invasiveness of neuroblastoma cell lines, consistent with what occurs following inhibition of endogenous miR-542-5p. We therefore conclude that miR-542-5p acts by indirectly up-regulating RAB1A through an unknown mechanism, leading to decreased cellular invasiveness.

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POB123

Inhibition Of Neuroblastoma Differentiation By CARM1-Induced Methylation Of The HUD Protein

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Background: Neuroblastoma (NB) is the most frequent extracranial tumor in children, and it derives from neural cells crest. NB cells have embryonic features, presumably as a consequence of a perturbed differentiation during development of the sympathetic nervous system. Therefore, molecular modulators able to induce neuronal differentiation may be effective in the treatment of this malignancy. Arginine methylation has been shown to regulate the differentiation of a number of cell lineages. In particular, it was found that coactivator-associated arginine methyltransferase 1 (CARM1) can negatively regulate neuronal differentiation by methylating the HuD RNA binding protein, a known determinant of the neuronal phenotype.

These observations suggest that the CARM1 inhibition can be a possible therapeutic approach in neuroblastoma treatment.

Methods: SKNBE(2) cells have been infected with a CARM1 shRNA lentiviral vector. The neuronal phenotype has been than analyzed in terms of neurite length and root number of neurite roots by means of an automatic high content screening system. A microarray analysis on total RNA extracted from shCARM1 and scramble SKNBE2 cells was performed. The expression of the most interesting genes was then confirmed with quantitative RT-PCR analyses. Stable SKNBE2 clones were generated transfecting three different vectors carrying HuD wt, HuD-R236W (a 'methylmimetic' form) and a HuD-R236K (not methylatable by CARM1).

Results: We first demonstrated that stable CARM1 silencing produces a significant neurite extension and branching in SK-N-BE(2) neuroblastoma cells. This morphological evidence has been confirmed at the molecular level by a microarray analysis that showed an overexpression of the neuronal markers NeuroD1, NeuroD2 and MAP1 in CARM1 silenced cells. In addition to this, higher level of p21 mRNA, an HuD target already known to play a pivotal role in cell cycle exit, were found. These data have been validated with quantitative RT-PCR.

To demonstrate if the above effects are mediated by CARM1 methylation of HuD, we analyzed the neuronal phenotype in HuD wt, HuD-R216K and a HuD-R216W SKNBE(2) stable clones. As expected, a clear neurite extension increase was observed in HuD-R216K cells compared to HuD wt and-R216W HuD clones.

Conclusions: Our data demonstrate that CARM1 can modulate HuD methylation and consequently the differentiation status of neuroblastoma cells. These evidences indicate that CARM1 and HuD can be two potential druggable targets in neuroblastoma therapy.

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POB124

Neuropeptide Y receptor 5 (NPY5R) as a novel survival factor for neuroblastoma

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Background: Neuropeptide Y (NPY) is a sympathetic neurotransmitter released from neuroblastoma cells. High systemic levels of NPY are associated with poor clinical outcome of the disease, which is in agreement with its proliferative effect in neuroblastoma cells and angiogenic properties. While all the above functions of NPY are mediated mainly by its Y2 receptors (Y2Rs) predominantly expressed in neuroblastoma and endothelial cells, some neuroblastoma cell lines additionally express NPY Y5Rs. Thus, the goal of our study was to elucidate their functions.

Methods: Gene expression was assessed by real-time RT-PCR, Western Blot (WB) and immunohistochemistry (IHC); activation of signaling pathways by WB, cell survival by MTS assays and apoptosis by caspase activity.

Results: Expression of Y5Rs in neuroblastoma cells was induced by their known survival factor – brain-derived neurotrophic factor (BDNF) and pro-apoptotic conditions (serum-free culture, chemotherapy). In agreement with this, in human neuroblastoma tissues, expression of Y5Rs correlated positively with expression of both BDNF and its receptor, TrkB (RT-PCR), while Y5R protein was detected preferentially in undifferentiated neuroblastoma cells (IHC). Moreover, expression of Y5Rs and NPY was elevated in cell lines derived from neuroblastoma patients at relapse, as compared to those at diagnosis.

On a functional level, blocking Y5Rs inhibited BDNF-induced TrkB phosphorylation and p44/42-MAPK activation. In line with this, Y5R antagonist significantly reduced BDNF-induced neuroblastoma cell survival and markedly increased cell death in chemotherapy treated cells. These observations were made in SY5Y-TrkB stable transfectants and confirmed in native cells derived from patients before and after chemotherapy.

Conclusions: Y5R serves as an inducible survival factor protecting neuroblastoma cells from chemotherapy-induced cell death. This effect is mediated by interactions between NPY and BDNF signaling and cross-talk of their TrkB and Y5Rs. These findings identify Y5R as a potential new target in neuroblastoma therapy directed specifically against the recurrent disease.

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POSTERS
TRANSLATIONAL
POT001 – POT117

POT001
The Receptor Tyrosine Kinase AXL Contributes to Resistance of ALK-F1174L to TAE684 in Neuroblastoma
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Background: Mutations in the ALK tyrosine kinase receptor provide a novel therapeutic target in neuroblastoma. The most common somatic mutation, ALK-F1174L, is resistant to the ALK inhibitor crizotinib, currently in pediatric trials, but is sensitive to another ALK inhibitor TAE684, a structurally unrelated, ATP-competitive, diaminopyrimidine derivative.

Methods: TAE684-resistant cells, SY5Y-TR, were established by serially exposing SH-SY5Y neuroblastoma cells (harboring the ALK-F1174L mutation) to increasing concentrations of TAE684 over time. IC50 values were determined using CellTiter-Glo Luminescent Cell Viability assays. Resistance was characterized by Phospho-Receptor Tyrosine Kinase (pRTK) arrays and immunoblotting.

Results: SY5Y-TR cells were found to have >10-fold reduced drug sensitivity to TAE684 as compared to parental SH-SY5Y cells. The SY5Y-TR cells exhibit decreased ALK phosphorylation compared to the parental SH-SY5Y cells, indicating that the drug resistance was neither due to a secondary ALK mutation nor due to drug efflux from the cell. In contrast to the parental cells, an increase in pERK1/2 expression was seen in the resistant line, suggesting the development of compensatory signaling pathways contributed to the emergence of resistance. pRTK arrays in one of the SY5Y-TR clones, SY5Y-TR1, revealed upregulation of pAXL as compared to parental cells. Knockdown of Axl in the SY5Y-TR1 cells resulted in decreased growth kinetics in these cells. In addition, SY5Y-TR1 cells were found to be sensitive to the Hsp90 inhibitor retaspimycin hydrochloride (also known as IPI-504) (IC50 35nM), as compared to parental SH-SY5Y cells (IC50=352nM). Exposure of SY5Y-TR1 cells to retaspimycin hydrochloride led to a decrease in pAXL and pERK1/2 with a concomitant decrease in the binding of AXL to Hsp90.

Conclusions: These data suggest that AXL activation contributes to the emergence of resistance to TAE684 in ALK-F1174L expressing neuroblastoma cells, and that Hsp90 inhibition could be used as a therapeutic strategy to overcome such resistance.

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POT002
The green tea compound Polyphenon E negatively modulates neuroblastoma-induced immuno-suppressive myeloid cells.
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Background: Green tea has been used for thousands of years in traditional Chinese medicine to treat human disease. Green tea catechins are natural polyphenolic compounds endowed with anti-oxidant and anti-cancer activity. In recent years a number of reports have shown that green tea catechins inhibit tumour proliferation and induce tumour cell apoptosis in vivo and in vitro. Polyphenon E is a green tea catechin compound, which has been shown to prevent tumourigenesis in cancer clinical trials. To further explore efficacy and mechanism, Polyphenon E was supplemented in the drinking water of neuroblastoma prone MYCN transgenic mice.

Methods: Mouse models of neuroblastoma: TH-MYCN transgenic; Neuro2A cells injected into AJ mice; shsy5y cells xenotransplanted into NOD-SCID mice. Patient derived blood and tumour samples were used to isolate suppressive myeloid cells.

Results: There was a significant decrease in neuroblastoma development in TH-MYCN mice exposed to the catechins, with approximately half of mice free from tumour after 8 months, compared with complete tumour penetrance in control mice. We identified reduced infiltration of myeloid suppressor cells (MDSCs) as a possible mechanism of the anti-tumour activity of Polyphenon E. MDSCs strongly inhibited the growth of syngeneic neuroblastomas in AJ mice, but not after treatment with Polyphenon E. We observed an increase of immunosuppressive myeloid cells circulating in the blood of children with neuroblastoma, compared to controls. Patient-derived tumour infiltrating lymphocytes could be activated in vitro by treatment with Polyphenon E, but only in the presence of infiltrating myeloid cells. Mechanistically, Polyphenon E stimulated the differentiation and impaired the immunosuppressive function of MDSCs by inducing the secretion of G-CSF.

Conclusion: Polyphenon E restricts neuroblastoma growth by inactivating MDSCs. Polypenon E is a safe, non toxic, clinical grade compound whose use could be particularly attractive in paediatric oncology.

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POT003
Salmonella application is the most effective DNA vaccine delivery method for a survivin-based vaccination in neuroblastoma
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Background: Recently, we generated a survivin minigene DNA vaccine (pU-Shigh) and demonstrated its efficacy in a syngeneic NB mouse model. Here, we addressed the importance of finding and characterizing the most effective application strategy among three potent DNA vaccine carriers, Salmonella typhimurium (SL), gene gun, and dendritic cells (DC).

Methods: Vaccination was performed (3x) by oral gavage of attenuated SL7207 carrying pUS-high, skin bombardment with pUS-high coated gold particles by gene gun or injection of mature minigene-transduced bone marrow-derived DCs (BMDCs) after s.c. NXS2 application (2x106). BMDC were generated using lentiviral vector particles (LV) made by HEK293T after CaCl2 precipitation. For DC maturation we added LPS (1 µg/ml) and TNF-alpha (250 U/ml) to the culture also containing GM-CSF (20 ng/ml). Tumor growth was calculated by calliper measurement and tumor target cell lysis by splenocytes was studied by Cr51 cytotoxicity assay.

Results: The most effective delivery system was the application of attenuated SL. Therapeutic vaccination of A/J mice with SL carrying pUS-high induced 100% suppression of primary tumor in more than 50% in contrast to application of SL carrying empty vectors. As a second-best delivery method emerged the application of DC. LV-transduced BMDC carrying survivin minigene showed 45% inhibition of primary tumor growth compared to vaccination with BMDC transfected with empty LV. Splenocytes from mice vaccinated with SL or DC carrying the survivin minigene were able to induce higher percentages of NXS2 lysis in contrast to mice immunized with appropriate vector controls. In contrast, gene gun application was not able to induce significant protective immune response against NB. Here, we found marginal suppression of primary tumor growth after skin bombardment with pUS-high versus pU (mock) coated gold particles.

Conclusions: In summary, vaccination with SL was most effective application strategy and should be favoured for future human DNA vaccination trails against NB.

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POT004
Histone deacetylase 10 causes neuroblastoma cell survival by promoting Hsc70-mediated autophagic flux
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Background: The group of advanced stage, high-risk neuroblastoma patients (INSS stage 4) remains to have a very poor prognosis despite continuous intensification of chemotherapy. Nevertheless, some patients can be cured by intensive treatment regimen for so far unknown reasons.

Methods: We examined HDAC1-11 expression levels and their correlation with clinical outcome of advanced neuroblastoma treated with multimodal chemotherapy (INSS stage 4). Functional assays were used to unravel a so far undescribed molecular function of the class IIb histone deacetylase family member HDAC10.

Results: Only HDAC10 mRNA expression significantly (bonferroni p-value = 0.020) correlated with poor overall survival in a cohort of 40 high-risk INSS stage 4 patients (10 year OS: 80% (HDAC10 low expression) versus 10% (HDAC10 high expression)). This result was confirmed in an independent cohort of 31 INSS stage 4 patients (bonferroni p-value < 0.005). Our functional analysis revealed that HDAC10 was directly bound to the lysosomal chaperone Hsc70 and regulated its acetylation status, which affected lysosomal Hsc70 localization. HDAC10-deprived tumor cells accumulated dysfunctional lysosomes, with consequent disruption of autophagic flux, accumulation of autophagosomes and p62, and lysosomal ROS release. Inhibition of HDAC10 re-sensitized neuroblastoma cells but not untransformed cells for cytotoxic drug treatment through inhibition of autophagic flux.

Conclusion: Our results identify HDAC10 as a regulator of neuroblastoma cell survival and potential biomarker for chemotherapy response prediction of INSS stage 4 neuroblastomas. Interruption of autophagic flux by selective inhibition of HDAC10 could represent a promising approach to re-sensitize tumors of high-risk neuroblastoma patients to chemotherapy.

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POT005

Nanoparticle (NP) Drug Delivery in Neuroblastoma (NB)

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Background: NB is a tumor with different genetic/biological subsets that correlate with clinical behavior. Many current chemotherapeutic agents have a narrow therapeutic window, and the toxic effects can be devastating. Here, we explore the use of NPs as a drug delivery system to increase the therapeutic effect on tumors and decrease the toxic effects on normal tissues.

Methods: We used the Trk-null SY5Y line transfected with TrkB. Polylactide-pegylated NPs were used to encapsulate several active agents for NB, including SN-38 (irinotecan), fenretinide and lestaurtinib. We used western blots to compare the effect of NP and free drugs on cell lines and RT-CES (Roche) to monitor cell proliferation with drug treatment.

Results: We tested SN-38 (1-200 nM), fenretinide (1-30 μM) and lestaurtinib (50-200 nM) both as a free drug and in NP form. With NP SN-38, 50% inhibition of cell growth was seen at 1 nM concentration and 100% inhibition of cell growth was seen at 10 nM. For NP fenretinide, 5 uM concentration resulted in near complete growth inhibition, whereas the same concentration of free drug resulted in just 50% growth inhibition. Combinations of free lestaurtinib with either NP SN-38 or NP fenretinide resulted in even greater inhibition of cell growth when compared with either drug alone. Studies are underway to treat NB xenografts with free and NP-encapsulated drugs and so far demonstrate greater efficacy and higher tumor/drug concentrations with NP-lestaurtinib than free drug alone.

Conclusions: NP encapsulation of active agents in NB resulted in improved inhibition of cell growth when compared to free drug alone. The combination of free lestaurtinib with either NP SN-38 or NP fenretinide resulted in better inhibition of cell growth than any single agent. NP encapsulation of cytotoxic drugs may result in better inhibition of tumor cell growth, especially when combined with a Trk inhibitor.

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POT006

Antitumoral effects of histone deacetylase 8 selective inhibitors

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Background: In order to improve the survival rate of neuroblastoma (NB) INSS stage 4 patients, novel specific anticancer drugs are currently under development. Previous expression studies of primary NB samples from the German Neuroblastoma Trial revealed histone deacetylase 8 (HDAC8) to be upregulated in stage 4 patients, and this was associated with a significantly worse prognosis. HDAC8 contains an unique secondary pocket adjacent to the main catalytic pocket, which has permitted the discovery of potent small molecule inhibitors (HDAC8i) highly selective for only this HDAC family member. Here, we investigated the anti-neuroblastoma activity of HDAC8-selective inhibitors in vitro and in vivo.

Methods: Treatment of neuroblastoma cell lines (BE(2)-C, IMR32, Kelly) and mouse xenografts were used to evaluate the therapeutic potential of HDAC8i against NB tumors. Selectivity for HDAC8 was verified by the absence of histone and tubulin lysine acetylation (targets of HDAC1-3 and HDAC6 respectively) as measured by Western Blot and FACS at the relevant in vitro and in vivo administration of HDAC8i. In vivo maximal tolerable doses (MTDs) were determined by toxicity studies including assessment of body weight, blood parameters and organ histology.

Results: HDAC8i treatment in vitro induced the expression of TrkA, neurofilament and CDK-inhibitor p21Waf-1/Cip-1 and decreased cell population growth to 18% (+/- 13%) of controls. Growth of non-transformed cell populations was not significantly inhibited (95% (+/- 10%)). Long term treatment resulted in apoptosis (Caspase activation BE(2)-C, 3-fold; IMR32, 6-fold; Kelly, 2-fold). In line with these findings, treatment of BE(2)-C xenografted animals with MTDs decreased tumor growth significantly compared to control groups ($p<0.001$).

Conclusion: Our data support the hypothesis that the selective inhibition of HDAC8 with small molecule compounds may provide a novel therapeutic strategy in treating advanced stage NB disease.

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POT007

Factors Involved in Resistance to Prolonged Antiangiogenic Therapy with Oral Metronomic Topotecan and Pazopanib in Neuroblastoma Mouse Model

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Background: Combination of daily oral Low Dose Metronomic (LDM) Topotecan (TP)and Pazopanib (PZ) significantly delayed tumor growth and enhanced survival in neuroblastoma (NB) mouse models. This regimen does not stop tumor growth and the animals are eventually succumbing due to tumor progression.

Objectives: To investigate the effect of prolonged TP+PZ therapy on tumor angiogenesis and its effect on perycite mediated drug resistance in NB mice xenograft model.

Methods: Mice bearing subcutaneous SK-N-BE(2) NB xenograft were treated with one of the four following regimens: daily oral administration of vehicle (untreated),PZ (150 mg/kg pazopanib),TP (1.0 mg/Kg topotecan), TP+PZ (150 mg/kg - 1.0 mg/Kg) using three different treatment durations: 28 days , 56 days and 80 days. Animals were sacrificed and tumors were sampled at these three different time points .End point criteria were tumor sizes exceeding 2.0 cm in diameter or animals showing signs of morbidity. Angiogenesis was measured by microvessel density (CD31 Immunofluorescence) and perycite presence measured by α- smooth muscle actin staining. Apoptosis and tumor hypoxia were also assessed.

Results: The animals in control ,PZ, TP reached the end point after 23d, 28d and 46d respectively. TP+PZ halted the tumor growth for up to 40 days, after which gradual growth was observed. At day 80 Micro-vessel densities was significantly reduced compared to control in all three TP+PZ treatment duration (CD31) ($P<0.05$) while tumor hypoxia (HIF-1 alpha) ($P<0.01$) and apoptosis (cleaved caspase-3) ($P<0.01$) increased. In addition the ratio of CD31: α-smooth muscle actin (pericytes marker) expression were significantly higher in animals treated with TP+PZ for 56 and 80 days, compared to control($P=0.0006$ and 0.004; respectively)

Conclusion: Long term TP +PZ administration is associated with drug resistance potentially related to perycite/tumor vessels maturation. Addition of vascular disrupting agent targeting periycte function could potentially reverse this drug resistance process.

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POT008

Preclinical Evaluation of Antitumor Efficacy of the Hypoxia-Activated Prodrug TH-302 and Sunitinib in Neuroblastoma Mouse Models

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Background: Hypoxia-activated prodrugs (HAPs) are activated under hypoxic conditions within tumors, targeting malignant cells in hypoxic zones, while having little toxicity to normoxic cells.

Objective: To evaluate the antitumor efficacy of HAP TH-302 as single agent and in combination with VEGFR TKI Sunitinib in neuroblastoma (NB) mouse models.

Method: SK-N-BE(2) subcutaneous xenograft model (XM) or IV metastatic model (MM) were developed in NOD/SCID mice.

Dose regimens were:,TH-302: 50 mg/Kg (TH), qdx5/wk x 2wks, IP; and sunitinib: 30 mg/Kg (SU30) or 80 mg/Kg (SU80), daily, orally.

Four combination therapy (CT) experiments were conducted: CT1: XM, SU30 starting 7 days before TH treatment; CT2: XM, SU30 and TH starting simultaneously; CT3: XM, SU80 and TH starting simultaneously; CT4: MM, SU80 and TH starting simultaneously. Efficacies of vehicle (control), single agent and combination treatments were compared. In CT1, CT2, and CT4, mice were sacrificed after completion of therapy. In CT3, mice were sacrificed at the end point. Pimonidazole and caspase 3 immunostaining were performed.

Results: Sunitinib, TH-302 and combination therapy all demonstrated significant superior efficacy ($P<.0.05$) compared to control in all models. In CT1, SU30 ($P=0.009$) and SU30+TH ($P=0.007$) significantly lowered tumor weights compared to TH. Immunofluorescence revealed more apoptotic cells (cleaved caspase-3) ($P=0.03$) in SU30+TH treated tumors compare to single agent. SU30 ($P=0.008$) and SU30+TH ($P=0.005$), but not TH, demonstrated an increase in tumor hypoxia (pimonidazole), compared to control. In CT3, SU80 +TH enhanced survival compared to SU80 ($P<0.05$) and TH ($P<0.05$). 2 weeks TH-302 monotherapy in MM significantly enhanced survival compared to control ($P=0.02$). In CT4, while survival was improved, no significant difference between liver tumor burden was observed between SU80 and SU80+TH.

Conclusion: TH-302 is an effective agent in localized and metastatic NB. TH-302 enhanced the efficacy of sunitinib therapy by increasing apoptosis in hypoxic zone in both models.

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POT009

In-vitro and in-vivo impaired proliferation and tumorigenicity of neuroblastoma cells after imetelstat treatment is dependent on telomere maintenance

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Background: Imetelstat is a novel antagonist of human telomerase which is expressed in almost all malignant cancers. Telomerase activity has previously been linked to progression and poor prognosis in childhood cancers, including neuroblastoma (NB).

Objective: To investigate the anti-tumor effect of Imetelstat on NB in vitro and in vivo.

Methods: Six NB cell lines, CHLA-15, CHLA-20, CHLA-90, SH-SY5Y, NUB-7 and SK-N-BE(2) were exposed to imetelstat treatment over a 6 week period at 2 dosages- 5uM and 10uM. Cell viability was evaluated with the Alamar Blue assay. Telomerase activity and telomere length were analyzed in all described cell lines. Population doubling capacity was assessed in vitro by continued tumor cell passaging. In order to study the tumorigenicity of viable cells surviving drug treatment, same numbers of imetelstat pretreated and untreated CHLA-20 cells were inoculated into NOD/SCID mouse and xenograft tumor development was monitored (n=8).

Results: In vitro, over the period of 6 weeks, 4 out of 6 NB cell lines, including CHLA-20, SH-SY5Y, NUB-7 and SK-N-BE(2) cells, showed reduced cell viability at both concentrations, while CHLA-15 and CHLA-90 showed no significant response to imetelstat treatment. CHLA-90 cells revealed lack of telomerase activity and a telomere length pattern compatible with alternative lengthening of telomeres (ALT).CHLA-20 was selected for further in vitro and in vivo studies. Declined telomerase activity and shortened telomere length was observed after in vitro Imetelstat treatment. In vivo tumor growth was significantly impaired following a 7-week pretreatment with Imetelstat ($p<0.01$).

Conclusion: Imetelstat inhibits telomerase activity and reduces telomere length in telomerase positive but not in ALT NB cell lines. Long-term treatment may be required to achieve a significant anti-tumor effect in children. Assessment of tumor telomere maintenance may be required before initiation of treatment with imetelstat for patients with NB.

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POT010

Identification of new MRP4 inhibitors from a library of FDA approved drugs

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Background: The multidrug transporter MRP4 is an attractive therapeutic target in neuroblastoma. It is transcriptionally regulated by N-Myc, is expressed at high levels in poor-outcome tumors and effluxes the cytotoxic drugs topotecan and irinotecan. MRP4 also regulates the pharmacokinetics of its drug substrates and its inhibition can substantially increase their bioavailability. Finally, we recently demonstrated that MRP4 inhibition slows neuroblastoma cell growth independently of its drug efflux role (JNCI, 2011). We therefore aimed to identify potent MRP4 inhibitors from approved drugs and bioactive compounds.

Methods: We developed a robust, high throughput, live cell-based bioluminescent screen ($Z'= 0.8$) based on our observation that MRP4 can efflux the bioluminescence substrate luciferin. We applied this screen to a combined library of 1200 FDA-approved drugs and 1280 known bioactive compounds, with subsequent validation of the top hits in multiple cell culture systems.

Results: From combined library, 86 compounds effectively inhibited MRP4 (based on a 2-fold bioluminescence increase) and 23 were substantially more effective than the established MRP4 inhibitor MK571 (>4-fold luminescence increase). Amongst the most effective compounds, inhibitors of receptor tyrosine kinases and phosphodiesterases were over-represented.

Conclusion: We anticipate that these inhibitors may be useful both as investigative tools and as potential future therapeutics that function either by reversing MRP4-mediated drug resistance, increasing the bioavailability of MRP4 substrate drugs, or directly targeting essential functions of MRP4. As the inhibitors identified are established drugs and bioactive compounds, preclinical testing is readily achievable.

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POT011

HDAC11 controls mitotic cell cycle progression of neuroblastoma cells

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Background: Expression of HDAC11, the most recently identified histone deacetylase, is restricted to the cell nuclei in poorly differentiated neuroblastomas. We have previously shown that HDAC11 depletion induces a prognostically favorable neuroblastoma transcriptome, partly by reverting BMP4 epigenetic silencing, thereby, triggering this developmental pathway. Here we aimed to decipher the functional relevance of further distinct alterations in gene expression caused by HDAC11 depletion.

Methods: Whole-genome expression was evaluated in time-course in p53-wildtype and -mutant MYCN-amplified neuroblastoma cells following HDAC11 depletion. Differential expression of candidate genes in primary neuroblastomas was assessed in three independent datasets from 468, 102 and 88 tumors. Cell cycle and death assays were conducted after target gene depletion in neuroblastoma cell lines.

Results: HDAC11 depletion caused the genome-wide differential expression of 259 and 167 genes in p53-mutant BE(2)-C and p53-wildtype IMR-32 cells, respectively. The biological functions of genes consistently regulated over time across each cell system were assessed by analyzing gene ontology term over-representation. Genes necessary for mitotic cell cycle progression and cell division were most prominently enriched. All ten of these genes were strongly repressed by HDAC11 depletion, followed by a G2/M arrest and apoptosis in functional assays. High candidate gene expression levels in primary neuroblastomas strongly correlated with unfavorable overall patient survival in all 3 datasets, demonstrating their clinical relevance. Depletion of 6 candidate genes, singly, reduced metabolic activity up to 90% and increased caspase-3/7-like activity up to 10-fold, mimicking the phenotype caused by HDAC11 depletion. HDAC11 depletion did not affect viability of nontumorigenic cells.

Conclusion: Here we investigate a group of cell cycle-promoting genes repressed by HDAC11 depletion, being both, predictors of patient outcome and essential for neuroblastoma cell viability. Our data further support HDAC11 inhibition as a novel targeted therapeutic approach for the treatment of high-risk neuroblastomas, regardless of p53 status.

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POT012

FTY720 interferes with sphingosine-1-phosphate signaling in neuroblastoma, inhibiting tumor growth and enhancing the tumor-suppressive effect of topotecan in preclinical models.

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Backgrounds: Neuroblastoma (NB) is the most common extra-cranial solid tumor in childhood. Despite improvements in outcome for those with low-risk NB, the outcome for children with high-risk NB is still poor, underscoring the need for novel therapeutic strategies. FTY720, an immunomodulating drug approved for multiple sclerosis, has been investigated in cancers with promising preclinical activities. Its effect in NB has not been explored. But FTY720's ability to occupy sphingosine kinase 2 (SphK2), which is uniquely overexpressed in NB and in-part responsible for the production of pro-survival factor sphingosine-1-phosphate (S1P), suggests a potential beneficial effect of FTY720 in NB. Herein we describe our preclinical experience with FTY720 as a single agent and combination strategies in NB.

Methods: Methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay was performed to assess the efficiency of FTY720 on cell viability. A NB xenograft model was employed to assess the efficacy of FTY720 on tumor growth. The liquid chromatography/tandem mass spectrometry (LC/MS/MS) method was conducted to measure sphingosine and S1P levels. Western blot analysis was employed to determine changes of protein activity.

Results: FTY720, but not FTY720-P (inactive at engaging SphK2), strongly induced cell death in vitro, inhibited tumor growth in vivo and enhanced the tumor-suppressive effect of topotecan in NB xenografts. Apoptotic sphingosine levels were dramatically increased while pro-survival S1P levels were significantly decreased in NB cells and NB xenografts after treatment with FTY720. Detailed mechanisms in NB cells revealed that FTY720-induced cell death was caspase-independent and involved the dephosphorylation of Akt and BAD at Ser136 as well as the subsequent release of cytochrome c.

Conclusions: Our data for the first time demonstrate that FTY720 has a potent preclinical anti-cancer activity in NB. Its unique death signaling acts cooperatively with that of topotecan suggesting that FTY720 and related compounds may become promising new anti-cancer drugs for NB treatment.

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POT013

Radiosensitization potential of bortezomib and SN38 in neuroblastoma

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Introduction: Both external beam and targeted radiotherapy with meta-iodobenzylguanidine (MIBG) have therapeutic benefits in treating neuroblastoma. The ability to augment radiation-induced cytotoxicity with novel radiosensitizers, including the irinotecan metabolite SN38 and the proteosome inhibitor bortezomib is now examined.

Methods: IMR-32 (MYCN amplified) or SY-5Y (MYCN non-amplified) cells were irradiated for 16 hrs at 10cGy/hr or 26.6cGy/hr in a device designed to simulate the low dose rate radiation delivered with MIBG therapy. Drug treatment was initiated 4hr (1nM SN-38) or 1hr (with 5 or 10 nM bortezomib) prior to the start of LDR-RT, continued during LDR-RT, and for 4hr post LDR-RT. At the end of the treatment, colony forming assays were performed to assess cell survival. Enhancement ratios were calculated as: [surviving fraction of cells receiving LDR-RT only/surviving fraction of LDR-RT+drug-treated cells], with a value >1.0 indicative of enhanced drug effect. Data are shown as the mean ±SEM of 3-5 experiments. Experiments with LDR-RT alone served as controls.

Results: In SY-5Y cells, the combination of 10nM bortezomib with LDR-RT at 26.6cGy/h resulted in the greatest radiosensitization, with an enhancement ratio (ER) 21.2. Enhancement ratios with SN38 + LDR-RT were comparable to LDR-RT alone, at both 10 cGy/hr (ER, 1.2±0.4) and 26 cGy/hr (ER, 1.2±0.7). The addition of SN38 to either 5 or 10 nM bortezomib did not affect enhancement ratios, when compared to bortezomib alone at those same concentrations. Under these same conditions, IMR-32 cells were not radiosensitized by SN38 ±bortezomib (data not shown).

Conclusion: The combination of bortezomib with low dose rate radiation therapy produced significantly greater cytotoxicity than LDR-RT alone in a MYCN non-amplified cell line. These data suggest that, for patients with MYCN non-amplified disease, combination therapy of bortezomib plus concurrent radiotherapy warrants clinical investigation.

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POT014

Synergistic induction of cell death mediated by ROS using allosteric Akt inhibitor MK2206 and rapamycin

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Background: Activation of Akt is a marker of decreased event-free or overall survival in neuroblastoma (NB) patients. MK-2206, a novel allosteric Akt inhibitor, is now tested in clinical trials in adult cancers. In this study, effect of MK-2206 on tumor growth and murine survival, alone or in combination with etoposide or rapamycin was evaluated.

Methods: The anti-cell proliferation effect of MK-2206 was tested in eight NB cell lines by MTS assay. Caspase 3/7 activity, cell cycle analysis and reactive oxygen species (ROS) production were determined. Effect of MK-2206 combined with etoposide or rapamycin was evaluated in vitro and in vivo. Akt phosphorylation was measured by Western blotting in NB cells and tumors.

Results: In vitro, MK-2206 treatment inhibited NB cell proliferation which was accompanied by a cell line selective G1 arrest of cell cycle or production of ROS. A synergistic effect between MK-2206 and etoposide was detected in 4 tested NB cell lines via caspase-dependent apoptosis, while increased inhibition of cell growth induced by combination of MK-2206 and rapamycin was mediated by ROS production. In vivo, MK-2206 alone decreased tumor growth and increased murine survival at dose which inhibited Akt phosphorylation in tumors. MK-2206, in combination with etoposide or rapamycin, caused a significant decrease in tumor growth and increase of murine survival compared to MK-2206 alone.

Conclusion: Akt inhibition by MK-2206 increased the efficacy of etoposide or rapamycin. Our study supports future clinical evaluation of MK-2206 in combination with conventional cytotoxic therapy or with rapamycin in high-risk NB patients.

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POT015

Inhibition of MYCN by M606, a novel small molecule inhibitor identified through chemical library screening

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Background: The MYCN oncogene is one of the most powerful prognostic markers identified for neuroblastoma and represents a potentially valuable target for the development of novel treatment approaches. We aimed to identify MYCN inhibitors using chemical library screening.

Methods: A 34,000 diverse chemical library of small molecules was screened using a cell-based assay and MYCN reporter construct. PCR, Western analysis, siRNA knockdown, and signal transduction pathway analyses were used to characterise hit compounds.

Results: Among a number of molecules identified as potential Myc inhibitors, M606 was found to reduce protein expression of MYCN and its downstream targets in MYCN-amplified neuroblastoma BE(2)-C cells. A similar effect was also observed in c-Myc over-expressing tumour cells. Analysis of signalling pathways affected by M606 using FACTORIAL™ technology (Attagene Inc) indicated that this compound inhibited Myc mediated transcription in a dose-dependent manner. Interestingly, hypoxia inducible factor (HIF1A) was induced ~100 fold in this assay. We observed HIF1A protein accumulation and nuclear translocation post-M606 treatment under normoxic conditions accompanied by transcriptional activation of the HIF1A targets, erythropoietin and VEGF-A. siRNA-mediated knockdown of c-Myc or HIF1A in HepG2 cells followed by M606 treatment demonstrated that c-Myc downregulation and HIF1A upregulation by M606 are two independent events. Furthermore, inhibition of HIF1A prolyl hydroxylases (PHDs) by dimethyloxalylglycine resulted in downregulation of c-Myc protein independent of any HIFA upregulation. M606 was found to be structurally related to a family of PHD inhibitors and inhibited PHD activity in vitro.

Conclusions: M606 represents a novel Myc inhibitor that targets PHD to downregulate Myc and upregulate HIF1A. Development of this compound may have clinical potential in the treatment of cancers overexpressing Myc oncoproteins, and also in the treatment of HIF1A-mediated disorders such as ischemia-reperfusion injury.

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POT016

Targeting the ABCC4 transporter in neuroblastoma

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Background: While multidrug transporter proteins are known for their contributions to chemoresistance and efflux of anti-cancer drugs from cancer cells, a significant body of evidence points to their fundamental roles in tumour biology, which we have recently outlined (Nat Rev Cancer, 2010). We have previously shown that ABCC1 and ABCC4 are both powerful independent prognostic indicators of clinical outcome (NEJM, 1996; JCO, 2006; JNCI, 2011). However, the agents used to treat the patients described in these studies are not known substrates of ABCC4. Furthermore, knockdown of ABCC4 results in reduced proliferation and enhanced morphological differentiation of neuroblastoma cells, in the absence of any cytotoxic drug treatment. (JNCI, 2011). This study investigated a potential new approach for the treatment of neuroblastoma through the development of ABCC4 small molecule inhibitors.

Methods: Cell-based screening of a 30k compound library was used to isolate potent small molecule inhibitors of ABCC4 that were able to sensitize HEK293 cells over-expressing ABCC4 to 6-mercaptopurine (6MP). Compounds were prioritized based upon their ability to mimic siRNA-mediated knockdown of ABCC4 as well as potentiating 6MP accumulation.

Results: We have identified several chemical structures able to specifically block ABCC4-mediated transport of 6MP and SN38, the active metabolite of irinotecan. In addition, two compounds inhibited cell growth in neuroblastoma cell lines and significantly potentiated morphological differentiation associated with exposure to all-trans retinoic acid. These inhibitors also affect growth and morphology in ABCC4-expressing prostate and ovarian cancer cell lines. The most stable compound is currently being tested in vivo, with initial results showing that it is able to inhibit tumorigenesis in the TH-MYCN mouse model.

Conclusions: These pharmacological compounds are potent inhibitors of ABCC4 with clinical potential for the treatment of neuroblastoma and other ABCC4-overexpressing cancers.

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POT017

Increased endogenous trapping of topoisomerase I to escape from irinotecan cytotoxicity in resistant neuroblastoma tumors

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Background: Acquired drug resistance is a major obstacle to successful treatment of neuroblastoma by chemotherapy. To study the resistance mechanisms in a therapeutic setting, we established an in vivo resistant neuroblastoma model. Resistance was induced by the topoisomerase I inhibitor irinotecan through repeated administration. Classical mechanisms of topoisomerase I inhibitors resistance were not involved (Calvet et al. 2004) and we explored here the occurrence of specific nuclear events induced by irinotecan in the sensitive (IGR-NB8) and resistant (IGR-NB8-R) models. We particularly evaluated the stabilization of topoisomerase I cleavage complexes (Top1cc) and their conversion into DNA damage, that both represent determinants for topoisomerase I inhibitors efficacy.

Methods: Top1cc stabilization was measured by slot blot quantification of topoisomerase I in DNA extracts. DNA damage formation and repair were evaluated using Comet assay. Western blot analyses were performed to monitor DNA damage signaling (by following gamma-H2Ax and the P53 pathway) and DNA repair (by evaluating the Base Excision Repair (BER) pathway).

Results: In IGR-NB8, Top1cc were induced thirty minutes after irinotecan treatment and resulted in DNA damage formation and signaling as measured by a significant increase of the comet tail moment as well as a recruitement of gamma-H2Ax and the P53 pathway two hours after irinotecan treatment. In contrast in IGR-NB8-R, Top1cc trapping was abnormally high already at basal level and decreased upon irinotecan treatment. Similarly, the level of DNA damage measured by the comet tail moment was significantly enhanced at basal level and diminished after irinotecan exposure. The BER pathway, particularly XRCC1 protein was overexpressed in IGR-NB8-R at basal level.

Conclusions: Prolonged irinotecan exposure may have rendered tumors genetically instable, resulting in the accumulation of Top1cc and DNA damage but also in the constitutive activation of DNA repair, representing therefore key mechanisms for acquired resistance to irinotecan.

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POT018

Hsp90 is a therapeutic target in TH-ALKF1174L/MYCN-neuroblastoma

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Background: Clinical resistance to the first generation ALK inhibitor crizotinib has been a problem in early phase adult cancer trials and is associated with the acquisition of mutations within the ALK tyrosine-kinase domain. The oncogenic ALK fusion proteins EML4-ALK and NPM-ALK are known clients of Hsp90. ALKF1174L is the most common somatic mutation in neuroblastoma and we hypothesize that ALKF1174L depends on Hsp90 for its function. Inhibitors of Hsp90, such as NVP-AUY922, have shown promising clinical activity in adult cancer trials, making combined inhibition of ALK and Hsp90 an attractive strategy by which resistance to crizotinib could be overcome.

Methods: A panel of neuroblastoma cell lines, expressing either ALKWT or ALKF1174L, were examined for sensitivity to treatment with either 17-AAG or the clinical inhibitor NVP-AUY922 and preclinical trials of 17-AAG and NVP-AUY922 were undertaken utilizing the novel TH-ALKF1174L/MYCN transgenic model of neuroblastoma (Berry et al).

Results: NVP-AUY922 was a more potent inhibitor of growth than 17-AAG in all tested cell lines. At the established GI50 for NVP-AUY922 and 17-AAG, inhibition of the heat shock response was confirmed by characteristic compensatory induction of Hsp72 and Hsp27. Within this dose range, down regulation of ALKF1174L and ALKWT was detected by immunoblotting in Kelly and BE2C cell lines respectively. Both exhibited simultaneous downregulation of MYCN. Finally, the ability of Hsp90 inhibitors to slow the progression of transgenic tumours with high-level expression of ALKF1174L was examined as monotherapy in a new murine model of neuroblastoma in which tumours are driven by expression of both ALKF1174L and MYCN.

Conclusion: Our early in vitro and in vivo studies show that ALKF1174L is a likely client of Hsp90 in neuroblastoma cells, indicating that inhibitors of Hsp90, especially NVP-AUY922, deserve thorough evaluation as potential therapy for treatment of NB tumors that carry ALKF1174L-mutations.

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POT019

Efficacy of mTORC1 inhibition in murine 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. I have previously demonstrated that the combined PI3K-mTOR inhibitor PF04691502 induces apoptosis in the TH-NMYC murine model of neurtoblastoma.

Methods: Homozygous TH-MYCN transgenic mice underwent serial abdominal US from four weeks of age until abdominal neuroblastomas greater than 50mm3 were detected. Tumour volumes were calculated and baseline FDG-PET scans were performed followed by a survival intervention using Temsirolimus, an allosteric mTORC1 inhibitor. Both US and FDG-PET were repeated at 48 hours. US were then performed twice weekly until mice were sacrificed. 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 pathway as well as markers of senescence, apoptosis and angiogenesis.

Results: A significant decrease in the FDG uptake was observed following treatment with Temsirolimus (T:B ratios 3.6±0.5 reduced to 2.2± 0.4) when compared with vehicle (T:B ratios 3.6± 0.7 compared to 4.2± 0.6 post treatment, $p<0.001$). Treatment with Temsirolimus also improved survival (median 43 days, n=7) when compared with vehicle (median 7 days, n=7, $p<0.001$), with a corresponding significant inhibition of tumour growth on US. WB and IHC analysis demonstrated decreased levels of MYCN. WB and IHC showed reduced tumour vascularity as evidenced by a significant decrease in CD31 staining at 96 hours. There was evidence of increased beta-galactosidase staining and morphological changes consistent with senescence in Temsirolimus treated mice.

Conclusions: Temsirolimus significantly decreased FDG uptake, suggesting inhibition of metabolism. Treatment with Temsirolimus prolonged survival and reduced tumour growth and vascularity. Temsirolimus reduced expression of MYCN and induced senescence. 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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POT020

Parvovirus H-1 Induces Oncolytic Effects In Human Neuroblastoma Cells In Vitro And In A Neuroblastoma Xenograft-Bearing Mouse Model

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Background: In the last two years oncolytic virotherapy approaches have entered pediatric clinical trials. H-1PV is an oncolytic rodent parvovirus currently under clinical investigation in a phase I/IIa trial in adult glioblastoma patients. Here, we evaluated its oncolytic activity on neuroblastoma in vitro and in vivo.

Methods: Non-transformed neuroectodermal infant cells and neuroblastoma cell lines were infected in vitro. The therapeutic efficiency of intra-tumoral H-1PV infection in vivo was determined in a subcutaneous xenograft mouse model, bearing human MYCN amplified, chemotherapy-resistant BE(2)-c cells. Animals were either treated by a single intra-tumoral injection of 109 p. f. u. of wild type H-1PV (treatment group, n=10), or by an equivalent dosis of empty, UV-inactivated capsids (control group, n=9). Clinical conditions of the tumor-bearing animals and their response to treatment were subsequently monitored.

Results: In vitro, viral entry, virus replication and cytotoxicity was confirmed in 13 NB cell lines with different MYCN status, including neurosphere cultures. In all neuroblastoma cell lines H-1PV induced a lytic infection at LD50s between 0.001 and 10 p. f. u. per cell.

In vivo no relevant virus-induced toxicity was observed in nude mice. In human neuroblastoma xenotransplant-bearing animals H-1PV significantly repressed tumor growth ($p < 0.001$) and significantly prolonged survival ($p < 0.001$). Mean survival could be increased from 19 days in the control group to 38 days in the virotherapy group. One out of 10 H-1PV treated mice showed complete long-term remission for more than 100 days.

Conclusions: Sensitivity to H-1PV infection and efficient virus replication were demonstrated in a broad variety of neuroblastoma cell culture models. In a neuroblastoma relapse animal model, a single intratumoral injection of H-1PV induced a significant treatment response, without causing toxic side effects. Thus, H-1PV deserves consideration as a promising oncolytic virus for clinical research on neuroblastoma patients.

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POT021

Wnt/beta-catenin signaling regulates the expression of O6-methylguanine DNA-methyltransferase in neuroblastoma cells: a new strategy to increase sensitivity for DNA alkylators in cancer therapy

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Background: A number of DNA-damaging agents attack the O6 position on guanine and thereby form the most potent cytotoxic DNA adducts known. The DNA-repair protein O6-alkylguanine (O6-AG) DNA alkyltransferase (AGT) encoded by the gene O6-Methylguanine (O6-MG)-DNA-methyltransferase (MGMT) repair DNA adducts caused by alkylating agents. MGMT has important implications in cancer treatment since its expression correlates inversely with sensitivity to agents that form O6-alkylguanine adducts, such as temozolamide. It is therefore of great interest to find agents that induce MGMT deficiency, increase the sensitivity and possible overcoming resistance to alkylating chemotherapeutic agents.

Methods: Cell lines from neuroblastomas, medulloblastomas and gliomas were examined for Wnt/beta-catenin activity and MGMT expression. We used western blot, Real-Time quantitative PCR (Q-PCR), siRNA knockdown, cDNA overexpression of beta-catenin and MGMT promotor reporter plasmids. Cell cytotoxicity and clonogenicity of chemotherapeutic drugs in combination with celecoxib were examined in cell lines using fluorometric microculture cytotoxicity assay and clonogenic assay, respectively. Compounds targeting Wnt signaling was investigated in combination with temolzolamide in vitro and in vivo.

Results: MGMT expression level was correlated to Wnt signaling activation both in primary tumors and cell lines of different origins. Transfection experiments revealed that beta-catenin directly regulates MGMT expression via Tcf/LEF binding. Wnt inhibiting drugs and compounds potentiates the cytotoxic effect of the DNA alkylating drug, temozolomide in cells with elevated MGMT expression in vitro and in vivo.

Conclusions: Our data demonstrate that MGMT is a direct Wnt/beta-catenin target, and agents that inhibit Wnt signaling reduces the transcription of MGMT through a prostaglandin E2-Wnt/beta-catenin route and thus increases the sensitivity to temozolomide. This provides a rational approach for improved efficacy of chemotherapeutic drugs inducing DNA alkylation in cancer treatment. The data also suggest that Wnt/beta-catenin is an important target for therapeutic interventions.

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POT022

Hypoxia and neuroblastoma: from bench to clinic

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Background: Hypoxia, a condition of low oxygen tension occurring in poorly vascularized areas, has a profound effects on tumor growth and resistance to therapy. We studied the relationship between hypoxia in neuroblastoma tumors and patients outcome.

Methods: We identified a gene expression signature, selective for hypoxic neuroblastoma cells. This signature is an independent risk factor and was used to build a classifier (NB-hypo classifier) predicting neuroblastoma patients outcome. We developed and validate the Multi Layer Perceptron classifier predicting outcome on the gene expression profiles of 182 neuroblastoma tumors. Immunohistochemistry validated gene expression and gene set enrichment analysis (GSEA) measured the enrichment of independent hypoxia-related gene sets in poor outcome patients.

Results: NB-hypo classifier predicted patients outcome with an accuracy of 87% which is greater than that achieved by current risk factors on the same cohort. NB-hypo classifier correctly predicted the outcome of all patients with localized and stage 4s tumors. The GSEA analysis was used to explore the relationship between NB-hypo outcome prediction and tumor hypoxia and showed a major enrichment of hypoxia related gene sets in tumors of poor outcome patients. The hypoxic status of these tumors was further validated by immunohistochemistry evaluation of hypoxia specific genes (CAIX, VEGF). A clinical protocol based on NB-hypo classifier was developed.

Conclusions: NB-hypo classifier derives from a biology-driven approach which allows the immediate identification of hypoxia as clinical target. NB-hypo classifier, while probing the hypoxic status of the tumor, is a new and robust predictor of neuroblastoma patient's outcome with very low error rate that decreases to negligible levels in localized tumors. NB-hypo classifier is ready to use and of immediate clinical application. We designed and implemented a clinical protocol that utilizes drugs targeting hypoxia (Avastin, Topotecan etc.) for NB-hypo predicted poor outcome neuroblastoma patients with recurrent disease.

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POT023

Development, Characterization And Cytotoxic Activity Of sTrail-Targeted Liposomes Against Neuroblastoma

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Background: The tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a promising anti-cancer agent. It's soluble form (sTRAIL) exerts anti-tumor activity in a variety of tumor cells in vitro and in vivo. However, the therapeutic usefulness of recombinant sTRAIL has several limitations such as its short biological half-life and the instability in physiologic environments.

Aim: To develop novel liposomal formulations carrying the sTRAIL at their outer surface in order to verify its preserved anti-tumor activity in vitro while improving its pharmacokinetic features and increasing its efficacy in vivo.

Methods: The expression of sTRAIL receptors was validated by FACS analysis in a panel of human neuroblastoma cell lines. For building up the sTRAIL-targeted Stealth Liposomes (sTRAIL-SL), coupling was allowed between a thiol of sTRAIL and a maleimido moiety on the pegylated liposomes. Dose dependent cellular association of sTRAIL-SL was analyzed by FACS. In vitro neuroblastoma cells cytotoxicity and selective apoptosis induction by sTRAIL-SL were examined by CytoTox 96® cytotoxicity and annexin V/PI assays for sTRAIL, either free or coupled to SL. In vivo experiments are on running.

Results: FACS analysis showed that sTRAIL-SL were able to bind to TRAIL receptors expressing cells in a dose dependent manner and in relationship to the level of expression of the receptors on the neuroblastoma cell surface. Treatments with sTRAIL-SL resulted in a dose dependent cytotoxicity and induction of apoptosis and necrosis in tumor cells. No effect was exerted on normal fibroblasts.

Conclusions: Our preliminary data show that sTRAIL-SL preserve the anti-tumor activity of sTRAIL in vitro. Ongoing experiments are focused in validating the improved pharmacokinetic profile of sTRAIL when attached to the liposome surface and its anti-tumor effects in vivo. The expected results will lead us to consider sTRAIL-SL a more efficient and stable tool for the anti-tumor efficacy of sTRAIL in vivo.

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POT024

Novel phage-display derived peptides for tumor- and vasculature-targeted therapies in neuroblastoma

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Background: The therapeutic index of anticancer drugs is increased by liposome encapsulation and further improvements is obtained by coupling tumor-targeting ligands to the surface of the lipidic envelop. Phage display technology is a powerful tool in discovering novel ligands specific to receptors on the surface of tumor epithelial and endothelial cells.

Methods: To find novel neuroblastoma (NB)-specific targeting moieties, we established a protocol for the isolation of heterogeneous cell populations by tissue fractionation of primary tumors and metastases from animal models of human NB. Cells extracted from corresponding healthy organs from mice were used both in a negative pre-selection step and as a negative control for specific phage enrichment. The NB cell suspensions were subjected to multi-step screenings with the phage-displayed peptide library X7 (X = any aminoacid).

Results: We globally isolated 121 phages displaying NB-binding peptides. Of these, 26 were selected for binding to the primary tumor mass, 15 to the metastatic mass, 57 to tumor endothelial cells, and 23 to endothelial cells of metastases. Among those, 5 phage-displayed peptides showed specific binding on NB tumors derived from mice and from stage IV, stroma poor, NB patients, and homing to tumor cells and tumor vasculature in orthotopic NB-bearing mice. Negligible healthy tissues distribution was observed. Compared with a scrambled peptide, the five corresponding synthetic peptides showed specific binding in in vitro and ex-vivo cellular association experiments, and when coupled at the external surface of doxorubicin-loaded nanocarriers, showed their anti-tumor activities evaluated against different models of human NB.

Conclusion: The availability of novel ligands binding to additional tumor-associated antigens and to targets on both endothelial and perivascular tumor cells will allow to design more sophisticated liposomal targeted anticancer strategies that exhibit high levels of selective toxicity for the cancer cells.

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POT025

Tumor-inhibiting and -promoting properties of drug-induced senescent neuroblastoma cells

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Background: Cellular senescence, a permanent state of proliferative arrest can be induced in tumor cells upon stress and is associated with inhibition of tumor initiation and tumor regression. Other reports claim tumor-promoting properties of senescent cells. We have shown that in MYCN-amplified (MNA) early passage neuroblastoma (NB)-cell lines derived from stage 3/4 patients, hydroxyurea (HU) treatment leads to senescence in vitro. The aim of this study was (1) to compare HU with 2 genotoxic drugs, Camptothecin (CPT) and Bromodeoxyuridine (BrdU) and analyse senescence markers, pathways and the secretory profile; And (2) to analyse their functional properties, i.e. angiogenesis and stimulation of immune effector cells.

Methods and Results: Two MNA low-passage NB cell lines were treated in vitro for 5 or 8 weeks with CPT and BrdU or HU, resp. Drug-treated cells were equal in senescence-associated-beta-Galactosidase activity and elevated p21, p16 and phospho-p53. MYCN copy number, MYCN protein expression, GD2 and CDK2 levels were significantly lower in drug-induced senescent NB-cells. Furthermore, gene expression profiling revealed a positive correlation with the profile of low risk tumors. However, the secretory profile differed significantly depending on the senescence inducer: only BrdU-treated cells highly secrete MCP-3, VEGF and MMP-9, implicated in inflammation, angiogenesis and metastasis. In functional analyses senescent NB-cells showed an increased angiogenic potential, whereas in co-cultures senescent cells reduce cell growth and GD2 levels of non-senescent tumor cells. Moreover, senescent NB cells facilitate CD8+ T-cell activation, probably through cell-bound factors.

Conclusion: Thus, drug-induced senescent NB-cells have pro-immunogenic and tumor-inhibitory as well as tumor-promoting properties in vitro. Further investigations are needed to clarify whether NB patients may benefit from treatment with senescence inducing drugs.

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POT026

The HDAC6 inhibitor Tubastatin A synergizes with Bortezomib in Neuroblastoma

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Background: Bortezomib, a proteasome inhibitor, has been used successfully to induce cell death in many cancer cell types, including neuroblastoma. We showed that, in neuroblastoma cells, HDAC6, a class 2 deacetylase found in the cytoplasm, acts as a survival factor such that inhibition or depletion of HDAC6 induces neuroblastoma cell death. The primary target of HDAC6 is Ku70-Bax complex in the cytoplasm, HDAC6 inhibition or depletion triggers Ku70 acetylation resulting in Bax-dependent cell death. However, besides regulating Ku70-Bax complex, HDAC6 also regulates protein degradation by proteasome. Here we demonstrate that bortezomib synergizes with HDAC6 specific inhibitor in reducing viability of neuroblastoma cells.

Methods: IMR32 and SY-SY5Y cells were treated for 24 hours with doses of bortezomib, SAHA (a class 1 and class 2 deacetylase inhibitor), or tubastatin A (a HDAC6-specific inhibitor) alone or in combination (deacetylase inhibitor plus bortezomib). Cell viability was determined by MTT assay. HDAC inhibition was monitored using histone H3 acetylation (an index of class 1 HDAC inhibition) and tubulin acetylation (a substrate of HDAC6 acetylation).

Results: Treatment of bortezomib, SAHA, or tubastatin A reduces dose-dependently neuroblastoma cell viability. However, co-treatment of SAHA or tubastatin A with bortezomib (10nM) shifted the IC50 of SAHA (2.5 to 0.5μM) or tubastatin A (8 to 2.5μM) while bortezomib alone has minimal effect (10% reduction). SAHA or tubastatin A increases tubulin acetylation, and that SAHA but not tubastatin A also induces histone H3 acetylation.

Conclusion: The synergistic effect of Bortezomib and tubastatin A seen in the reduction of neuroblastoma cell viability may be the results of targeting the two cell death pathways (Ku70-Bax complex and proteasome degradation) of HDAC6. These results warrant further investigation of combination therapy using HDAC6 specific inhibitors and bortezomib in neuroblastoma.

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POT027

Targeting overexpression of XIAP in neuroblastoma by Smac mimetic LBW242 sensitizes for TNF-α independent apoptosis

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Background: High risk Neuroblastoma (NB) is associated with poor outcome despite intensive treatment regimens. Based on reports that overexpression of X-linked inhibitor of apoptosis protein (XIAP) is associated with chemotherapy resistance in several malignancies we investigated its role as a potential target in neuroblastoma.

Methods: XIAP protein and mRNA expression was determined in a panel of human and murine NB cell lines, murine adrenal glands, and primary NB samples. Effects on proliferation and on apoptosis were analyzed after treatment with Smac mimetics (LBW242, Novartis) alone or in combination with cytotoxic drugs typically used in neuroblastoma therapies.

Results: XIAP protein but not mRNA expression was found to be highly increased in NB cells compared to healthy adrenal gland tissue consistent with a post-transcriptional regulation of XIAP expression. Treatment with LBW242 sensitized human and murine NB cell lines for chemotherapy-induced apoptosis mediated by the activation of both, intrinsic and extrinsic apoptosis pathways. Smac mimetics have been reported to stimulate TNF-α induced apoptosis by degradation of cIAP-1/2. Interestingly, despite induction of cIAP-1/2 degradation and TNF-α expression, LBW242-mediated sensitization of NB cells for chemotherapy occurred in a TNF-α independent manner.

Conclusions: In summary we demonstrate for the first time that sensitization of NB for chemotherapy by targeting increased XIAP expression using LBW242 may provide a new venue to further improve treatment results.

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POT028

Biologically driven gene-set association analysis identifies new neuroblastoma susceptibility common DNA variations at region downstream of the NEFL gene

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Background: Using genome-wide association studies (GWAS) we identified several genetic risk loci associated with neuroblastoma (NB), in LINC00340 (NEJM, 2008), BARD1 (Nature Genetics, 2009), LMO1 (Nature, 2010), DUSP12 (PLoS Genetics, 2011), HSD17B12 (PLoS Genetics, 2011), NBPF23 (Nature, 2009). Other risk loci are probably hidden among signals discarded by the multiple testing correction needed in the analysis of genome-wide data.

Methods: To identify additional risk factors for NB, we have adopted a strategy to find new associated loci starting from a set of genes identified in our previous proteomic 2D-DIGE-based study on NB (J Proteome R, 2007). Thirty-two genes that encode proteins differentially expressed during neuronal differentiation induced by all-trans-retinoic acid treatment underwent gene-set association analysis in a GWAS dataset of 1627 cases and 2575 controls.

Results: The NEFL gene showed significant association with NB (P=0.01). A microarray gene expression analysis using public data showed that NEFL was over-expressed in 11 ganglioneuroblastomas compared to 51 neuroblastomas (P=0.002). Genetic association of two SNPs downstream of the gene that showed the most robust signal in the discovery set was confirmed in two independent replication sets of European American (490 cases and 1507 controls) and Italian (350 cases and 800 controls) origin, respectively (rs169061, P=0.008 – OR=0.82 and P=0.02 – OR = 0.80; rs11994014, P= 0.08 – OR=0.85 and P= 0.03 – OR=0.80). SNP rs11994014 is in linkage disequilibrium (r2=0.63) with rs2979704 in NEFL 3'UTR region that was also associated with NB in both populations (European Americans: imputed P=0.055-OR=0.88; Italians: real P=0.02 – OR=0.74). Recently, NEFL downregulation has been associated with resistance to cisplatin-based chemotherapy in head and neck cancer (Mol Cancer Res, 2012).

Conclusions: Taken together, these observations suggest that DNA variants downstream of NEFL influence NB susceptibility and demonstrate how genetic and functional datasets can be merged to maximize discovery efforts.

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POT029

Exome and transcriptome sequencing of multiple tumors reveals a stable expressed somatic mutation profile in a patient with metastatic neuroblastoma

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Background: Previous studies have reported that metastatic tumor cells acquire additional mutations to those present in the primary due to an unstable cancer genome. However, it is not clear if all malignancies follow a similar pattern.

Methods: To identify potential driver mutations and investigate genomic changes that occur during tumor progression, we performed comprehensive genomic analyses including exome and transcriptome sequencing of a primary and two metastatic tumors from a patient with neuroblastoma taken at different time points during therapy.

Results: We found that despite 3.5 years of multimodal cytotoxic therapy the genomes of all three cancers remained relatively stable using SNP genotyping and array comparative genomic hybridization. Remarkably, there were only 5 expressed somatic non-synonymous mutations and all of them were present in the three tumors with no additional protein-disrupting mutation arising in the metastatic samples. One of these mutations was in the potentially druggable G-protein coupled receptor LPAR1 which resulted in a significant increase in cell motility, but had no effect on cell growth.

Conclusions: The chromosomal structural stability together with the lack of evidence for progressive accumulation of expressed mutations indicates that this cancer arose from a single catastrophic chromosomal event combined with a small number of somatic mutations. Parallel whole exome and transcriptome sequencing identified a cell motility driver mutation in the LPAR1 gene, and this combinatorial approach may be leveraged for precision therapy in patients with cancer by targeting expressed driver mutations.

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POT030

Genomic Evolution in Relapsed Neuroblastoma

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Background: Genomic evolution has been hypothesized to occur in neuroblastoma as a tumor progresses. To test this hypothesis, we

analyzed the genomes of paired tumor samples obtained at the times of initial diagnosis and relapse.

Methods: From 1990 to 2010, a total 100 patients with NB were treated in our institution. Twelve of these patients suffered relapses and paired tumor samples were obtained from six of them. Five of these patients had MYCN amplification. Pangenomic data for each of the tumor samples were analyzed by neuroblastoma-specific multiplex ligation-dependent probe amplification.

Results: Segmental chromosome alterations were found in all six patients at diagnosis. At the time of relapse, new chromosomal alterations (on 1q, 2p, 7p, and 9p) were observed in addition to those observed at diagnosis in five of the patients. On the other hand, in the sixth patient, some segmental chromosomal alterations detected in the tumor at diagnosis had disappeared at the time of relapse. The reason is unclear but one possibility is that the disappeared alterations were associated with a cell population that was killed by initial treatment, and that a minor population of chemo-resistant cells at the time of diagnosis proliferated and caused the relapse.

Conclusion: Our data provide evidence of genomic evolution of neuroblastoma, and suggest that tumor progression is linked to the accumulation of chromosomal aberrations. We also speculate that tumors showing chemo-resistance at relapse may be derived from minor subpopulations that had already existed at the time of initial diagnosis.

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POT031

Next-generation sequencing: Integrated exotome analysis in human multiple neuroblastoma

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Background: Although multifocal neuroblastoma (NBL) is rare, its susceptibility is due to germline aberration and this predisposition gene may also be involved in sporadic neuroblastoma tumorigenesis. Sequence capture enrichment strategies and massively parallel next generation sequencing (NGS) were used for gene discovery because of their alterations.

Methods: Twenty samples derived from 10 multifocal neuroblastoma cases were analyzed by a method for whole-exome sequencing coupling Agilent whole-exome capture to the Illumina DNA-sequencing platform. Using this technology, we investigated consistent point-mutations and insertions-deletions in these cases. Then, we analyzed the expression levels of these candidate genes in 298 sporadic NBL samples.

Results: Among these 10 cases, one case showed ALK mutation in both of his multifocal tumors and germline mutation of ALK exon 23 (F1174L) was identified. These cases showed 57 candidate genes with consistent point-mutations and insertions-deletions including genes correlated with embryonal development and cell cycle. The expression levels of these candidate genes revealed that several genes including ID3 and ALOX15 genes were significantly decreased in these multifocal NBL tumors, except for one ALK mutated case. Then, we examined the expression levels of these two genes in sporadic NBL tumors. The expression levels of ID3 and ALOX15 genes were significantly low in 42 and 61 NBL tumors, respectively but the low expression of these two genes were detected in only three sporadic NBL tumors.

Conclusions: Whole-exome sequencing using NGS in multifocal neuroblastoma revealed several candidate germline-mutated genes including ALK in such NBL-susceptibility cases. The expression levels of these genes also indicated that heterogeneous subtypes exist in sporadic NBLs. NGS can be used in research and diagnostic settings to screen for mutations and deletion/insertion in number of loci in genetically heterogeneous neuroblastoma. Further NGS analysis provided important candidates of indicators for diagnostic and therapeutic targets for NBLs.

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POT032

Next-generation sequencing reveals differential expression of MYCN target genes in neuroblastoma (NB) and suggests the mTOR pathway as a promising therapy target in MYCN-amplified NBs

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Background: Deep sequencing of mRNA promises to identify all transcripts expressed in a given system transcriptome. MYCN is known to be a master regulator of the neuroblastoma (NB) transcriptome, causing massive reprogramming of the RNA-producing machinery. Here we investigated the MYCN-driven transcriptome using next-generation RNA sequencing of primary NBs with and without MYCN amplification, and compared the results to those from an in vitro NB cell model for inducible MYCN.

Methods: Total RNA was prepared and depleted of rRNA then reverse transcribed from 20 primary NBs and the SHEP-MYCN-ER cell line with and without MYCN induction. The resulting cDNA was sequenced using paired-end sequencing, and reads were mapped to the Human Genome RefSeq Hg19 using BioScope v1.21. Statistical analyses and clustering was performed using R2.14 (r-project.org). Potential MYC targets were verified using either existing databases or by applying MosDI, which identifies MYCN-binding motifs in the promoter regions of differentially expressed transcripts.

Results: Transcriptome sequencing produced 30 - 90 million mappable reads for each dataset. The most abundant RNA species was mRNA, but snoRNAs, pseudogenes and processed transcripts were also recovered. A total of 223 genes were significantly differentially expressed between MYCN-amplified and single-copy tumors. In total, 32% of MYCN upregulated and 37% of MYCN downregulated genes were verified either as previously identified MYCN targets or as having MYCN-binding motifs. GO and KEGG pathway analyses revealed that proteins involved in ribosome complexes and proteins of the mTOR pathway were significantly upregulated both in MYCN-amplified tumors and after MYCN induction in vitro.

Conclusions: Next-generation RNA sequencing allows in principle for the identification of all MYCN regulated transcripts in neuroblastoma. As our data suggest MYCN involvement in mTOR pathway activation on the transcriptional level, mTOR inhibitors should be evaluated as a promising option for the treatment of MYCN-amplified NBs.

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POT033

Replication and cumulative genetic risk of GWAS-identified common variations for neuroblastoma in an Italian population

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Background: Our recent genome-wide association studies (GWAS) have identified diverse susceptibility loci to neuroblastoma (NB). Replication of GWAS findings remains the gold standard for results validation.

Methods: Our aim was to test the association of 12 SNPs (intronic rs6939340, rs4712653 in LINC00340, intronic rs6435862, rs3768716, 3'regutaltory region rs7585356, coding rs2070094, rs2229571, rs1048108 in BARD1, intronic rs110419 and rs4758051 in LMO1, rs1027702 in DUSP12 and rs11037575 in HSD17B12) in Italian population composed of 350 cases and 800 controls. We further evaluated their cumulative effect on NB development and high-risk phenotype.

Results: All SNPs were confirmed except for rs4758051 in LMO1, and the most significant SNP was rs6435862 in BARD1 (P=3.38x10-15). rs6939340 in LINC00340, rs6435862, rs7585356, in BARD1, rs110419 in LMO1, rs1027702 in DUSP12 and rs11037575 in HSD17B12 were not in LD (r2 <=0.10), so we undertook a cumulative genetic risk analysis. Interestingly, BARD1 showed two independent signals (rs7585356 and rs6435862; r2=0.07) and rs7585356 influenced gene expression in LCL cell lines according to the analysis of three public datasets (P=1.04x10-4; 7.74x10-8; 3.01x10-4). Individuals with multiple risk alleles had higher risk of developing NB and a clinically aggressive phenotype in Italian (risk-alleles 9-11 OR:6.50 CI:3.55-11.90 for case-control and OR:2.23 CI:0.85-5.84 for high-risk vs. intermediate-low-risk) and US population (1627 cases and 2575 controls) (risk-alleles 9-11 OR:3.25 CI:2.35-4.48 for case-control and OR:3.46 CI:1.98-6.03 for high-risk vs. intermediate-low-risk)

Conclusion: These results provide further evidence that risk loci identified in GWAS studies contribute to NB susceptibility in distinct populations. Moreover, this study showed that the confirmed loci confer a significantly greater effect once the effect of additional risk variants are accounted for, but a large proportion of the heritability remains undefined. Ongoing work will focus on epistatic effects at known risk alleles and other loci genome-wide.

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POT034

Identification of a new hereditary neuroblastoma predisposition locus at chromosome 2p25

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Background: Familial predisposition to neuroblastoma is evident in ~1% of cases. The majority of familial cases harbor germline mutations in ALK or PHOX2B, but in the remaining kindreds the genetic etiology is unknown. We performed a linkage analysis to determine whether the remaining kindreds shared a common genetic etiology, and to identify genes for resequencing.

Methods: We identified 17 families with 2 or more affected individuals who tested negative for germline mutations in ALK or PHOX2B. We performed a genome-wide scan for linkage in eight informative families where DNA was available from 2 or more affected cases, and/or obligate carriers other than parent-child pairs. Constitutional DNAs were genotyped using the Illumina OmniExpress chip (700,000 SNPs). A total of 64 DNA samples passed quality control, including confirmation of family relationships, and were retained for analysis. Correlated SNPs (r2>0.1) were removed to reduce the probability of a false positive finding. Linkage analysis was performed using the non-parametric Merlin-rapid method.

Results: We identified a significant linkage signal at chromosome 2p25 with a maximum non-parametric likelihood ratio (LOD) score of 3.28 observed at SNPs rs1358135 and rs6431763. LOD scores > 3 identified a gene dense 10.7 Mb region of interest delimited by rs11679592 and rs4668795. The signal was present in all families studied, and no other region of the genome showed LOD >2.

Conclusions: A third familial neuroblastoma locus is located at chromosome 2p25. Next-generation exome sequencing efforts are ongoing to discover the causal gene with implications for genetic counseling and genetic testing algorithms, and will likely provide new insights into the biology of this complex disease.

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POT035

Genetic alterations are related to immune response in Neuroblastoma

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Background. Clinical translation of neuroblastoma (NB) immunobiology has contributed to improve patient treatments, such as monoclonal antibody technology. Understanding how neuroblasts interact with the immune response (IR) is important to develop new therapeutic strategies.

Purpose. Quantification of cellular population related to IR and the relationship with neuroblasts genetic alterations.

Methods. Sixty-six primary NBs were immunohistochemically characterized, using anti-CD3, anti-CD-20 and anti-CD45 antibodies (Dako, Denmark) to detect leukocyte lineage cells (LLC). The stained slides were digitized by ScanScope XT and the number of positive cells (PC) per area was quantified using Image Analysis ToolBox (Aperio Technologies Inc.). FISH and MLPA were performed to ascertain the genetic profile.

Results. The mean of CD20 PC was low in 11q-deleted (5.9 vs 48.7; M-W p=0.036) and 17q-gained tumors (32.7 vs. 52.3; M-W p=0.027), and advanced stages patients (33.1 vs. 47.7; M-W p=0.004). Patients > 18 months suffered tumors with less CD3 and CD45 PC (413.6 vs. 1310.3, M-W p=0.043; 2324.2 vs. 4848.5, M-W p=0.003). MYCN-amplified (MNA) tumors presented low number of CD3 (255 vs. 1228.2; M-W p=0.002), CD20 (26.5 vs. 45.8; M-W p=0.001) and CD45 (2302.2 vs. 4390.6; M-W p=0.02) PC. The quantity of CD3, CD20 and CD45 PC were higher in tumors with segmental chromosomal aberrations (SCA) without MNA profiles than in tumors with numerical chromosomal aberrations or with MNA profiles (K-W: p=0.015; p=0.006; p=0.055).

Conclusions. There is a group of NBs capable of suppressing IR, with reduced number of LLC infiltrating the tumor mass. Those tumors presented genetic alterations as MNA and other factors indicating poor prognosis (> 18 months of age at diagnosis and/or advanced stages of disease). Tumors with several SCA without MNA profiles showed an increased number of infiltrating immune cells, reflecting an unusual and possibly ineffective IR.

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POT036

Therapeutic Targets For High-Risk Neuroblastoma By Functional Genomics

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Background: Amplification of MYCN is a predictor of poor prognosis, but a significant percentage of advanced cases present without amplification with less understood molecular drivers. We previously identified synthetic lethal genes with MYC overexpression by functional genomics and validated inhibition of one gene, CSNK1e in MYCN amplified neuroblastoma xenografts. This example demonstrated the power of unbiased screens to identify novel targeted therapies.

Aims: Our goal is to broadly identify druggable targets for high-risk neuroblastoma utilizing functional genomics. Here, we focus on survival pathways that distinguish MYCN amplified versus non-amplified neuroblastoma.

Methods: We employed automated high-throughput siRNA and drug screening to test in parallel neuroblastoma with and without MYCN amplification. The screens focused on the druggable genome and the NCI collection of 88 oncology drugs.

Results: Overlap of "Hits" from MYCN amplified neuroblastoma with MYC-synthetic lethal genes confirmed common "Hits". Among non-kinases, but potentially druggable, was CECR2, a bromodomain containing protein involved in neuroectodermal development as well as PES1, the Pescadillo orthologue. Kinases with selectivity from MYCN amplified were NEK2,3,4, FGFR2, TIE1, PIK4CB and PIM3. All but PIM3 were also among the MYC-synthetic lethal genes, thus validating the context dependent toxicity of their knock-downs. With selectivity for non-amplified neuroblastoma, among others, RYK, a transmembrane receptor tyrosine kinase that functions as a receptor of WNT ligands, and DLK1, an inhibitor of NOTCH signaling, emerged. Results from the drug screening also indicated dramatic differences in sensitivity with topoisomerase inhibitors being selective for MYCN amplification, while Cladribine, was selective for non-amplified neuroblastoma.

Conclusions: siRNA screening and broad drug screening integrated with microarray data from tumor samples indicated novel targets for therapeutic development and demonstrate that high-risk MYCN amplified and non-amplified neuroblastoma are functionally distinguishable cell lineages. Therapeutics approaches derived from these findings have the potential to be highly selective and less toxic.

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POT037

Genetic variants associated with poor outcome in high-risk neuroblastoma are enriched with variants associated with in vitro cyclophosphamide resistance

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Background: High-risk neuroblastoma (HRNB) is a deadly disease, but some patients are able to achieve long term cure with intensive chemotherapy-based regimens. We sought to evaluate the role of germline genetic variants associated with resistance to an active metabolite of cyclophosphamide from a human cell-based model in high-risk patients with poor event-free survival (EFS).

Methods: After quality control, we analyzed 511,836 germline variants for association with the occurrence of an event within 3 years from diagnosis in 1,070 children with HRNB using chi-square tests. SNPs with nominal association with EFS ($P < 0.001$) were included in this analysis. To identify SNPs associated with cellular sensitivity to cyclophosphamide, we exposed 431 International HapMap human lymphoblastoid cell lines to concentrations ranging from 10 to 200 μM of phosphoramide mustard (PM), a metabolite of cyclophosphamide that is thought to be responsible for its antitumor activity. Top SNPs from the analysis of HRNB EFS were evaluated for their association with PM IC50. A null distribution of overlap SNPs for the two datasets was generated in 1,000 simulations by using random SNPs with minor allele frequencies matching those of the observed overlap SNPs to assess for degree of overlap.

Results: 455 SNPs were associated with EFS and 28 of these SNPs were associated with PM IC50. Based on the permuted null distribution of expected overlapping SNPs, there was a significant enrichment of PM-associated SNPs in patients with poor EFS ($P = 0.039$).

Conclusions: These data support cyclophosphamide resistance as a contributor to high-risk neuroblastoma treatment failure. Integration of our cell-based modeling with GWAS data offers a means to identify clinically relevant SNPs associated with chemotherapeutic resistance in neuroblastoma. Functional studies to determine the role of the overlapping SNPs in cyclophosphamide resistance are underway.

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POT038

AADC gene expression, 18F-FDOPA uptake, and tumor differentiation in neuroblastic tumors

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Background: Neuroblastic tumors (NTs) originate from the sympathoadrenal lineage and possess amino acid decarboxylase (AADC) activity, which functions in catecholamine metabolism and serves as the molecular target of 18F-FDOPA positron emission tomography (PET) imaging that has been successfully applied in NTs recently. In this study, we examined the relationship among AADC expression, 18F-FDOPA accumulation, and tumor differentiation in NTs.

Methods: Patients with NTs treated at National Taiwan University Hospital during 2006-2010 were enrolled. Real-time PCR was performed to determine AADC expression level in NT samples.

Results: Thirty-one NTs from 26 patients (median age 2.3 [range, 0.2-6.9] years; male:female 19:7; including 16 [62%] stage 4 patients in whom 3 [12%] with MYCN amplification) were eligible for analysis. Histopathology showed 7 undifferentiated neuroblastomas (UNB; 23%), 9 poorly-differentiated neuroblastomas (PDNBs; 29%), 5 differentiating neuroblastomas (DNB; 16%), 8 ganglioneuroblastomas (GNB; 26%), and 2 ganglioneuromas (GN; 6%). Using GAPDH mRNA as internal controls, the 25th%, 50th%, and 75th% relative AADC expression levels of the 33 tumors were 2.109, 7.166, and 23.416 *0.001 folds, respectively. The AADC expression of PDNB followed by DNB was significantly higher than that of the least differentiated UNB or the more differentiated GNB and GN (median, UNB/PDNB/DNB/GNB/GN, 6.849/35.235/20.913/2.108/1.755 *0.001 folds; P=0.0076 by Kruskal-Wallis test). In NTs with relevant 18F-FDOPA PET imaging (n=17), PDNB followed by DNB also showed higher tumor uptake of 18F-FDOPA (median tumor-to-liver SUV ratio, UNB/PDNB/DNB/GNB/GN, 1.126/3.465/3.216/1.807/1.868, P=0.05). The tumor uptake of 18F-FDOPA was strongly correlated with AADC expression (Spearman's Rho=0.77, P=0.0003). There was no significant correlation between histology and urinary vanillylmandelic acid (VMA) (P=0.12), or AADC expression and VMA (P=0.30).

Conclusions: AADC expression and 18F-FDOPA uptake showed a non-linear relationship with NT differentiation, in that PDNB followed by DNB had the highest levels. 18F-FDOPA PET imaging has the potential to predict the underlying tumor biology in vivo.

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POT039

FDG:FDOPA uptake ratio by PET scans at diagnosis correlates with genomic type and treatment outcome of neuroblastoma

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Background: Molecular imaging is fundamental for the diagnosis and surveillance of neuroblastoma (NB), but the biological and prognostic implications of novel positron emission tomography (PET) scans are less understood. In this study, we examined whether the signal intensity of NB on 18F-FDG and 18F-FDOPA PET at diagnosis is associated with genomic types and treatment outcome.

Methods: As MIBG scans are still not available for routine clinical practices in Taiwan, NB patients were enrolled for 18F-FDG and 18F-FDOPA PET scans since 2008. Each primary NB's maximal standard uptake value (SUVmax) was normalized to liver uptake, generating a tumor-to-liver SUV ratio (T/L) for each scan. The ratio between 18F-FDG and 18F-FDOPA T/L at diagnosis (FDG:FDOPA) was used as a novel biomarker to compare with major clinical parameters.

Results: Twenty-one patients (median age 2.0 [range 0.2-6.9] years; male:female 16:5) were eligible for analysis, with a median follow-up of 21.6 months. Ten (83%) of the 12 NBs with array comparative genomic hybridization (array-CGH) and/or chromogenic in situ hybridization for MYCN status belong to one of the major genomic types, including type 1 (gains and losses of whole chromosomes; n=5), subtype 2A (segmental alterations involving 11q and/or 17q; n=3), and subtype 2B (MYCN amplification; n=2). In contrast to type 1 NBs, type 2 NBs were associated with older age (P=0.024), stage 4 (P=0.008), higher 18F-FDG uptake (P=0.0163), lower uptake on 18F-FDOPA PET (P=0.0283), and higher FDG:FDOPA (P=0.009). Among all 21 eligible patients, FDG:FDOPA >=1 significantly correlated with stage 4 (P=0.007) and worse progression-free survival (2y-PFS, 68% vs. 0%, P=0.0348), but not with age (P=0.64) or overall survival (2y-OS, 88% vs. 86%m P=0.23).

Conclusions: Major genomic types of NB can be distinguished by diagnostic 18F-FDOPA and 18F-FDG PET scans. NB patients with initial FDG:FDOPA ratio >=1 might have an inferior outcome.

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POT040

Effects of different corticosteroids on neuroblastoma imaging and therapy

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Background: Radiolabelled meta-iodobenzylguanidine (mIBG) is taken up by norepinephrine transporters (NET) in neuroblastoma cells and probably mainly by organic cation transporter 3 (OCT3) in healthy tissues. In this study we investigated the short term and long term effects of clinically used corticosteroids on OCT3 and NET in vitro and in vivo.

Methods: In vitro experiments were carried out using neuroblastoma cells (SK-N-SH, Kelly, IMR-32) and HEK-293 cells transfected with human OCT3 (hOCT3). Different corticosteroids were tested in several concentrations for their effects on the incorporation of [^{3}H]norepinephrine, [^{3}H]dopamine or [123I]mIBG in cell culture. The influence of hydrocortisone and prednisolone pretreatment on [123I] mIBG scintigraphy was controlled by in vivo imaging using small animal SPECT. Mice without corticosteroid treatment served as control.

Results: In vitro application of corticosteroids directly prior to radioactivity caused a significant inhibition of hOCT3, which might be mediated by phospholipase C. The radioactive uptake in neuroblastoma cells by NET however was only slightly reduced. SPECT imaging of mice, which were injected intravenously with corticosteroids prior to [123I]mIBG scintigraphy, showed no reduced uptake in mouse OCT3 (mOCT3) expressing tissues. Although hOCT3 and mOCT3 are very similar, corticosteroids obviously only inhibit mIBG uptake by hOCT3. Under long term treatment with corticosteroids in vitro no inhibition of hOCT3 could be detected, whereas the radioactive incorporation in neuroblastoma cells was increased. This enhanced uptake might be associated with an elevated expression of NET.

Conclusion: Clinically used corticosteroids are specific inhibitors of the human OCT3 under short-term treatment and are able to increase the uptake of [123I]mIBG in neuroblastoma under long-term treatment. They might be used in combination with [123I]mIBG scintigraphy to improve specific imaging of neuroblastoma and to reduce radiation dose of non-targeted organs in [131I] mIBG treatment.

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POT041

Functional analysis of miRNA in drug resistant neuroblastoma cell lines

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The acquisition of multidrug resistance is the principle obstacle to the successful treatment of neuroblastoma. Thus, the elucidation of mechanisms involving multidrug resistance, along with the development of novel approaches for sensitizing cells to therapy, would be of translational benefit. To this end, we have developed cisplatin resistant neuroblastoma cell lines (SKNASCis24, KellyCis83 and CHP212Cis100), and determined that two of the lines are cross-resistant to etoposide (SKNASCis24, KellyCis83). The drug resistant and parental lines were profiled for the expression of 770 miRNA. 41, 51 and 72 miRNAs were differentially expressed in KellyCis83, SKNASCis24 and CHP-212Cis100 respectively, with an enrichment of differentially expressed miRNA from the miR-379/656 cluster on chromosome 14q32. Array CGH identified a region of focal gain in SKNASCis24 that contained the NAIP (neuronal apoptosis inhibitory protein) gene, which is over-expressed 15 fold relative to parental cells. Interestingly, 13 miRNA down-regulated in SKNASCis24 are all predicted to target NAIP, indicating that both DNA copy number gain and silencing of targeting miRNAs contribute to increased expression in SKNASCis24. Preliminary results using siRNA inhibition confirm a functional role for NAIP in neuroblastoma cell drug resistance, though the precise mechanism of action is not completely understood. Two lines, KellyCis83 and CHP-212Cis100, exhibited enhanced cellular proliferation when grown in the presence of low-dose cisplatin, relative to cells grown in normal media. MiRNA expression profiles of cells challenged with 0.2 μM cisplatin were also obtained and 31 miRNAs differentially expressed in KellyCis83 and CHP-212Cis100 were identified. Several differentially expressed miRNAs have been previously associated with drug resistance, and/or related molecular pathways, confirming the efficacy of this approach. Our results support the conclusion that miRNAs contribute to the acquisition of a drug resistant phenotype in neuroblastoma, and we are now working to elucidate the mechanism of miRNA involvement in the process of multidrug resistance.

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POT042

Expression of 14q32.31 microRNA cluster is a new biomarker of relapse in favourable neuroblastoma

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Background: Combination of age at diagnosis, stage and MYCN amplification, and genomic alterations, stratifies neuroblastoma into low- and high-risk groups. We aimed at establishing whether a miRNA signature could be associated with prognosis in both groups.

Methods: Microarray expression profiling let to select miRNAs for qRT-PCR analysis on a cohort of 226 patients. The relationship between miRNA expression and the overall (OS) or disease-free survival (DFS) was analysed using the log-rank test and the multivariable Cox proportional hazard model.

Results: The imprinted human 14q32.31 miRNA cluster was significantly down-regulated in the high-risk group. Multivariable analyses show 14q32.31 miRNA expression is an independent prognostic factor of the disease (OS and EFS). Importantly, miRNA expression of 14q32.31 locus is significantly associated with DFS of the non-MYCN-amplified favourable neuroblastoma, e.g. localized (stage 1, 2 and 3) and stage 4 of infant <18 months. As expected, the methylation status of this locus is inversely correlated with miRNA expression in tumors. Moreover the treatment of neuroblastoma cell lines with 5-aza-2'deoxycytidine or 4-Phenyl butyrate strongly increases the miRNA cluster expression.

Conclusions: Expression of 14q32.31 miRNAs shows predictive value beyond the classical high/low risk stratification and constitutes a biomarker of relapse in favourable neuroblastoma. The combination of this new biomarker with nonrandom chromosomal abnormalities is under investigation to provide its input on risk stratification of the disease. The mechanism of miRNA expression regulation is currently investigated using various neuroblastoma cell lines to elucidate the physio-pathological properties of this cluster.

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POT043

MIR-192 DIRECTLY REGULATES DICER1 IN NEUROBLASTOMA, LEADING TO A MORE AGGRESSIVE DISEASE

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Neuroblastoma (NB) arises from the embryonic neural crest and is the most common extracranial solid tumor in children under 5 years of age. microRNAs (miRs) are non-coding, single-stranded RNA molecules that base-pair with target mRNAs and negatively regulate their stability and translation efficiency. Dicer1 together with Drosha, catalyze the sequential cleavage of miR maturation.

We studied the involvment of Dicer1 and miRs that regulate Dicer1 in 47 NB. Low levels of Dicer1 expression correlated with poor outcome (p=0.032). One of the miRs that were predicted to target Dicer1 was miR-192. Overexpression of miR-192 correlated with poor outcome (p=0.028). Multivariate analysis identified miR-192 as an independent prognostic marker, with an increased risk to relapse of 3 fold in NB patients (p=0.04). A significant inverse correlation was detected between miR-192 and Dicer1 expression levels (p=0.018). Overexpression of miR-192 significantly reduced the expression of Dicer1 levels (p=0.001) in NB cell lines, suggesting that miR-192 regulates Dicer1. Indeed, we were able to show that miR-192 directly binds the 3' UTR of Dicer1. Furthermore, overxpression of miR-192 resulted in a significant increase in cell proliferation (p=0.044) and in the migration ability of NB cells (p=0.00036).

Our findings suggest that miR-192 is a novel regulatory oncomiR in NB, that regulates Dicer1 expression, leading to a more aggressive disease.

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POT044

In vivo modelling of early growth and spread of embryonic neural tumors.

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Background: Key knowledge is still missing for the successful treatment and cure of embryonic neural tumors, including neuroblastoma. Traditionally, rodent xenograft models recently supplemented with transgenic models, have supplied a basis for in vivo pre-clinical exploration of new therapies. Existing animal models entail however either the study of non-human cancer, or human cancer in a non-human environment, i.e. conditions that do not fully mimic the circumstances in the patient. For optimization of novel candidate treatments, improved pre-clinical prediction of the efficacy in patients is needed.

Methods: Pluripotent stem cells (PSC) were used to develop a human embryonic in vivo microenvironment in mice. Such in vivo growth of PSC is commonly referred to as benign teratoma, but could also be described as a failed embryonic process. Although the structural organisation is chaotic by nature, common findings in mature teratomas are areas of advanced tissues and even organoid embryonic structures, with striking similarities to human development including strong components of neural differentiation [PLoS One 6(11):e27741 2011]. Such substitute human embryonic environment was used for injections of fresh or frozen clinical tumor samples or cell lines of human embryonic neural tumor origin, and engraftment compared to xenografts and to information on original tumor from the patient.

Results: Successful engraftment was obtained using fresh or frozen material from childhood embryonic neural tumors as well as cell lines. Histology and immunohistochemistry, using a large panel of markers, revealed several differences between the models tested.

Conclusion: This model opens up for an in vivo analysis of early growth and spread, including migratory characteristics, of embryonic neural tumors in a developing human neural environment. Thus providing an opportunity to an experimental set up functionally representing developing neuroectodermal tissues, potentially with similarities to that in the young developing patient.

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POT045

Tumor-associated macrophages promote neuroblastoma growth through upregulation of MYC and COX2 expression in a novel murine neuroblastoma model.

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Background: Tumor-associated inflammation predicts poor outcome in children with high risk MYCN non-amplified neuroblastoma (NBL-NA). The mechanisms responsible for this effect are largely unknown.

Methods: Growth kinetics of tumors (n=22) from a novel murine transgenic neuroblastoma model (NBL-Tag) were assessed by MRI. Gene expression profiles from tumors of this large T-antigen driven murine model were compared with those of human neuroblastomas using Affymetrix microarrays. Expression of inflammation-related genes in the murine neuroblastomas and normal age-matched adrenal glands were analyzed in 4, 8, 12, and 16 week-old mice (n=3 per group) using mouse immune-arrays. Frequency and characteristics of tumor-associated macrophages (TAM) cells were analyzed using flow cytometry and cytokine profiling. The effects of murine peritoneal macrophages on NBT2 cells (NBL-Tag tumor-derived) were assessed using proliferation (BrdU) and protein assays, and gene expression profiling (Nanostring-Arrays).

Results: Global gene expression profiling reveal close clustering of NBL-Tag and human neuroblastomas. The tumors of NBL-Tag mice are detected by MRI only after 12 weeks of age, which coincide with IL6 levels becoming detectable and increasing in blood. NBL-Tag tumors show high IL6 expression, and infiltration by IL6-producing TAMs. NBL-Tag adrenal glands show age-dependent increase in expression of monocyte chemotactic protein 1 and fibronectin starting at 4-6 weeks prior to detection of tumors by MRI. In vitro co-culture of macrophages and NBT2 cells increases tumor cell proliferation by 50%. This effect does not require cell-cell contact and is associated with a four-fold and two-fold increase in COX2 and c-MYC protein expression, respectively. Gene expression profiles of the co-cultured cells show alterations in the CCR5/CCR7 pathway and genes associated with M2-like polarization of macrophages.

Conclusions: Alterations in inflammation-related genes occur early in the development of NBL-Tag tumors and are associated with tumor growth in vivo and IL6 production by TAMs. Macrophage and neuroblastoma cross-talk leads to proliferation of tumor cells and associated with induction of COX2 and MYC.

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POT046

Minimal residual disease monitoring in neuroblastoma patients by a set of real-time RT-PCR markers

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Background: Minimal residual disease (MRD) is derived from tumor-initiating cells (TICs) consisting of only a small population of tumor, and its activation leads to tumor relapse. Because over 50% of high-risk neuroblastoma patients experience tumor relapse that is lethal in most cases, accurate MRD monitoring is essential for their better outcome. To overcome tumor heterogeneity and achieve sensitive detection, several sets of multiple real-time RT-PCR markers for MRD monitoring in neuroblastoma patients have been reported from different centers.

Methods: To determine previously reported 11 real-time RT-PCR markers (CHRNA3, CRMP1, DBH, DCX, DDC, GABRB3, GAP43, ISL1, KIF1A, PHOX2B, TH) expression in bone marrow (BM), peripheral blood stem cell (PBSC), and peripheral blood (PB) samples, we first generated standard curves of the 11 markers by serially diluting neuroblastoma TICs into normal PB and then deduced normal range for each marker. Neuroblastoma TICs were isolated as spheres of neuroblastoma BE(2)-C cells and normal PB was taken form healthy adult volunteers. Based on the standard curves, the 11 markers expression was quantitated in 29 BM, 4 PBSC, and 1 PB samples obtained from 8 neuroblastoma patients treated at Hyogo Children's Hospital between February 2011 and January 2012. MRD was scored positive when one of the 11 markers expression exceeded normal range.

Results: MRD was positive in 100%, 71%, 67%, and 70% of BM cytology-positive (n=1), elevated VMA (>15μg/mgCr, n=7), elevated HVA (>30μg/mgCr, n=6), and elevated NSE (>20ng/ml, n=10) samples, respectively. MRD was also positive in 57%, 54%, 56%, and 52% of BM cytology-negative (n=28), normal VMA (≤15μg/mgCr, n=26), normal HVA (≤30μg/mgCr, n=27), and normal NSE (≤20ng/ml, n=21) samples, respectively.

Conclusion: MRD quantitation based on a set of the 11 real-time RT-PCR markers may achieve accurate MRD monitoring in neuroblastoma patients treated at a single center.

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POT047

GENETICALLY VERIFIED TUMOR CELLS IN THE BONE MARROW OF PATIENTS WITH LOCALIZED NEUROBLASTOMA – FREQUENCY AND CORRELATION WITH KNOWN RISK FACTORS

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Background: By definition, localized tumors have no tumor cells in the BM based on cytomorphological examination. Applying an ultrasensitive and specific technique able to confirm the neoplastic nature of GD2 positive cells, we investigated BMs of INSS stages 1 to 3 NBs to evaluate the actual frequency of tumor cells in the BM.

Methods: 176 patients with localized NBs were consecutively registered in Austria (1987-2008). The tumors were analyzed by FISH, by a multilocus technique (MLPA) and by FCM. BMs were tested with a fully automatic fluorescence based device combining GD2 immunocytology and molecular-cytogenetics (automatic immunofluorescence plus FISH, AIPF). The minimal analyzed MNC number was 3x10e6.

Results: From 176 patients with localized disease, 6 patients died of disease (4 with MNA tumors; median observation time: 6.5 years). The AIPF method verified tumor cells in 9.5% (12/126) of patients with available and evaluable BMs (0.26 to 200 in 10e6 MNCs; 2 stage 1, 3 stage 2, 7 stage 3). 11 of them were below 18 months and all but one (with an MNA tumor) are alive and in CR (four without chemotherapy). Positive BMs were more frequently found in unresectable tumors (25% vs 7.4%; p=0.049), in MNA tumors (18.8% vs 8.6%), in patients below 18 months (11.6% vs 3.2%) and with increasing frequency from stage 1 to 3 (4.4%, 9.4%, 14.9%). However, neither a correlation with segmental chromosome aberrations (22.2% vs 29.4%) nor with survival was found for patients with tumors lacking MNA (5-years EFS 0.89+0.1 vs 0.93+0.03; p=0.42).

Conclusion: Verified BM positivity in localized NBs is rare. In this patient cohort, it occurred especially in patients below 18 months, independent of the presence of SCA and - in case of a normal MYCN status - had no influence on the patients' outcome within the context of the respective trial treatments.

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POT048

Alternatively spliced NKp30 isoforms affect outcome in patients with metastatic neuroblastoma and minimal residual disease after induction chemotherapy

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Background: Neuroblastoma (NB) is sensitive to natural killer (NK) cells cytotoxicity. NKp30 is a natural cytotoxicity triggering receptor with three different splice variants and functional immune differences: NKp30a isoforms display the highest cytotoxicity while NKp30b or

c isoforms have respectively Th1 cytokines or IL-10 secretion with defective cytotoxic activity.

Aim: To evaluate NKp30 splice variants in metastatic NB (HR-NB) patients and their influence on disease prognosis.

Methods: Patients treated in Gustave Roussy Institut for a HR-NB underwent NKP30 genotyping by quantitative RT-PCRs in peripheral mononuclear blood cells. Relatives ratios, defined as the amount of each isoform related to the other ones were correlated with progression-free survival (PFS). Two groups were defined according to the median (higher/lower). Transfected NKL with each NKp30 isoform were also co-cultured with NB cell line NB8. NKP30 ligand (B7H6) expression at baseline and after co-culture was monitored on NB cells by FACs analysis.

Results: Among the 110 HR-NB patients, NKP30 predominant isoforms were distributed as follow: 28% NKP30a, 40% NKP30b and 32% NKP30c . NKP30 isoforms at diagnosis did influence neither response to treatment nor outcome. By contrast, in 70 patients with minimal residual disease after induction chemotherapy, those with a high Nkp30c/NKP30b ratio (n=36) had a higher rate of progression when compared with those with a low Nkp30c/NKP30b (n=34) ratio (3 years-PFS: 15% versus 60% p value= 0.01). Moreover, B7H6 expression on NB cells was significantly up-regulated after co-culture with NKL expressing the immuno-stimulatory NKP30a and b isoforms when compared with those expressing the immunosuppressive NKP30c isoform, indicating a sensibilization to NK lysis due to induction by NKp30a and/or b isoforms.

Conclusion: NKP30 immunogenetics represents a novel prognostic marker in patients with metastatic NB. These findings highlight the importance of immune cells, particularly NK, in elimination of minimal residual disease in NB. Finally, drugs that modulate alternative splicing of NKp30 receptor may represent a new therapeutic approach in NB.

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POT049

A three-gene expression signature model for risk stratification of patients with neuroblastoma

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Background: Neuroblastoma is an embryonal tumor with contrasting clinical courses. Despite elaborate stratification strategies, precise clinical risk assessment still remains a challenge. The purpose of this study was to develop a PCR-based predictor model to improve clinical risk assessment of neuroblastoma patients.

Methods: The model was developed using real-time PCR gene expression data from 96 samples, and tested on separate expression data sets obtained from real-time PCR and microarray studies comprising 362 patients.

Results: Based on our prior study of differentially expressed genes in favorable and unfavorable neuroblastoma subgroups, we identified three genes, CHD5, PAFAH1B1 and NME1, strongly associated with patient outcome. The expression pattern of these genes was used to develop a PCR-based single score predictor model. The model discriminated patients into two groups with significantly different clinical outcome (Set 1 5-year overall survival [OS]:0.93±0.03 vs 0.53±0.06, 5-year event free survival [EFS]:0.85±0.04 vs 0.042±0.06, both P<0.001; Set 2 OS:0.97±0.02 vs 0.61±0.1, P=0.005, EFS:0.91±0.8 vs 0.56±0.1, P<0.001 and Set 3 OS:0.99±0.01 vs 0.56±0.06, EFS:0.96±0.02 vs 0.43±0.05, both P<0.001). Multivariate analysis showed that the model was an independent marker for survival (P<0.001, for all). In comparison with accepted risk stratification systems, the model robustly classified patients in the total cohort, and in different clinically relevant risk subgroups.

Conclusion: We propose for the first time in neuroblastoma, a technically simple PCR-based predictor model that could help refine current risk stratification systems.

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POT050

CREB-binding protein regulates Ku70 acetylation in response to ionization radiation in neuroblastoma

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Background: Ku70 was originally described as a nuclear DNA repair factor, but it also functions in the cytoplasm as an anti-apoptotic protein by binding to Bax, blocking Bax-mediated cell death. In neuroblastoma cells, Ku70's binding with Bax is regulated by Ku70 acetylation such that increasing Ku70 acetylation results in Bax release, triggering cell death. While regulating cytoplasmic Ku70 acetylation is important for cell survival, the role of nuclear Ku70 acetylation in DNA repair is unclear. Here we demonstrated that Ku70 acetylation in the nucleus is regulated by the CREB-binding protein, and that Ku70 acetylation plays an important role in DNA repair in neuroblastoma cells.

Methods: We treated neuroblastoma cells (SH-SY5Y and SH-EP1) with ionization radiation and measured DNA repair activity (γH2AX levels) as well as Ku70 acetylation status using anti-pan-acetyl-lysine antibodies. The CREB-binding protein was knocked down in some cells using specific siRNA.

Results: Cytoplasmic and nuclear Ku70 were acetylated after ionization radiation in neuroblastoma cells. Interestingly, cytoplasmic Ku70 was redistributed to the nucleus following irradiation. Depleting the CREB-binding protein in neuroblastoma cells results in reducing Ku70 acetylation and enhancing DNA repair activity in neuroblastoma cells suggesting that nuclear Ku70 acetylation may have an inhibitory role in DNA repair.

Conclusion: These results provide support for the hypothesis that enhancing Ku70 acetylation, through deacetylase inhibition, may potentiate the effect of ionization radiation in neuroblastoma cells.

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POT051

Mechanisms of CHD5 Inactivation in Neuroblastomas (NBs)

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Background: NBs have genomic, biological and clinical heterogeneity. High-risk NBs are characterized by several genomic changes, including MYCN amplification and 1p36 deletion. We identified the chromatin-remodeling gene CHD5 as a tumor suppressor gene that maps to 1p36.31. Low or absent CHD5 expression is associated with a 1p36 deletion and an unfavorable outcome, but the mechanisms of CHD5 inactivation in NBs are unknown.

Methods: We examined 1) the CHD5 sequence in 188 high-risk NBs investigated through the TARGET initiative to identify coding sequence or splice site mutations; 2) the methylation status of the CHD5 promoter in 108 NBs with or without 1p36 deletion and/or MYCN amplification; and 3) mRNA expression of CHD5 and MYCN in 814 representative NBs using TaqMan low-density array microfluidic cards. We correlated expression with clinical (age, INSS stage) and biological (MYCN amplification, 1p deletion, 11q deletion, ploidy, histology) variables, as well as outcome (event-free survival and overall survival).

Results: We found no examples of somatically acquired CHD5 mutations, even in cases with 1p36 deletion, indicating that homozygous genomic inactivation is rare. Methylation of the CHD5 promoter was common in the high-risk tumors, and it was generally associated with both 1p deletion and MYCN amplification. High CHD5 expression was a powerful predictor of favorable outcome, and it showed prognostic value even in multivariable analysis after adjusting for MYCN amplification, 1p36 deletion, and/or 11q deletion.

Conclusions: We conclude that 1) somatically acquired CHD5 mutations are rare in primary NBs, so inactivation probably occurs by deletion and epigenetic silencing; 2) CHD5 expression and promoter methylation are associated with MYCN amplification, suggesting a possible interaction between these two genes; and 3) high CHD5 expression is strongly correlated with favorable clinical/biological features and outcome.

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POT052

The significance of Wip1 in neuroblastoma

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Background: In neuroblastoma (NB) 17q+ is the most powerful genetic predictor of adverse clinical outcome. We found aberrations of chromosome 17 in 85% in a national study of NB with 17q+ in 46% and whole 17 gain in 39%. Gain of 17q correlated with poor survival in our population-based material. In NB gain of the putative oncogene wild-type p53-induced phosphatase 1 (Wip1) at 17q23 is frequently detected. Wip1 is a serine/threonine phosphatase and was described as a gatekeeper in the Mdm2-p53 regulatory loop by promoting Mdm2-mediated proteolysis of p53.

Methods: Comparative genomic hybridization (CGH) was used to analyze primary NB tumors and cell lines. NB cells were examined for Wip1 expression using immunoblotting, immunohistochemistry and qPCR. Stable Wip1 knock-down NB cells were generated by shRNA transfections. H2AX phosphorylation and clonogenicity of Wip1 knock-down were examined using flow cytometry and clonogenic assay, respectively.

Results: Detailed CGH analysis revealed that Wip1 is present in at least one extra genomic copy in all 54 tumor samples containing 17q gain. Results from our wide range of NB cell lines demonstrate 17q aberrations in all samples. These findings have been confirmed by analyzing the expression pattern of Wip1 using different immunostaining techniques. Moreover, mRNA levels of Wip1 correlate to Wip1 protein expression. Transfection experiments with shRNA against Wip1 showed decreased cell viability, proliferation and colony formation in NB cells. In addition, substantial increase of gamma-H2AX expression after Wip1 knock-down compared to the non-silencing transfection was observed.

Conclusions: Our results showed that Wip1 is present in at least one extra genomic copy in the majority of primary NB. We identified NB cell lines expressing high, moderate and low levels of Wip1 where Wip1 knocked cells exhibited decreased cell growth and clonogenic capacity compared to non-silenced cells. Moreover, knock-down of Wip1 induced phosphorylation of histone H2AX, indicating a significant role of Wip1 in the DNA damage response of these tumors. Our data suggest that Wip1 expression may have significant oncogenic function in neuroblastoma development.

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POT053

Human Neuroblastoma Cell lines with the Alternative Lengthening of Telomeres (ALT) Phenotype Manifest High Levels of Cytotoxic Drug Resistance

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Background: Telomeres are terminal eukaryotic chromosomal elements that progressively shorten as a cell divides. Most cancer cells maintain telomeres to enable continual cell proliferation by expressing the ribonucleoprotein telomerase. A subset of cancers maintain telomeres by activating a telomerase-independent alternative lengthening of telomeres (ALT) mechanism. Recent reports suggest that 10-20% of neuroblastoma (NB) tumors appear to use the ALT mechanism and are associated with a significantly poor prognosis.

Methods: We screened a panel of 40 human NB cell lines for ALT by measuring mRNA expression of TERT (the catalytic component of telomerase) by RT-PCR and telomerase activity (TA) by RQ-TRAP. Telomere length (TL), MYCN amplification, MYCN, and c-MYC mRNA expression were measured by RT-PCR. The C-Circle (CC) telomere plasmid assay was performed by PCR. Cell line identities were confirmed using short tandem repeat (STR) genotyping. Response to cytotoxic drugs was assessed using DIMSCAN.

Results: Four of the 40 lines were found to have significantly low mRNA levels of TERT ($p<.05$): LA-N-6, SK-N-FI, CHLA-90, and COG-N-291; these 4 lines had 50 to 100-fold less TA when compared with telomerase-positive NB cell lines. The average TL in the four ALT lines was > 2-fold that of telomerase-positive NB cell lines ($p<.005$). All four ALT lines had telomere C circles (lacking in TA-positive lines). All ALT lines were MYCN non-amplified, and had low mRNA levels of MYCN and c-MYC when compared to MYCN-amplified and high c-MYC expressing NB lines, respectively ($p<.05$). All ALT lines were established at progressive disease and the concentrations cytotoxic to 90% of cells (IC90) were higher than those clinically achievable for melphalan (1.5-fold), etoposide (4-fold), topotecan (5-fold), and carboplatin (4-fold).

Conclusions: ALT-based telomere maintenance in NB is infrequent, and is associated with high levels of resistance to DNA-damaging chemotherapy. This may account for the poor prognosis associated with this subset of NB tumors. Characterization of the ALT phenotype in NB cell lines may provide novel insights into mechanisms of drug resistance.

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POT054

Tumor sphere specific CD133 regulation in neuroblastoma

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Background: A stem cell marker CD133 (Prominin-1) was expressed in several neuroblastoma (NB) cells at protein and mRNA levels. We previously reported that CD133 suppressed differentiation via RET suppression and p38MAPK and AKT phosphorylation in NB cells (ONCOGENE, 2011). However, the role of CD133 in stemness and its regulatory mechanism of transcription in NB tumor cells remain to be elucidated.

Methods: Gene knockdown and over-expression were performed by lentiviral systems in both NB cell lines and primary NB tumor sphere cells.

Results: CD133 was induced in tumor sphere-forming NB and brain tumor cells which are supposed to be including Tumor Initiating Cells. To study the regulatory mechanism of CD133 transcription in NB tumor sphere, we analyzed CD133 P1-P5 promoter activities. In CD133 highly-expressing adherent NB cells, CD133 was expressed by both P1 and P2 promoters. P2 promoter upstream of exon 1B was silenced in CD133 low-expressing NB cells by DNA methylation and 5-aza treatment cancelled the suppression. Intriguingly, CD133 was mainly transcribed by P1 promoter upstream of exon 1A in NB tumor sphere. ChIP analysis in NB tumor sphere indicated that histone H3K4 methylation and H3 acetylation were upregulated in P1 promoter region. Luciferase assay of P1 promoter denoted considerable transcriptional activity in NB cells. To identify the sphere formation-specific transcription factors, we examined RNA expression of several transcription factors in NB spheres. The expression pattern of the TFX1 transcription factor was correlated with that of CD133 during NB tumor sphere formation. Furthermore TFX1 directly bound to CD133 P1 promoter region and upregulated CD133 expression and NB sphere forming efficiency.

Conclusion: These results suggest that TFX1-related activation and epigenetic changes of P1 promoter region have a role in tumor sphere-specific CD133 expression which may contribute to maintenance of tumor initiating cells and their characteristics.

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POT055

TrkA3 Isoform Expression Upregulates Stem Cell Markers and Correlates with Worse Outcome in Neuroblastomas (NBs)

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Background: TrkA is the receptor for nerve growth factor (NGF). High TrkA expression is generally associated with favorable clinical outcome in NB. TrkA3 is an isoform with a deletion in the extracellular domain that causes constitutive activation. Here we studied the effect of TrkA3 expression on cell growth, morphology and expression of stem cell markers. We also determined the clinical significance of TrkA3 expression in primary NBs.

Methods: We stably transfected TrkA3 into SH-SY5Y, a Trk-null NB line. We determined the expression of neural markers (tyrosine hydroxylase, synaptophysin), a Schwann cell marker (S-100) and stem cell markers (Nestin, CD117, CD133 and SOX-2) using RT-PCR, Western blot and immunofluorescence. High TrkA expression was observed in 208 of 500 primary NB. Of the 208, 104 were selected to be clinically and biologically favorable (e.g., stage 1, no MYCN amplification), and 104 were unfavorable (stage 3 or 4, over 1 year of age). Validation of total TrkA and TrkA3 was determined by real-time RT-PCR (TaqMan). T-tests were used to compare expression between favorable and unfavorable groups.

Results: Constitutive expression of TrkA3 increased the expression of Nestin, CD117, CD133 and SOX-2. Increase in TrkA3 expression causes a change in cell morphology from neuronal to a more undifferentiated appearance. In the primary NBs, there was no difference in total TrkA expression between favorable and unfavorable groups (P=0.38). However, the mean expression of TrkA3 was significantly higher in the unfavorable high-TrkA group compared to the favorable high-TrkA group (P<0.0001).

Conclusions: Our data suggest that TrkA3 overexpression upregulates expression of stem cell-like markers and leads to a more undifferentiated morphology. This supports the association between TrkA3 expression and unfavorable features and outcome, which could result from reversion to a more undifferentiated, stem cell-like state.

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POT056

Side population analysis and gene expression profiling identify Notch pathway genes in neuroblastoma

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Background: High-risk neuroblastoma (NB) has a very poor prognosis. We previously investigated NB cancer stem-like cells using side population (SP) analysis of three pairs of NB cell lines derived from single patients at the time of diagnosis and after relapse. We reported that the proportion of SP cells in the relapsed lines was increased compared with its paired pretreatment line. Thus, we hypothesized that signaling pathways would be differentially expressed in the pre- versus post-relapse SP cells.

Methods: Gene expression profiling was assessed using Affymetrix arrays on the SP fraction of the cell lines SMS-KCN (pre-treatment) and SMS-KCNR (relapse). Gene expression changes of 9 Notch pathway genes were confirmed by RT-PCR in six paired cell lines (SMS-KCN and KCNR, SMS-KAN and KANR, and CHLA-122 and 136). Cell viability was analyzed using Vi-cell Beckman counter.

Results: 126 genes including members of the Notch signaling pathway were up-regulated at least 2-fold in the post-relapse SP cells compared to pre-relapse SP. Multiple notch genes were confirmed by qRT-PCR to be upregulated from ~1.5-fold (NOTCH1) to 15-fold (HES1) in the post-relapse compared to the pre-treatment SP cells, as well as in the unsorted post-relapse cell lines. Treatment of four cell lines with a γ-secretase inhibitor (DAPT, 5μM) resulted in >50% decrease in viability. Evaluation of the NCI NB gene expression database (http://home.ccr.cancer.gov/oncology/oncogenomics/) showed that increased expression of NOTCH1, 2, and 3 correlated with a worse clinical outcome compared to tumors with low Notch expression levels.

Conclusions: These results suggest that side population analysis coupled with gene expression profiling is a useful method for identifying novel pathways in NB and that upregulation of the Notch pathway may be important in the biology of NB cancer stem-like cells. Inhibition of the Notch pathway using γ-secretase inhibitors may provide new therapeutic options in NB.

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POT057

Phosphorylation status of Ascl1 regulates neuroblast self-renewal and differentiation

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Background: NB (NB) is a tumor arising from improper development of the sympathetic nervous system. High-risk forms, often associated with MYCN amplification, still face dire prognoses. Achaete-scute homolog 1 (Ascl1) is a proneural transcription factor responsible for inducing neuroblast differentiation. We hypothesized that phosphorylation status of Ascl1, by cyclin-dependent kinases, critically regulates its ability to induce differentiation in developing neuroblasts.

Methods: A phosphomtuant form of Ascl1 (Ascl1-6SA) where serines, of 6 putative Cdk phosphorylation sites, were mutated to alanine was generated. Ascl1-WT and Ascl1-6SA mRNA, alone or in combination with either Cdk2/Cyclin A or N-Myc, were injected into Xenopus laevis embryos and expression of downstream noradrenergic markers were assayed by in situ hybridization. SY5Y, SKNAS, and KCNR cell lines were treated with retinoic acid (RA) and/or Cdk inhibitors to determine Ascl1 phosphorylation status and neural differentiation.

Results: Ascl1-WT induces ectopic differentiation in Xenopus laevis which is inhibited when co-expressed with Cdk2/Cyclin A or MYCN. Ascl1-6SA is resistant to inhibition. In NB, Ascl1 was found to be expressed at the mRNA and protein level, associated with poor prognosis, and heavily phosphorylated in SY5Y, SKNAS, and KCNR cell lines. Low level treatment with Cdk inhibitors and RA causes increased differentiation in RA-susceptible cell lines, KCNR and SY5Y, and induces differentiation in RA-resistant SKNAS cells.

Conclusions: Ascl1 plays a vital role in noradrenergic neuron differentiation, however, Ascl1 is highly expressed in NB. We propose a model by where high Cdk activity maintains Ascl1 in a phosphorylated state, preventing Ascl1 from inducing neural differentiation. This provides a mechanistic tie between cell cycle exit and differentiation and suggests inhibiting cell cycle progression when combined with pro-differentiation drugs will be clinically active.

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POT058

A Pilot Study On The Use Of Metabolomics In Neuroblastoma: Preclinical And Patient Metabolite Biomarker Profiles

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Background: The current risk-group classification system used to determine therapy fails to accurately place a proportion of intermediate risk patients who subsequently are over- or undertreated. In addition, it does not identify the subset of high-risk patients who will not respond to therapy. Serum metabolomic analysis measures hundreds to thousands of small-molecule metabolites and generates a metabolic "fingerprint" of a patient. It has proven utility in several types of cancer. We hypothesize that serum metabolomic analysis will enhance accuracy of risk-group classification for NB patients.

Methods: In Vitro n-Myc status comparison: Supernatants from six NB cell lines(3 n-Myc amplified) were compared. Tumour detection in vivo: Flank tumors were established in Nu/Nu mice by injection of NB cell lines with varied levels of n-Myc amplification (IMR-32, SH-EP, SK-N-AS, 3-4 mice/group, 1x106 cells/ mouse). Serum was drawn pre-injection, at 1 week after injection when there was no visible tumour, and again once tumours were grossly visible (6-8 weeks after injection). Risk-group comparison from human patients: An institutional pilot study was carried out on 5 patients. Sera obtained during bulk disease or minimal disease (CR/VGPR) of the same patient was compared using metabolomic analysis. A second pilot study was done on COG tumour bank sera from10 patients (5 high-, 5 low-risk).

Metabolomic analysis: Supernatants or sera were analyzed by NMR

and/or GC-MS. Multivariate data analysis was conducted using SIMCA-P (Umetrics).

Results: Supernatant from n-Myc amplified cells showed a characteristic metabolomic signature. Mouse sera developed an identifiable metabolomic pattern before tumors were grossly visible (p=0.004) but could not detect differences in n-Myc status. The metabolomic fingerprint of patient sera with bulk disease or CR/VGPR was distinguishable, as was sera from high- and low risk patients.

Conclusion: Serum metabolomic analysis can distinguish several characteristics of NB. A larger retrospective analysis of COG banked sera is warranted.

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POT059

Comparison of DNA methylation markers in advanced stage, high risk Neuroblastoma patients

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Background: Most of the high risk, stage 4 Neuroblastoma (NB) patients experience a poor outcome despite novel multimodal therapeutic protocols, but the favorable outcome observed in a subgroup of patients at high risk indicates that additional prognostic markers must be identified and validated to assign the patients to the most appropriate risk category. Methylation of the Protocadherin B (PCDHB) cluster analyzed in a cohort of patients spanning from Stage 1 to Stage 4 seemed to be a really promising biomarker in a Neuroblastoma. We analyzed this biomarker specifically in stage 4 patients at high risk.

Methods: We developed a pyrosequencing assay to measure the methylation level of 17 genes of the PCDHB cluster and we analysed 106 tumors of high risk stage 4 NB patients comparing the results with stage 4 at lower risk and stage 1 at very low risk NB patients.

We assessed a multivariate analysis considering all clinically important parameters in stage 4, high risk NB patients such as MYCN amplification and including also the methylation of Stratifin (SFN) gene a biomarker statistically related to survival in this group of patients.

Results: DNA methylation of PCDHB cluster is lower in stage 1 respect to stage 4 patients, but in stage 4 high risk patients its predictive power is absorbed by other clinically relevant parameters while methylation of SFN gene appears as an indipendent predictor of outcome which identifies high-risk patients surviving more than 60 months with methylation levels comparable to tumors derived from lower risk patients.

Conclusion: Methylation level of PCDHB cluster doesn't act as a surviving biomarkers in high risk stage 4 NB patients and we hypothesized that a subset of patients considered at high risk—but displaying low levels of SFN methylation—could be assigned at a lower risk group.

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POT060

Development and Evaluation of Pharmacodynamic Biomarker Assays for Children with Neuroblastoma

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Background: Pharmacodynamic (PD) biomarkers provide proof-of-principle of target modulation and evaluate downstream effects of targeted therapeutics. Generally the tumour itself is the source of tissue for biomarkers, so repeat biopsies are required. We have reported PI3K/AKT/mTOR kinase pathway inhibition is a therapeutic strategy for MYCN-driven neuroblastoma. Development of PD biomarkers providing proof of PI3K pathway inhibition is essential for trials of agents targeting this pathway. We have developed strategies for analysis of PI3K pathway inhibition in surrogate -platelet-rich plasma (PRP) and neuroblastoma bone marrow (BM) cells.

Methods: For PRP assays, blood samples from 24 children with solid tumours (8 neuroblastoma) were collected and compared to adults. Total and phospho-protein signals for AKT, GSK3-β and p70S6K were analysed using MesoScale Discovery® technology, phospho-PRAS40 was measured using an ELISA.

For BM assays, pre-clinical assay development was by spiking blood with Kelly neuroblastoma cells and comparing two immunomagnetic (MACS MicroBead technology [Miltenyi Biotech]) separation strategies: CD45 depletion and GD2 positive selection. Eight BM from patients were analysed; purity (proportion neuroblastoma cells), recovery and total protein content before/after separation determined.

Results: PRP: Total AKT levels were comparable between adult and children but phospho-AKT was lower (3088±2699 vs. 7853±10056, p=0.03). Children had higher total and phospho GSK3- and p70S6K. Phospho-PRAS40 signals were also detectable.

CD45 depletion compared to GD2 positive selection achieved superior purity of tumour cells from bone marrow (85.0%±0.1 vs. 51.5%±0.3, p=0.04) and recovery (12.3%±2.6 vs. 1.5%±0.2 total cells recovered). CD45 BM depletion resulted in enriched cell suspensions (76.0%±20 purity neuroblastoma). Cell suspensions from BM contained sufficient protein for assays (722.2±347 micrograms of total protein).

Conclusions: The PRP assay is ready for phase I trial agents targeting the PI3K/AKT/mTOR pathway. Immunomagnetic separation with CD45 depletion of BM results in cell suspensions enriched for neuroblastoma cells and is ready for clinical pharmacodynamic biomarker assays.

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POT061

The epigenetic modifier CHAF1A regulates neuroblastoma differentiation and is a novel prognostic indicator for poor survival.

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Background: Novel approaches to identify high-risk neuroblastoma may improve risk stratification schemes and identify new therapeutic targets. We hypothesized that altered regulation of specific p53-mediated transcriptional targets contributes to the aggressive phenotype of high-risk neuroblastoma.

Methods: Microarray expression profiling and GSEA (Gene Set Enrichment Analysis) defined the transcriptional response to p53 activation upon MDM2 inhibition with Nutlin-3a. Multivariate logistic regression analysis identified a molecular signature for high-risk cases which was validated in multiple independent patients cohorts (n=888 total patients). Quantitative-PCR assays were used for validation and correlation with outcome in the SIOPEN cohort. Loss-of-function studies in neuroblastoma cell lines with inducible and stable CHAF1A knockdown were performed in vitro and in vivo. Gene expression profiling of knockdown and control cell lines was performed on the Affymetrix U133+2.0 arrays.

Results: We identified a novel risk classifier consisting of four genes (CHAF1A, RRM2, MCM3 and MCM6) transcriptionally repressed by p53 and over expressed in high-risk neuroblastoma, which strongly predicts poor survival (Progression-Free and Overall Survival, $p<0.001$) in 5 independent patient cohorts. Importantly, this predictive signature is independent of current risk stratification schema including MYCN, age and stage. A primary component of this signature is CHAF1A, a subunit of CAF1 (Chromatin Assembly Factor-1) which directly participates in histone recruitment and modification during DNA synthesis and repair. CHAF1A is highly over expressed in high-risk neuroblastoma and in vitro and in vivo silencing dramatically induces differentiation and reduces tumor growth and vascularity.

Conclusions: Cumulative expression of CHAF1A, RRM2, MCM3 and MCM6 represents a novel powerful prognostic gene signature for neuroblastoma independent of current risk classification schema. Furthermore, we provide a biologic rationale for the prognostic correlation of high CHAF1A expression with poor clinical outcome. Prospective evaluation of this signature is required to determine its potential impact on clinical risk assessment.

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POT062

In vivo and in vitro characterization of three radiolabeled anti-GD2 Antibodies to be used as Biomarkers for Neuroblastoma Imaging and Radioimmunotherapy

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Immunotherapy using GD2-specific-antibodies can improve the prognosis of neuroblastoma (NB) patients. Additionally, radiolabeled anti-GD2-antibodies may constitute a highly specific tool for diagnostic imaging and targeted radiation therapy. The ch14.18 mutant ch14.18-Δch2 (Δch2) is an anti-GD2-antibody with reduced immunoreactivity and might form an excellent biomarker for this approach. Here we study the in vivo and in vitro characteristics of this antibody and two clinically used anti-GD2-antibodies to evaluate their benefit for NB diagnostics and radioimmunotherapy (RIT).

For in vitro characterization of Δch2, ch14.18 and hu14.18, binding, blocking and internalization studies were accomplished. An unspecific antibody was used as control. Binding capabilities of I-123-Metaiodobenzylguanidine (mIBG) were studied in addition. Small animal PET was used to analyze the in vivo behaviour of the antibodies. Immunodeficient mice bearing subcutaneous NBs were injected with 20µg [Cu-64]DOTA labeled antibody. In vivo PET and MR images were acquired 3h, 24h, and 48h post injection. Ex vivo biodistribution and autoradiography studies were performed after 48h.

The in vitro binding of Δch2 is superior to that of hu14.18 and ch14.18. Internalization of Δch2 is highest among the antibodies. Blocking studies approve GD2-specificity for Δch2 and hu14.18. The three anti-GD2-antibodies show stronger binding to NB cells than mIBG and an unspecific antibody. The in vivo accumulation of the radiolabeled antibodies in GD2 expressing tumors is highest for Δch2. Tumor uptake of the unspecific control antibody is inferior in comparison to the anti-GD2-antibodies at all time-points. Biodistribution and autoradiography studies support these findings.

It is the first time that anti-GD2-antibodies were compared by in vivo PET. Our in vitro and in vivo data show improved binding and internalization capabilities of Δch2 in comparison to hu14.18, ch14.18 and mIBG. Due to these favourable binding characteristics, the low immunoreactivity and its small size Δch2 is a promising candidate for NB imaging and RIT.

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POT063

Safety profile of Radioimmunotherapy (RIT) in Patients with Central Nervous System (CNS) Neuroblastoma (NB)

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Background: Survival after relapse with CNS NB has improved with the addition of intrathecal antibody-based RIT using 131I-3F8 or 131I-8H9 targeting GD2 or B7-H3 (J NeuroOncology 97: 409-18, 2010). We now summarize RIT toxicity.

Methods: Patients with radiographic and/or pathologic confirmation of CNS NB received RIT under IRB approved MSKCC protocols (2000-2012). 111In-CSF flow and cytology studies, and MR of brain/spine were obtained. Following a 2 mCi dosimetry study, patients received serial 131I-3F8 (10 mCi 131I-3F8/injection, maximum 40 mCi) or 131I-8H9 (20-100 mCi, dose-escalated) via intraventricular catheters. Toxicity was per NCI CTCAE criteria. Through 2008, treatment was inpatient over 48 hours. After 2008, treatment was outpatient with daily follow-up x3 days, weekly x4 and then every ~3 months.

Results: 56 patients received 176 injections, 131I-3F8 (n=27) or 131I-8H9 (n=149). Mean follow-up was 27 mon (5 mon- 8 years). 2 patients (4%) needed catheter revisions for placement before treatment. 131I-3F8 was commonly associated with self-limited headache, vomiting and fever, but no grade >3 toxicities. Adverse events related to 131I-8H9 included grade 3 elevation ALT/AST (n=1, after 2nd injection), and grade 3/4 myelosuppression (n= 10, thrombocytopenia, neutropenia, lymphopenia and/or anemia). One patient had chemical meningitis (headache, vomiting and CSF pleocytosis over 72 hours after the 3rd injection), and another had fever/pneumonia. No deaths occurred on study. Although association with RIT is unclear given extensive prior chemoradiotherapy, long-term issues included secondary leukemia (n=2, one MLL-associated, one t(3,8)), hypothyroidism (n=5) and growth hormone deficiency (n=3) corrected by replacement.

Conclusions: RIT using 131I-3F8 or 131I-8H9 can be safely administered in the outpatient setting to children with CNS NB. For such patients, RIT is associated with acute and manageable toxicities. Transient headache and vomiting are common with 131I-3F8, and myelosuppression with 131I-8H9.

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POT064

Synthetic lethal siRNA Screening to Identify Novel Combinational Therapies with Topotecan in Neuroblastoma

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Despite advances in multimodal treatment, the outcome of neuroblastoma (NB) is still often fatal for children with advanced-stage disease. The topoisomerase-1 inhibitor topotecan is currently a mainstay for both up-front and salvage regimens for NB patients. High throughput loss of function siRNA screens have been used to identify genes whose inhibition sensitizes cell lines to drug treatment. The transmutation of these silenced-gene-drug interactions into potent synergistic drug-drug combinations for cancer therapy is the ultimate goal. In our previously published siRNA screen of 418 apoptosis-related genes in the NB cell line, SKNAS, inhibition of the NF-κB pathway emerged to be synergistic to topotecan treatment.

The aim of this current study is to expand the screen by including siRNAs against the human druggable genome in four NB cell lines, two MYCN-amplified (IMR5 and IMR32) and two non-amplified (SKNAS and NBEB) lines. We utilized the QIAgen human druggable genome kit containing 13910 siRNAs to target 6878 genes. Off-target binding of the seed region of siRNAs (2nd -8th base) to multiple gene transcripts is a major cause for false positives in these screens. Thus, we performed common seed analysis to eliminate siRNA exhibiting this effect. We then ranked the siRNAs by their activity applying the RSA algorithm and identified 150 genes whose silencing caused a significant decrease in cell survival in combination with topotecan in any of the four cell lines. This list was further filtered to contain only curated drug targets.

Furthermore, Haystack analysis allowed us to predict target genes based on the siRNA off-target effects in our screens. By this method, 39 additional transcripts whose 3'-UTR regions contain off-target binding sites for the seeds of multiple, active siRNAs were identified. Additionally, we picked transcription factors predicted to regulate our target genes by Ingenuity Transcription Factor Analysis.

These hits are currently being validated by testing 3 additional siRNAs. Once validated, the drugs that inhibit these gene protein products will be tested for synergetic activity with topotecan. Using this strategy we will identify novel combinational therapies for treating patients with currently incurable refractory or relapsed neuroblastoma.

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POT065

The effect of Nutlin-3 and Cisplatin in p53 wild-type and mutant neuroblastoma cell lines

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Background: Less than 40% of patients with high-risk neuroblastoma are long-term survivors. Therefore, novel therapeutic strategies are needed to increase the survival of these patients. p53 is the most commonly mutated gene in human cancer occurring in >50% of tumour types. In neuroblastoma, p53 mutations are rare, however p53 pathway inactivation through MDM2 amplification and inactivation of p14ARF has been reported. Therefore, reactivating wild-type p53 using MDM2/p53 inhibitors offers a novel therapeutic strategy for the treatment of neuroblastoma. The emergence of p53 mutations in vitro after treatment with MDM2/p53 inhibitors alone, suggests that combination with conventional chemotherapy may be more effective in circumventing resistance as well as reducing the toxicity of conventional therapies.

Aims: To undertake combination studies of Cisplatin and Nutlin 3 in neuroblastoma to determine whether there is synergistic activity

Methods: Cell viability was determined using the XTT cell proliferation assay. GI50 values (50% growth inhibition) of Nutlin-3 and Cisplatin were determined in 3 p53 wild-type and 3 mutant neuroblastoma cell lines including an isogenic pair of wild-type/mutant p53. Cell lines were then treated with a combination of Nutlin-3 and Cisplatin, and the median-effect-analysis of drug combinations was used to determine whether the interaction was synergistic in p53 wild-type cells.

Results: In comparison to p53 wild-type neuroblastomas cell lines, p53 mutant cell lines had an approximately 60 and 8 fold higher Nutlin-3 and Cisplatin GI50 value, respectively. Combination studies found that Nutlin-3 potentiates the cytotoxic killing effect of Cisplatin in p53 wild-type neuroblastoma cell lines but not in p53 mutant cell lines.

Conclusions: The data shows that Nutlin-3 is highly effective at killing neuroblastoma cells with wild-type p53 in vitro, and could be used in combination with existing chemotherapy agents such as Cisplatin in the treatment of neuroblastoma, in particular, to improve the survival of patients with high-risk disease and possibly reduce toxicity.

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POT066

Starvation cycles sensitize neuroblastoma to chemotherapy and retards its growth.

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Background: Short-term starvation (STS) provides protection to normal cells, mice, and possibly patients from a variety of chemotherapeutic drugs, but its effect on cancer cells is poorly understood. Aim of this study was to investigate the mechanisms of STS-dependent differential stress resistance and the therapeutic effects of STS in combination with different chemotherapeutic agents in experimental NB models.

Methods: In vitro cytotoxicity of STS + chemotherapy was tested by Trypan Blue, BromoDeoxiUridine, and MTT assays. In vivo STS protocol allowed mice to consume water but not food for 48-60 hours prior to chemotherapy. The in vivo therapeutic effects of STS + chemotherapy were evaluated in term of toxicity, tumor burden and survival in A/J mice injected with NB cells. The antitumor effect of STS alone was evaluated by measuring the volume of subcutaneous NB tumors. The effect of STS alone or in combination with chemotherapy on the regulation of Akt, mTOR, S6K, p38 MAPK, caspases and heme-oxygenase-1 (HO-1) in NB cell lines was evaluated by Western Blot.

Results: The reduction of serum and glucose sensitized NB cell lines to chemo-drugs by decreasing viability, blocking cell cycle S-phase and inducing cell death. Similarly, in vivo experiments demonstrated that STS in combination with chemotherapy decreased the toxicity and prolonged the survival of NB bearing mice. Reduced expression of phospho-Akt, HO-1 and increased levels of caspases 3 and 8 were involved in STS-mediated sensitization of NB cells to chemotherapy. In addition, we demonstrated that STS alone could reduce NB cell growth through downregulation of Phospho-Akt, Phospho-mTor, Phospho-S6K, p38-MAPK and HO-1. On going experiments showed that STS dramatically downregulated serum levels of proinflammatory cytokines.

Conclusions: These studies suggest that fasting has the potential to replace certain toxic chemotherapeutic drugs in the treatment of various tumors including NB and to promote chemotherapy-dependent Differential Stress Sensitization.

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POT067

Oligonucleotide-mediated gene targeting: a powerful technique for preclinical studies in cancer treatment

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Oligotargeting (gene targeting by single-stranded oligodeoxyribonucleotides (ssODNs)) emerges as an important tool for introducing subtle gene modification in mouse embryonic stem cells (mESCs) that can be used for the generation of mutant mice.

This can be useful for mimicking human diseases in mice and for pharmacological or toxicological studies in which a drug target sequence in humans differs from the homologous sequence in mice. E.g., MYCN plays an essential role in high-risk neuroblastoma and may be used as a target for therapy. However, studying the efficacy and toxicity of a drug targeting a specific sequence in human MYCN in mice, required replacement of a single-base-pair in murine Mycn.

Here we show the humanization of Mycn gene can be achieved by oligotargeting.

We designed an antisense ssODN to substitute G33 of the Mycn open reading frame for C. After transient suppression of MLH1 and transfection of the ssODN, mESCs were cultured in small pools of cells that were analysed for the presence of the desired modification. We developed a realtime PCR assay combined with MAMA assay to monitor the subcloning of correctly targeted cells from an excess of untargeted cells. The obtained pure mutant clone was used to obtain chimeric mice.

Amplification of mutant sequences was compared to amplification of wild-type sequences. During subcloning, the wild-type-to-mutant ratio of the seeded pools showed a strong enrichment of mutant cells. Sequencing of PCR-amplified genomic Mycn fragments from a pure cell clone confirmed the G to C substitution. We obtained chimeric mice with the same point mutation.

We demonstrate the possibility of oligotargeting to change a single basepair in the mouse genome and to obtain chimeric mice. Our technique allows routine substitution of any basepair or codon of interest in the mouse genome and is an important tool in modelling human cancer in mice.

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POT068

YM155 inhibits survivin-mediated survival, migration, and tumor growth in neuroblastoma

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Background: Neuroblastoma is the most common extracranial solid tumor in children, with poor prognosis in children diagnosed after 12-15 months of age. Even with current multimodal therapies, the 5-year event-free survival is below 50%, necessitating the development of new, targeted therapies. Survivin is a developmentally regulated gene that is highly upregulated in many cancers. In neuroblastoma, survivin is upregulated by PI3Kinase-AKT signaling and its expression correlates with poor prognosis. Survivin is a member of the Inhibitor of Apoptosis family of proteins and is involved in cell survival through caspase dependent and independent pathways. Survivin is also involved in mitosis at multiple checkpoints. Knockdown of survivin in melanoma cells reduces cell migration in vitro and its expression correlates with metastasis in Ewings Sarcoma.

Methods: Cell viability was determined by Calcein AM, following 48 hr exposure to YM155 alone or in combination with other chemotherapy. Cell migration was measured by wound assay over 20 hr ± YM155. Effects on tumor volume were determined by xenograft implant of neuroblastoma cell lines followed by treatment with YM155.

Results: Treatment with YM155 causes a dose-dependent decrease in survivin expression in neuroblastoma cells. YM155 reduces cell viability in neuroblastoma cell lines, however IC50 values vary by cell line and range from 50-500 nM. Low levels of YM155, without cytotoxicity, reduced cell migration 40-50%. Treatment with YM155 reduced tumor growth in vivo by 25-70%.

Conclusions: YM155 inhibits survivin expression, reduces cell viability, and migration in vitro, and reduces tumor volume in vivo, making it an attractive therapeutic agent in neuroblastoma.

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POT069

Therapeutic targeting of the DNA damage mediators Chk1 and Wee1 in neuroblastoma

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Background: High-risk neuroblastoma accounts for only 4% of all pediatric cancer diagnoses, yet despite intense therapies, results in 12% of pediatric cancer deaths. We previously identified checkpoint kinase 1 (Chk1) as a therapeutic target in neuroblastoma (PNAS, 2010), and single agent Chk1 sensitivity is likely due to myc-induced replication stress. In order to identify potential mediators we analyzed other pathway members, with Wee1 emerging as an additional target. Like Chk1, Wee1 is constitutively phosphorylated in many primary tumors, and RNAi depletion causes apoptosis in neuroblastoma cell lines. To translate these findings, we evaluated small molecule inhibitors of these kinases in pre-clinical models of neuroblastoma.

Methods: A panel of 10 human and 2 murine neuroblastoma cell lines were selected to screen for Wee1 (MK-1775) & Chk1 (SCH 900776) growth inhibition using the CellTiter-Glo viability assay. These cell lines were chosen to represent clinically relevant genomic variation in neuroblastoma. Single-agent efficacy was determined across a 6-log range, followed by combination indices with each other and with chemotherapy agents.

Results: Neuroblastoma cell lines were sensitive to single agent Chk1 and Wee1 inhibition and demonstrated synergistic cytotoxicity in combination with each other or chemotherapy, with the most potent combination being dual Wee1/Chk1 inhibition. In several representative neuroblastoma lines treated with SCH 900776 or MK-1775, we observed decreased phosphorylation of the respective downstream targets: Chk1(S296) and Cdc2(Y15). However, induction of DNA damage (via p-γH2A.X), was highest when the two inhibitors were combined.

Conclusion: Single-agent inhibition of Chk1 and Wee1 is significantly cytotoxic in neuroblastoma in vitro, and this is enhanced in combination with each other or chemotherapy at doses that are likely to be physiologically feasible. Current efforts are focused on optimizing combinatorial strategy in murine models, including biomarker determination for anti-tumor activity with the intention of translating these findings to early phase clinical trials.

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POT070

Targeted BCL2 inhibition effectively inhibits Neuroblastoma tumor growth

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Background: The intrinsic apoptotic pathway is at the core of cell faith determination. As a rule this signaling is strongly impaired in any tumor type.

Methods: We used high throughput mRNA expression and protein data to identify promising targets in the apoptotic signaling cascade. The BCL2 gene was further tested as potential drug target using various interference techniques and the ABT263 compound was validated in vitro and in vivo.

Results: We detected high BCL2 mRNA and protein levels in the majority of neuroblastoma tumors by Affymetrix expression profiling and Tissue Micro Array analysis. This BCL2 mRNA expression is strongly elevated compared to normal tissues and other malignancies. Most neuroblastoma cell lines lack this high BCL2 expression. Only two neuroblastoma cell lines (KCNR and SJNB12) show BCL2 expression levels representative for neuroblastoma tumors. To validate BCL2 as a therapeutic target in neuroblastoma we used lenti-viral mediated shRNA. Silencing of BCL2 in KCNR and SJNB12 resulted in massive apoptosis, while cell lines with low BCL2 expression were insensitive. Identical results were obtained by treatment of the neuroblastoma cell lines with the small molecule BCL2 inhibitor ABT263, which is currently in phase1/2 trials in adults. Combination assays of ABT263 with most classical cytostatics showed strong synergistic responses. Subcutaneous xenografts of a neuroblastoma cell line with high BCL2 expression in NMRI nu/nu mice showed a strong response to ABT263.

Conclusion: These findings are in contrast with previous reports on BCL2 inhibition in neuroblastoma cells. This might relate to the sub types of cell lines used. Our findings re-establish BCL2 as a promising drug target in neuroblastoma and warrant further evaluation of ABT263 and other BCL2 inhibiting drugs.

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POT071

Microsomal prostaglandin E2 synthase-1 is expressed in neuroblastoma and provides a novel specific therapeutic target

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Background: Neuroblastoma (NB) cells are enriched in the omega-6 fatty acid arachidonic acid, the substrate for the cyclooxygenase (COX) enzymes and prostaglandin biosynthesis. The inducible isoform, COX-2 is overexpressed in NB and NB cells produce prostaglandin E2 (PGE2) that acts as an autocrine and/or paracrine survival and proliferation factor. Downstream of the COX enzymes, specific synthases are responsible for the production of the respective prostaglandins. Microsomal prostaglandin E2 synthase-1 (mPGES-1) specifically converts PGH2 to PGE2 and is thought to primarily couple to COX-2. The aim of this study was to investigate if inhibition of mPGES-1 could represent an alternative therapeutic approach to COX- inhibition in NB.

Methods: Western blot and immunohistochemistry were used for protein detection. Cell viability of seven NB cell-lines treated with the mPGES-1 inhibitor CAY1052 was determined by MTT-assay. Stable mPGES-1 knockdown SK-N-BE2 clones were established using shRNA and the clonogenic capacity was analysed by clonogenic assay. To study the in vivo effect of COX inhibition, four week old homozygous TH-MYCN mice were treated with 10mg/L diclofenac for two weeks. Ex vivo analyses of COX-metabolites in tumors were performed by LC-MS/MS.

Results: Expression of mPGES-1 in our panel of human NB cell lines was detected. Inhibition of mPGES-1 reduced NB cell growth in vitro and knockdown of mPGES-1 significantly reduced the clonogenic capacity. Expression of COX-1, COX-2 and mPGES-1 in TH-MYCN tumors could be detected and treatment with the dual COX-inhibitor diclofenac significantly reduced tumour weight, compared to untreated animals. Ex vivo analysis of tumor tissues from treated animals revealed a significantly decreased level of COX metabolites compared to controls. The expression of mPGES-1was not affected by the treatment. Furthermore, cells staining positive for cleaved caspase-3 were more prevalent in treated tumors indicating apoptosis Induction.

Conclusion: We found that mPGES-1 is expressed in NB, with a potential role for PGE2 synthesis and tumor growth. mPGES-1 represents an alternative therapeutic target for inhibiting NB growth through specific PGE2 inhibition and the TH-MYCN model is well suited for in vivo studies with this purpose.

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POT072

Identification of Novel Anti-Cancer Agents Targeting the Neuroblastoma Kinome

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Background: Neuroblastoma is the leading cause of childhood cancer mortality in North America, necessitating the development of new and effective therapies. In this regard, kinase inhibitors that target ALK, Chk1, and PLK1, have shown promising results inhibiting neuroblastoma growth in preclinical models.

Methods: To identify novel anti-neuroblastoma agents, we screened a unique library developed by the Ontario Institute for Cancer Research of 300 compounds of high purity targeting human kinases, including compounds in clinical trails. IMR-32 and SK-N-BE(2) cells were seeded in 96 well plates, treated with compounds for 48 hours prior to a further 24 hour incubation in the presence of alamarBlue and subsequent fluorometric reading. Hits were defined as compounds that resulted in a signal decrease of > 45%.

Results: Using this screening approach, we identified a number of compounds that markedly decreased the viability of neuroblastoma cancer cell lines. Inhibitors of PLK1, Chk1, Cdks, and Aurora kinase, kinases that have been previously implicated in neuroblastoma pathogenesis, were found to be cytotoxic. In addition, inhibitors selective for Lim kinase-1 (Limk1), TGF-beta associated kinase1 (TAK1), and Ras-Erk-Net(Elk3) pathways identified these targets as potential novel regulators of neuroblastoma growth and survival. Secondary screens (cytotoxicity, apoptosis assays) confirmed TAK1 and Elk3 inhibitor activity in 4 neuroblastoma cell lines. We are currently validating these inhibitors, analogues, and additional hits, using a panel of 10 neuroblastoma cell lines with different genetic backgrounds (eg MYCN and p53 status), and by performing siRNA knockdown experiments of the candidate kinases. In vivo efficacy of prioritized hits is being tested using xenograft models.

Conclusion: Identification of novel compounds that target neuroblastoma cell survival by inhibition of specific kinases will help elucidate critical signaling pathways in neuroblastoma and lead to the development of new therapies.

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POT073

Dual Inhibition of Notch and VEGF Signaling Paradoxically Increases Liver Metastases in Experimental Neuroblastoma

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Background: Inhibitors of VEGF can suppress tumor growth in a numerous pre-clinical models, but are insufficient to prevent tumor progression. In attempt to increase efficacy, we used combined blockade of two angiogenic pathways, VEGF and Notch.

Methods: A novel soluble Notch1 decoy receptor (N1D) was used to inhibit Notch signaling. The neuroblastoma cell lines NGP and SH-SY5Y were engineered to express N1D, and were implanted intrarenally in athymic mice. Mice were treated with the anti-VEGF antibody (bevacizumab) and tumor growth monitored by bioluminescence. To further examine of Notch inhibition we used Notch decoys that selectively inhibit DLL4 or Jagged1. Microarray analysis was performed to examine the change in gene expression profile between metastatic cells and NGP-N1D.

Results: Dual inhibition of Notch and VEGF significantly reduced NGP (p=0.048) and SH-SY5Y (p=0.005) tumor growth. Surprisingly, dual inhibition resulted in markedly increased liver metastases in both NGP and SH-SY5Y. Immunohistochemical staining showed increased disruption of tumor vasculature with Notch-VEGF blockade. Measurement of circulating tumor showed no change among the groups suggesting that modulation of the liver niche is required for the phenotype. Using ligand specific Notch decoys, we found that blockade of both DLL4 and Jagged1 signaling isrequired for increased hepatic metastasis. Gene expression profile analysis revealed upregulation (fold change>2) of 38 genes during liver metastases.

Conclusion: Our study indicates that the inhibition of VEGF and Notch promotes hepatic metastatic spread in experimental neuroblastoma and identifies new potential targets for improved therapeutics.

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POT074

Potent antitumor activity of fenretinide/-LYM-X-SORBTM (4-HPR/LXS) oral powder in combination with ketoconazole and vincristine against recurrent neuroblastoma xenografts

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Background: Recurrent neuroblastoma poses a therapeutic challenge. Fenretinide (4-HPR) is a synthetic retinoid that acts in part via overproducing dihydroceramides achieved multiple complete responses in a phase I clinical trial in recurrent NB when formulated as an oral powder using the LYM-X-SORBTM (LXS) organized lipid matrix (4-HPR/LXS). We have shown that ketoconazole (keto) increased 4-HPR plasma concentrations in mice by > 2-fold over 4-HPR alone.

Methods: Human NB xenografts in nu/nu mice were established from patients with progressive disease, CHLA-119 and CHLA-90 (both TP53-mutant, multidrug-resistant lines), COG-N-415x, and Fu-NB-2006. 4-HPR/LXS was dosed at 240 mg/kg/day, keto at 38 mg/kg/day (both by oral gavagex5d/wk). Vincristine (VCR) was dosed at 0.5 mg/kg given twice a day every other week by intravenous injection. Tumor and plasma concentrations of 4-HPR and metabolites (4-oxo-4-HPR and 4-MPR) were quantified by HPLC and sphingolipids by MS/MS.

Results: In CHLA-119 tumors, 4-HPR/LXS + keto increased 4-HPR and 4-oxo-4-HPR tumor concentrations by > 2-fold over 4-HPR/LXS alone ($P<0.04$) and increased tumor concentrations of C16-dihydrosphingosine, total dihydroceramide, and total dihydro-1-ceramides over 4-HPR/LXS alone ($P<0.05$). In mice with CHLA-119 xenografts, keto increased the complete responses (CR) rate of 4-HPR/LXS from 1/10 to 5/10 mice. Combining VCR with 4-HPR/LXS increased mouse survival (CHLA-119m) to >300 day, with 50% of 4-HPR/LXS+keto+VCR mice surviving vs 38% with 4-HPR/LXS+keto ($P<0.01$) and 10% for VCR alone ($P<0.003$). Survival of COG-415x mice was for >180 days with 4-HPR+keto+VCR for 1/9 mice (11%) vs 0% for the other treatments ($P<0.001$). Survival of CHLA-90m mice was >147 days with 4-HPR/LXS+keto+VCR with 1/5 surviving (20%) vs 0% for VCR or 4-HPR/LXS+keto. In Fu-NB-2006 xenografts, 4-HPR/LXS+keto+VCR achieved 3 CRs, and 5/ (83%) of mice survived >113 days vs 0% of mice treated with for VCR or 4-HPR/LXS±keto.

Conclusions: These data support the ongoing NANT trial of 4-HPR/LXS + keto and suggest that a future clinical trial exploring 4-HPR+keto+VCR should be undertaken in patients with recurrent NB.

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POT075

AMXT-1501, a Novel Polyamine Transport Inhibitor, Synergizes with DFMO in Inhibiting Neuroblastoma Cell Proliferation by Targeting both Ornithine Decarboxylase and Polyamine Transport

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Background: Neuroblastoma (NB) is associated with MYCN oncogene amplification occurring in approximately 30% of NBs and is associated with poor prognosis. MYCN is linked to a number of genes including ornithine decarboxylase (ODC), the rate-limiting enzyme in polyamine biosynthesis. ODC expression is elevated in NB. Alpha-difluoromethylornithine (DFMO), an ODC inhibitor, is currently being used in a Phase I clinical trial for treatment of NB. However, cancer cells treated with DFMO may overcome their polyamine depletion by the uptake of polyamines from extracellular sources. A novel polyamine transport inhibitor, AMXT-1501, has not yet been tested on NB. We propose that targeting inhibition of ODC with DFMO, coupled with polyamine transport inhibition with AMXT-1501 will result in increased NB growth inhibition.

Methods: Independent and combination drug therapy was conducted in vitro on three NB cell lines. Calcein AM assay was used to assess cell viability and determine IC50 values. Western blot analysis was utilized to evaluate markers of proliferation and cytotoxicity. Proliferation inhibition was confirmed by the xCELLogence assay. HPLC analysis quantified intracellular levels of spermidine, spermine, and putriscine. CyQuant assays were combined to quantify intracellular levels of ATP.

Results: DFMO IC50 values ranged from 20.76-33.3 mM in NB. AMXT-1501 IC50 values ranged from 14.13-17.72 uM in NB. Combination treatment resulted in hypo-phosphorylation of pRb and increased expression of p27Kip1, suggesting cell growth inhibition. Combination treatment resulted in intracellular polyamine pool depletion as well as decreased intracellular ATP levels, further verifying growth inhibition.

Conclusion: With the current lack of effective therapies for relapsed/refractory NB patients and the preclinical effectiveness of DFMO + AMXT-1501, this combination has been advanced to study in xenograft models. DFMO and AMXT-1501 may be a potential new therapy for children with NB.

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POT076

A Sphingolipidomic Analysis of Neuroblastoma Tumors: Implications for Novel Therapeutic Targets

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Background: Bioactive sphingolipids such as ceramide and sphingosine-1-phosphate have been demonstrated to have important effects on cancer cell proliferation, differentiation, chemosensitivity, and apoptosis. In neuroblastoma, ceramides have been shown to stimulate differentiation of tumor cells, thereby decreasing their malignant potential. Recent studies implicated the amounts of specific ceramide species within tumors to be associated with tumor progression and responses to chemotherapy.

Methods: We studied whether the levels and types of sphingolipid species and the expression of sphingolipid metabolizing enzymes present in neuroblastoma tumor tissues correlate with neuroblastoma risk factors and prognosis. Sphingolipid levels (ceramide dihydroceramide, glucosylceramide, lactosylceramide, sphingomyelin species and sphingoid bases) were measured via liquid chromatography/mass spectrometry (LC/MS) in 69 neuroblastoma tumors from low/intermediate risk and high risk patients which were obtained from the Children's Oncology Group (COG) Neuroblastoma Tumor Bank and 40 ganglioneuromas obtained from the Cooperative Human Tissue Network. The mRNA levels of 26 key sphingolipid enzymes were examined using quantitative real-time PCR using custom designed PCR array plate (SABiosciences) in 106 tumor samples from low/intermediate risk and high risk patients and 29 ganglioneuromas.

Results/Conclusions: In general, much higher sphingolipid levels and gene expression were found in neuroblastomas than in ganglioneuromas. Within the cohort of neuroblastoma patients, we found strong associations between higher levels of some sphingolipids with favorable neuroblastoma risk factors, particularly MYCN non-amplification with ceramide, dihydroceramide, and sphingomyelin classes. Furthermore, the levels of C20:0 and C20:1 ceramides and C18:0 dihydroceramides were shown to be highly related with INSS stage, MYCN status, and COG risk group. Most importantly, lower levels (≤median value) of C18:0 ceramides (p=0.026), C18:1 ceramides (p=0.03), and C18:0 dihydroceramides (p=0.0041) were associated with adverse survival as compared with higher levels.

Conclusions: These results are in agreement with recent studies implicating C18 ceramide as a "tumor suppressor" lipid and key function of ceramide synthases.

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POT077

Novel Dihydroceramide Desaturase Inhibitors for Neuroblastoma Therapy

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Background: Sphingolipids play important roles in regulation of cell cycle, stress responses, apoptosis, inflammation, and cell migration. Our group has demonstrated that inhibition of dihydroceramide desaturase (DES-1), a key enzyme in sphingolipid metabolism, is associated with cell cycle arrest and growth inhibition. Fenretinide induced cytotoxicity in neuroblastoma is part mediated by DES-1 inhibition. Thus, development of novel DES-1 inhibitors with low cell toxicity and greater efficacy is crucial.

Methods: 17 potential sphingolipid based DES-1 inhibitors were screened using a rat liver microsomal in-vitro assay for DES-1 activity. Of these 3 compounds were found to inhibit DES. They were: LCL-235, LCL-47, and LCL-447.

Results: LCL-235, an 8 carbon ceramide analogue, was the most potent of the compounds studied thus far. It inhibited DES-1 activity in a dose dependent manner better than the retinoid DES-1 inhibitors 4-HPR and 4-OXO-4-HPR with an IC50 value of 0.579 μM versus 2.32 and 1.68 μM for 4-HPR and 4-OXO-4-HPR. In addition, LCL-235 was as cytotoxic as 4-HPR in MTT viability assays using SMS-KNCR cells. The compounds LCL-47 and LCL-447 were weak inhibitors as they were effective only at much higher concentrations with IC50 values of 38.5 and 32.5 μM. In order to determine the reversibility of the compound LCL-235, a dilution was performed, by incubation of 100X concentrated protein with the inhibitor in a concentration equal to 100X of its IC50 value. The test showed a rapid recovery of enzyme activity after dilution of 100X concentrated protein with other assay components including the substrate, indicating that the action of LCL-235 on DES-1 activity was reversible. Additionally, incubation with different concentrations of LCL-235 at different time points did not show time-dependent inhibition, supporting its reversible action. Furthermore LCL-235 inhibited DES-1 activity in SMS-KCNR cell extracts by 95%.

Conclusions: DES inhibitors are promising agents for neuroblastoma treatment.

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POT078

Inhibition of Sphingosine Kinase 2 in Neuroblastoma

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Background: Sphingosine-1-phosphate (S-1-P) is a bioactive sphingolipid which stimulates cell proliferation and is involved in angiogenesis and inflammation. S-1-P is generated exclusively by the action of sphingosine kinases. In mammals, two isoforms of sphingosine kinase (SK1 and SK2) have been cloned and characterized. Sphingosine kinase is an attractive target for cancer treatment because its product, S-1-P inhibits cell proliferation and induces apoptosis in cancer cells. ABC294640 is a novel SK-2 inhibitor that has been demonstrated to inhibit the growth of breast cancer, hepatocellular carcinoma, pancreatic adenocarcinoma, and renal carcinoma in vitro and in-vivo in mouse xenografts. It has not been tested in any pediatric cancers.

Methods: SMS-KCNR neuroblastoma cells were treated with increasing concentrations of ABC294640 and chemotherapeutic agents such as vincristine, doxorubicin, cisplatin, etoposide, and cyclophosphamide. Cell viability and growth were measured by MTT assays and trypan blue staining. Combined drug effects such as synergy or antagonism were determined using the median effect method of Chou and Talalay. Measurement of endogenous sphingolipids was performed by LC/MS.

Results: Treatment with ABC294640 decreased neuroblastoma cell viability in a dose dependent manner. There were modest increases in endogenous dihydroceramides as measured by LC/MS, indicating that dihydroceramide desaturase enzyme was also inhibited.

Conclusions: Targeting the SK2 enzyme with the inhibitor ABC294640 lead to the inhibition of neuroblastoma cell growth. Modulation of sphingolipid signaling pathways may provide an effective approach for neuroblastoma treatment.

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POT079

Low-dose aspirin treatment targets tumor-associated inflammation and inhibits aggressive neuroblastoma tumor growth in vivo

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Background: Tumor-promoting inflammation is considered an important enabling characteristic for all different hallmarks of cancer and a driving force in several adult malignancies, for which intake of low-dose aspirin has proven to reduce cancer incidence. However, remarkably little is known about tumor-associated inflammation in pediatric neoplasms and no in vivo data exists on the effectiveness of low-dose aspirin on established tumors. Our study evaluates inflammatory patterns paralleling neuroblastoma (NB) tumor growth in vivo and low-dose aspirin as a potential novel therapeutic option for high-risk NB.

Methods: Spontaneously arising tumors in the well characterized transgenic TH-MYCN NB mouse model were monitored for tumor-associated inflammation at various stages of disease using flow cytometry, immunohistochemistry and qRT-PCR. Homozygous pups received daily low-dose aspirin using oral gavage (10mg/kg, n=8) or no treatment (n=15), from the age of 4.5 weeks to 6 weeks of age.

Results: Ex vivo analysis of tumors revealed a transition from an adaptive immune response predominated by CD8+ T-cells in neoplastic lesions from 5 week old homozygous mice, towards enrichment in immature cells of the innate immune system, including myeloid-derived suppressor cells, dendritic cells (DCs), and tumor-associated macrophages (TAMs) during tumor progression. An M1 to M2 transition of TAMs was demonstrated, paralleled by a deterioration of DC status. Ten days of anti-inflammatory treatment with low-dose aspirin significantly reduced tumor burden ($p<0.01$) and the presence of tumor-associated cells of the innate immune system ($p<0.01$).

Conclusions: Tumor-associated inflammation progresses during NB growth and anti-inflammatory treatment with low-dose aspirin reduces tumor burden in the highly aggressive TH-MYCN transgenic mouse model of NB, suggesting a new treatment option for high-risk NB patients.

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POT080

TrkB/Akt: Potential targets of purine scaffold Hsp90 inhibitors for neuroblastoma (NB)

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Background: Expression of brain-derived neurotrophic factor BDNF and its tyrosine kinase receptor TrkB are associated with high risk NB. TrkB activates the downstream PI3K/Akt pathway and promotes chemotherapy resistance. Hsp90 inhibitors (PU-H71 and DZ-13) with high affinity for Hsp90 are potent anti-cancer agents.

Methods: Inhibition of cell proliferation was tested in 12 human neuroblastoma cell lines (SK-N-BE(1)N, IMR32, SH-SY5Y, LAN1, SK-N-BE(2)N, SKNAS, SKNJC2, NB1691, NGP, LS, SHEP1 and SKNLP) using WST-8 colorimetric assays following a 72-hour drug exposure. IC50 was calculated using SigmaPlot software. TrkB was induced in SH-SY5Y cells by all-trans retinoic acid. Interaction between Hsp90 and TrkB, and the effects on downstream signaling were studied by affinity pulldown using PU-H71-coupled beads, followed by western blotting.

Results: IC50 for PU-H71 on 12 NB cell lines ranged from 155 to 823 nM (271 ± 184, median 195 nM), and 56-548 nM (163 ± 132, median 118 nM) for DZ-13. TrkB expression was induced in SH-SY5Y after exposure to 8 μM retinoic acid for 5-7 days, and confirmed by western blot. PU-H71 (or control drug) affinity beads were used to isolate Hsp90-associated protein complexes. Among these Hsp90-associated proteins, TrkB and Akt were identified by western blotting. Treatment of SH-SY5Y cells with increasing concentrations of PU-H71 for 24 and 48 hours was associated with down-regulation of TrkB expression and its downstream signaling protein p-Akt.

Conclusions: NB is sensitive to Hsp90 inhibitors, and TrkB/Akt is a target. These inhibitors may have potential for the subset of high risk neuroblastoma where TrkB plays a critical role.

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POT081

Inhibition of MYCN-Max signaling with a small molecule induces apoptosis and TrkA-mediated differentiation in human neuroblastoma

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Introduction: MYC expression is deregulated in a wide range of human cancers, and targeting of Myc hence represents a promising therapeutic approach. In neuroblastoma, MYCN-amplification is strongly related to poor clinical outcome, with low survival rates despite novel advances in treatment strategies. An alternative treatment option for children with MYCN-amplified neuroblastoma is therefore urgently needed. Here, we present data on one small molecule that selectively targets neuroblastoma cells with high MYCN expression.

Methods: The in vitro outcome of treatment with the molecule on cell death induction and differentiation was evaluated in MYCN-amplified and non-amplified neuroblastoma cell lines. The effect on MYCN/Max interaction was studied by co-immunoprecipitation and proximity ligation assays. The in vivo efficacy was analyzed in the TH-MYCN-driven transgenic mouse model of neuroblastoma. To assess global changes in protein expression a quantitative mass-spectrometry based proteomic analysis was carried out.

Results: We observed a concentration-dependent cell cycle arrest and apoptosis induction in MYCN-amplified cells upon treatment with the small molecule whereas no significant effect was found in non-amplified cell lines. Furthermore, treatment of MYCN-amplified cell lines resulted in reduced MYCN-Max interaction followed by a decrease in MYCN protein levels. Importantly, prolonged incubation resulted in morphological and biochemical differentiation, including neurite outgrowth and up-regulation of the neurotrophin receptor TrkA. In addition, we found that survival of transgenic TH-MYCN mice was significantly enhanced in treated as compared to control animals. Proteomic analysis confirmed MYCN as the most significantly inhibited transcription factor.

Conclusions: We provide proof of concept that small molecules, which interfere with MYCN/Max signaling, could be beneficial for the treatment of neuroblastoma. Importantly, such molecules may also be used as tools to identify novel critical MYCN-regulated pathways that could serve as targets in this pediatric cancer.

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POT082

Genetic alterations in neuroblastoma associated with opsclonus myoclonus syndrome

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Background: Opsoclonus-myoclonus syndrome (OMS) is observed in 1-2% of patients with neuroblastoma (NB), and nearly always associated with favorable clinical features. OMS is probably triggered by an abnormal immune response, but the tumor antigen potentially responsible for this immune response remains elusive. In NB with a good outcome, numerical chromosome alterations (NCA) are more frequent, whereas NBs with a poorer outcome are associated with segmental chromosome alterations (SCA).

Methods: The genomic profile of 35 NBs associated with OMS was studied using an in-house BAC/PAC array (n=10), Nimblen®75k array (n=4), Agilent® 1Mb (n=1) or 105k array (n=20). Seventeen patients had stage INSS 1, 14 stage 2, 3 stage 3 and 1 stage 4 disease. Median age at diagnosis was 21 months (range 2-47 months), with a median follow-up of 64 months.

Results: Genomic profiling showed NCA in 14 cases (40%) and SCA in 21 cases (60 %). No tumor had MYCN amplification, but one case had an amplicon of chromosome 12q13.3, encompassing the STAT6 and CDK4 genes. The frequency of SCA in NB with OMS was not significantly different from that of patients with localized NB of the same age group (60 vs 45%,NS). The search for recurrently altered chromosome regions showed a small interstitial region on chromosome 10q deleted in 8/ 21 SCA cases, harboring amongst others the GPRIN2 gene which plays a role in neurite growth. No tumor-related event was observed. Although over half of all children had neurological sequelae, no correlation was observed between genomic profile and neurological outcome.

Conclusion: These data indicate that the genomic profiles of NB with OMS are not significantly different from NB without OMS. The observation of an excellent outcome even in cases with SCA underline the hypothesis that an immune response might be involved in tumor control in NB with OMS.

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POT083

The correlation between the number of segmental chromosome aberrations and the age at diagnosis of neuroblastomas with or without MYCN amplification

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Background: In neuroblastoma (NBs) without MYCN amplification, the segmental chromosome aberrations (SCAs) such as 11q loss and 17q gain were suggested to be associated with the prognosis of the patients. We assessed the correlation between the number of SCAs and the age at diagnosis in NBs with or without MYCN amplification.

Method: The status of SCAs in 54 primary NBs samples was analyzed using a SNP array (Human CMV370-Duo, Illumina). The status of MYCN amplification was determined by a SNP array and the FISH method. The DNA ploidy was determined by flow cytometry.

Results: Nine of 54 samples showed MYCN amplification. All 9 samples with MYCN amplification and 20 of 45 samples without MYCN amplification showed diploidy/tetraploidy, and other 25 samples without MYCN amplification showed aneuploidy. The most frequent SCAs were 17q gain (26/54; 48.1%) and 11q loss (16/54; 29.6%), followed by 1p loss (15/54; 27.8%). The number of SCAs in diploidy/tetraploid NBs without MYCN amplification (7.00±4.67) was higher than that in NBs with MYCN amplification (4.78±2.82) and in aneuploid NBs (1.64±2.78) ($p<0.05$). In diploid/tetraploid NBs without MYCN amplification, there was a significant difference between an age at diagnosis less than 12 months (n=7) and over 12 months (n=13) (4.14±3.63 v.s. 8.54±4.54; p=0.04). Moreover, the number of SCAs correlated with the age at diagnosis in diploid/tetraploid samples without MYCN amplification ($r=0.70$, $p=0.0006$). On the other hand, the number of SCAs did not correlate with the age at diagnosis in NBs with MYCN amplification,

Conclusion: In contrast to the NBs with MYCN amplification, the number of SCAs significantly increased in proportion to age at diagnosis in diploid/tetraploid NBs without MYCN amplification. The increase in the number of SCAs may play an important role in the prognosis of patients without MYCN amplification.

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POT084

Analysis of genomic alterations in neuroblastoma tumours by MLPA and aCGH : comparison of results

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Background: In neuroblastoma (NB), the presence of segmental chromosome alterations (SCAs) is associated with a higher risk of relapse. The alterations - losses of 1p, 3p, 4p and 11q and/or gains of 1q, 2p and 17q chromosome arms - occur recurrently in NB and have been retained to define the therapeutic strategy in new protocols for low- and intermediate-risk patients.

Methods: Different genome-wide techniques such as array-CGH, or the multiplex ligation-dependent probe amplification (MLPA) technique have been suggested for detecting segmental abnormalities. The aim of our study was to compare the array-CGH (Nimblegen DNA array containing 72000 oligonucleotide probes) and MLPA platforms for the analysis of genetic alterations in a large series of neuroblastoma tumors that contained >60% of tumor cells. The DNA of tumour samples from 91 patients have been analyzed by the two methods.

Results: Similar results were obtained with the two techniques for 75 samples (82%). Discrepancies were observed for 11 cases. In 3 cases the array-CGH showed a segmental profile, whereas the MLPA reported a numerical profile for the chromosome arms analysed. Among the alterations, a gain of 18q21.2-qter, and losses of 16p11.2 and 11q14.1-q14.3 have been detected by array-CGH. In 8 cases, a numerical profile was detected by array-CGH procedure whereas the MLPA indicated segmental alterations. SCAs affected the 7p, 7q and 14q arms in 6 cases, and 2p and 17q gains were observed in the two other cases. For these last cases, the FISH analysis confirmed the results obtained by array-CGH. Finally, in 5 cases, the results were not interpretable by MLPA whereas they were by array-CGH.

Conclusion: Array-CGH is recommended for analysis of genomic alterations in NB, because of its genome-wide information, its robust and easily interpretable results, and its reasonable cost in real time analysis.

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POT085

Prognostic gene expression profiling in MYCN-nonamplified high-risk neuroblastoma

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Background: Approximately 40% of children with high-risk neuroblastoma achieve long-term survival. Currently, it is not possible to identify patients who will be cured at diagnosis. Microarray studies have proposed gene expression profiles associated with outcome within high-risk cohorts. However, integrating this technology in the clinical settings has been challenging, due to the lack of available frozen tissue and high quality RNA. The nCounterTM overcomes this obstacle, using formalin-fixed paraffin embedded tissue (FFPE).

Objective: To test the correlation of a previously published "ultra high-risk" microarray gene signature developed in the MYCN-nonamplified high-risk neuroblastoma group (Asgharzadeh et al, J Natl Cancer Inst, 2006) with the gene expression signature obtained with the nCounterTM (NanoString Technologies) using RNA isolated from FFPE MYCN-nonamplified high-risk neuroblastoma samples.

Design/Method: FFPE tumor samples linked to clinical outcome data were obtained from 6 collaborative institutions. Tumor content of each sample was assessed morphologically. RNA was isolated using the RNeasy® FFPE-kit (Qiagen). Customized probes corresponding to the candidate genes were designed by NanoString. Hybridization reactions were performed in duplicate using 100 ng of RNA. Positive and negative control probes as well as housekeeping probes were included in every reaction and were used to normalize data for differences in purification, hybridization, and capture efficiencies.

Results: Forty-two MYCN-nonamplified high-risk neuroblastoma samples were analyzed by the nCounterTM. The cohort 5-year event-free-survival and overall survival were 44.6 +/- 8.0% and 53.8 +/- 8.4% respectively. Highly degraded RNA (RIN~1.5-2.8) was obtained. Unsupervised clustering and principle components analysis on normalized expression data showed differential expression of most of the genes with clustering of cases depending upon outcome (FDR=0.05).

Conclusions: Our results demonstrate that the nCounterTM can yield a gene expression profile that correlates to microarray gene signatures. Further investigation of the clinical utility the nCounterTM technology to prognosticate outcome in patients with high-risk neuroblastoma is warranted.

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POT086

Genetic characterization of an ultra-high-risk group of exclusive neuroblastomas with MYCN amplification plus 11q loss. An study of 18 cases

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Background: Amplification of MYCN (MNA) and 11q loss are two of the most critical genetic markers in Neuroblastoma (NB), being associated with unfavorable cases with advanced stage disease, aggressive behavior and relapse. Coexistence of both alterations in NB is highly infrequent and points out the existence of two distinct genetic subtypes of aggressive NB. These cases represent a valuable model to analyze the chaotic impact of the presence of both specific high risk genetic alteration markers. In a large set of NB tumors we identified a subset of NB having both these high-risk features. We aimed to characterize segmental chromosome alterations (SCA) and breakpoints in these unusual cases, and describe their effect on high chromosomal instability and genetic cell heterogeneity.

Methods: In our series of 905 NB, MNA and 11q status was examined by FISH. Multiplex Ligation-dependent Probe Amplification (MLPA) (NB Kits MRC-Holland) and SNP arrays (Affymetrix GeneChip Human Mapping 250K array) were further performed in tumors carrying MNA and 11q loss (18 cases). Clinical and histopathology evaluation as well as ploidy study were carried out.

Results: A high number of SCA were detected (median 10.7). Chromosome losses were more frequent than gains. A great diversity in MYCN amplicon and heterogeneous amplification was found. 11q shortest region of overlap was from 111.7 Mb to -qter. Additional rearrangements were detected.

Conclusion: We have identified a set of rare NB tumors with the potentially ultra-high-risk features of both MNA and 11q-deletion. This subgroup of NB, that is singular and especially unstable, reflects the chaotic dynamics. In some cases, the instability generates MNA and/or of 11q loss heterogeneity and this must be taking into account in routine genetic diagnosis. Each one of the used techniques has had its essential role. We considered that its application is important in the translational investigation of NB.

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POT087

MORPHOLOGIC AND GENETIC STUDIES OF NEUROBLASTIC TUMORS WITH SILENT MLPA PROFILE. A CONTROVERSIAL PROGNOSTIC IMPACT

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Background: Cytogenetic aberrations have been associated with neuroblastoma (NB) prognosis and are used increasingly in risk stratification systems. Using high resolution techniques different subgroups of patients defined by genomic alterations have been described. One subgroup is constituted by the infrequent neuroblastic tumors with a silent profile, in which no genetic changes are detected. Unclear or poor clinical outcome has been described. The aim of this study was to describe clinical, histopathological and genetic characteristics in a set of unusual NBs without aberrations in the 10 chromosomes most recurrently altered.

Methods: Chromosomal aberrations of 620 samples were determined using MLPA NB kits (MRC-Holland). Clinical data were collected. Paraffin slides were stained with hematoxylin-eosin for the histopathological study. Ki-67 expression was analyzed by immunohistochemistry. Ploidy was assessed by static cytometry. The MYCN status and the integrity of 1p36 and 11q22 regions were evaluated by FISH.

Results: We found 19 samples (3%) with ≥60% tumor cells and a silent or flat MLPA profile. Most of the patients were ≤18 months with localized tumors, good EFS and OS (median follow-up >5 years). The majority of the tumors were poorly-differentiated NB with low amount of necrosis. Tumor cells, without evident nucleoli, presented low mitosis-karyorrhexis index and high expression of Ki-67. Ploidy results were obtained in 15 samples, being mainly triploid. The samples were divided into 3 groups based on chromosome FISH somy and related with ploidy as: with disomic cells only (2/3 with diploidy); plus presence of tetrasomics cells (4/6 with triploidy) and plus presence of trisomic cells (5/10 with triploidy).

Conclusions: This group of tumors requires an extensive histopathological study with continued exploration using massive sequencing technologies and epigenetics research to acquire an understanding of their controversial prognostic impact.

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POT088

Comparison of exon-level and gene-level expression analyses for prediction of outcome and biological characterization of primary neuroblastoma

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Background: The identification of mRNA patterns related to neuroblastoma aggressiveness has been suggested to result in useful tools to predict patient outcome. This study addressed the question whether enhanced resolution by exon-level analyses could improve the robustness and accuracy of gene expression based classifiers for neuroblastoma. Factors governing alternative exon expression were also analyzed.

Methods: In a patient cohort comprising 113 primary neuroblastoma specimens expression profiling using exon-level analyses was performed. For prediction of outcome, patients were divided into a training and a test set. A Shrunken Centroids model was used to define predictive signatures. Alternative transcript use was calculated from relative exon expression and differential expression between classes was determined by Welch's t-test, followed by false discovery correction according to Benjamini-Hochberg. Validation of alternative transcripts was achieved using qPCR-based approaches.

Results: Both predictors derived from the gene or the exon levels resulted in prediction accuracies >80% for both event-free and overall survival and proved as independent prognostic markers in multivariate analyses. Alternative transcript use was most prominently linked to the amplification status of the MYCN oncogene, expression of the TrkA/NTRK1 neurotrophin receptor and survival.

Conclusions: As exon level-based prediction yields comparable, but not significantly better prediction accuracy than gene expression-based predictors, gene-based assays seem to be sufficiently precise for predicting outcome of neuroblastoma patients. However, exon-level analyses provide added knowledge by identifying alternative transcript use, which should deepen the understanding of neuroblastoma biology.

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POT089

Correlation between Pathology Classification and Genomic Signature in Neuroblastoma.

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Background: Several studies revealed genomic signature of neuroblastoma (NB) would be a prognostic factor. The correlation between genomic signature and pathology classification has not been analyzed.

Method: Histopathology of 92 NB cases from 314 cases already classified into 3 genomic groups by array-CGH, 1) Silent(S): no losses and gains except MYCN amplification, 2) Partial(P): partial chromosomal gains and/or losses, 3) Whole (W): whole chromosomal gains and/or losses, were evaluated based on International Neuroblastoma Pathology Classification (INPC). Correlation between pathology and genomic group and clinicopathological features of the 3 genomic groups were analyzed.

Results: Thirty seven NBs were classified into the Favorable Histology (FH) and 55 were into the Unfavorable Histology (UH). In FH, 27(73%) were in W, 5 in S, and 5 in P. In UH, 44(80%) were in P, 3 in S, and 8 in W. Twenty six NBs (90%) out of 29 MYCN amplified tumors were classified into P. In 43 cases of NBs showed the Poorly differentiated subtype with Low MKI (P-L), 15 were P group and 28 were W group. In P, 10 NB-P-L (67%) were UH, on the other hand, 22 of NB-P-L (78.5 %) in W were FH. These data showed the statistically correlation between INPC and genomic group. In 16 MYNC non-amplified tumors in P (Ps), 14 (88%) were UH and 2 were FH. The 10 cases of UH (71%) had 1p loss and/or 11q loss with 17q gain. Within those 10, 8 died and 9 showed histological pleomorphism.

Conclusion: Genomic signature of NBs significantly correlated with INPC. The UH tumors without MYCN amplification may be divided into 2 prognostic categories by genomic status of 1p loss and/or 11q loss with 17q gain.

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POT090

Merging the prognostic potential of independent gene signatures into a single, highly accurate classifier predicting neuroblastoma patients' outcome

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Background: Gene expression-based signatures are important to stratify neuroblastoma (NB) patients. Unfortunately, there is little overlapping among signatures and their prognostic potential can not be combined. We developed a new strategy to merge NB related gene signatures into a single, Multi-Signature Ensemble (MuSE)-classifier of patients' outcome.

Methods: Gene expression on Affymetris U133 p2 of 182 NB tumors, were used in the various phases of development and validation of NB NB-MuSE-classifier. Thirty three signatures were evaluated for patients' outcome prediction using 22 classification algorithms each and generating 726 classifiers and prediction results. The best-performing algorithm for each signature was selected, validated on an independent dataset and the 20 best performing signatures were retained. Gene expression by Affymetrix Exxo arrays of 250 NB tumors were also used.

Results: We combined the 20 predictions associated to the corresponding signatures through the selection of the best performing algorithm into a single outcome predictor. The best performance was obtained by the Decision Table algorithm that produced the NB-MuSE-classifier characterized by an external validation accuracy of 94%. Kaplan-Meier curves and log-rank test demonstrated that patients with good and poor outcome prediction by the NB-MuSE-classifier have a significantly different survival ($p<0.0001$). Survival curves constructed on subgroups of patients divided on the bases of known prognostic marker suggested an excellent stratification of localized tumors.

Conclusions: NB-MuSE-classifier relies on an ensemble approach merging twenty NB-related gene signatures to blend their discriminating power into a single, highly accurate patients' outcome predictor. The novelty of our approach derives from the integration of signatures, that are optimally associated with a single paradigm before merging into a single classifier. This model can be exported to make comparison across platform and we were successful in merging information from Exxon and U133 p2 Affymetrix platforms to validate the robustness of our NB-MuSE-classifier.

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POT091

NTRK1 gene transcripts in human neuroblastoma – preliminary results.

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Background: TrkA, the high-affinity tyrosine kinase receptor for neurotrophins encoded by NTRK1 gene, is the key player in neuronal development regulating processes leading to differentiation, maturation and apoptosis. Although TrkA expression is associated with favorable prognosis, its role in neuroblastoma pathogenesis is not fully understood. Therefore search for putative TrkA isoforms which might modulate particular protein functions and trigger selected intracellular signaling pathways was carried out.

Methods: NTRK1-related mRNA transcripts from public EST (expressed sequence tag) databases have been digitally analyzed. Sequence variants, including alternative splicing, truncated transcripts and novel promotor motifs have been identified. The sequences identified as most promising by in-silico studies have been verified experimentally in a series of primary neuroblastoma tumors and in neuroblastoma cell lines (SY5Y, IMR32). RNA was reversely transcribed and cDNAs were subject to a number of independently designed PCRs specific for selected fragments of the gene. PCR products were analyzed and semi-quantified by capillary electrophoresis. Identification of novel variants was confirmed by direct sequencing.

Results: Several truncated transcripts of the gene, an alternative promoter and a few ESTs with segmental deletions and/or insertions have been identified. Most of the transcripts have been found to be expressed in the cells expressing also the wild-type TrkA.

Conclusions: Preliminary structural and functional in silico predictions suggest that the identified transcripts might encode novel isoforms of the TrkA receptor. Their co-expression with the wild-type might interfere with the correct operation of the internal signal transduction dependent of TrkA-ligand binding and hence promote tumor growth instead of triggering regression. This discovery challenges generally acknowledged opinion that TrkA expression in neuroblastoma is solely favorable marker of the disease. Further analyses on enlarged group of patients as well as functional tests will be performed in order to better define the role of the putative TrkA isoforms in neuroblastoma pathogenesis.

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POT092

High-resolution arrayCGH profiling of germline and tumor-specific copy number alterations in a novel family of neuroblastoma

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Neuroblastoma (NB), the most common solid cancer in early childhood, usually occurs sporadically. However, like other embryonal childhood cancer, it can be inherited, although the genetic aetiology is largely unknown. Germline mutations in the ALK, PHOX2B and LMO1 genes have been shown to explain the majority of hereditary NBs. However, because some individuals harbouring mutations in these genes never develop NB, additional genetic alterations are required for a tumor to be generated. Herein, we studied a novel Italian family of NB with 3 affected individuals (stages 3, 4 and 4S) inheriting NB for ALK, PHOX2B and LMO1 mutations and compared differences of DNA copy number changes in blood and tumor tissues from affected and unaffected family members using an high resolution array-based Comparative Genomic Hybridization (CGH) technology. All data were confirmed by qPCR.

All NB patients carried the R1192P mutation in the ALK gene that was inherited from unaffected components. Of note none of them harbour mutations in PHOX2B or LMO1 genes. Array-CGH assay, displayed either gains or losses of whole chromosomes in stage 3 NB, and partial chromosome imbalances in stage 4 NB.

When profiling constitutional DNA, we identified 3 new genes inherited in chromosomal regions devoid of copy number variations (CNVs): PCNX (pecanex homolog) and CASC4 (cancer susceptibility candidate 4) located respectively in 14q24 and 15q15 were duplicated in two patients, while NKAIN2 (Na/K transporting ATPase interacting 2) located in 6q22 was duplicated only for stage 3 patient. qRT-PCR confirmed all data from Array-CGH.

Surprisingly, high NKAIN2 mRNA levels were found also in stage 4 and stage 4S, suggesting an additional mechanism of gene regulation. Noteworthy we found that 19 out of 20 NB patients show NKAIN2 mRNA up-regulation compared to healthy individuals. We suggest that NKAIN2 may play a role in NB pathogenesis.

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POT093

Differential metastatic patterns and gene expression profiles between BE(2)-C and SK-N-BE(2) cell lines

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Background and Objectives: BE(2)-C is a clone of the SK-N-BE(2) neuroblastoma (NB) cell line established from a bone marrow biopsy taken from a child with disseminated neuroblastoma post chemotherapy and radiotherapy. The differential behavior of the clone and parent line in xenograft models may allow the identification of molecular mechanisms underlying the ability to grow in different metastatic sites.

Methods: Metastatic tumor models were developed by tail vein injection of 1x10^6 SK-N-BE(2) or BE(2)-C cells into NOD/SCID mice. Metastases were confirmed by histology and compared between both cell lines. Tumor growth was monitored using bioluminescence imaging with luciferase+ lines. Gene expression profiles were analyzed with angiogenesis and metastasis pathway-specific microarrays. Independent studies of high throughput gene expression analysis on both cell lines were performed with the Illumina HT12 BeadChip system.

Results: SK-N-BE(2) cells formed metastases in multiple organs, including liver, kidney, lung and bone marrow. A higher incidence of metastases occurred in liver (100%), while the incidence of bone marrow, lung and kidney metastases was lower (50-60%). BE(2)-C cells metastasized to multi-organs in our tumor model. However, BE(2)-C cells had a higher incidence of bone marrow metastases (100%), with a smaller incidence and size of liver metastases by comparison with the SK-N-BE(2) model. Lung and kidney metastases were not noted. For BE(2)-C, hind limb metastases were detectable by bioluminescence within 1-2 weeks post injection, while liver metastases were not detected until much later, if at all. For SK-N-BE(2), liver metastases were observable within 1 week. By microarrray analysis, we identified significant differences in the expression of several genes related to tumor angiogenesis/metastases including CXCR4, NRP1, PGF and EFNB2 and SERPINF1.

Conclusion: The candidate genes identified should be further studied to determine their functional roles in the colonization of bone marrow and liver in NB.

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POT094

Gene expression and genomic aberration signatures cooperatively work to improve tumor risk stratification of neuroblastoma

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Background: We previously proposed the tumor risk classification of neuroblastoma (NB) based on the genomic and gene expression profiling. The genomic classification uses three genomic groups (GGs) of copy number aberrations in NB: silent (S), partial (P) and whole (W). Each GG was further segregated into subgroups with different prognostic risk clearly defined by MYCN amplification (MYCN-amp), 1p loss, 11q loss and 17q gain. On the other hand, gene expression (GE)-based classification uses posterior value, which corresponds to the probability of good prognosis (posterior>0.5 as favorable prognosis) calculated by using 200 genes-expression values on the diagnostic mini-chip. In this study, we conducted prospective validation of these classifiers.

Methods: 126 sporadic NBs obtained from 2005 to 2008 in Japan (27 stage 1or 2; 10 stage 4s; 29 stage 3; 55 stage 4) were analyzed by the 200-genes mini-chip as well as the 44K CGH microarray. Clinical impact of each signature was assessed by using the outcome information of the patients (median follow-up duration was 30 months).

Results: Statistical analysis indicated that GE signature exhibited good potential to classify prognosis of 126 patients (logrank-test, p=0.006) as well as of 57 intermediate-risk type patients (stage 3 or 4 with no MYCN-amp, p=0.014). 74 patients were with GG information, and multivariate statistical analysis showed that GE and GGs were significant, mutually independent prognostic markers for survival (p=0.036 and 0.012, respectively). GE signature showed good potential in Ps patients (GG-P with no MYCN-amp, p=0.023), while our previous study using 343 NBs indicated that GG could divide patients with MYCN-amp as high- or ultra-high-risk (P1a vs. others, p=0.07).

Conclusions: These results suggested that the combination of GG and GE signatures can efficiently predict prognosis of the patient with NB. Clinical studies with these molecular markers are further ongoing in Japan.

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POT095

Heme oxygenase-1 is a novel immune modulator in neuroblastoma

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Background: Heme oxygenase (HO)-1 is overexpressed in neuroblastoma (NB). Our recent data on peptide vaccination strategies that failed to combat NB in mice revealed increased HO-1 expression in metastasized livers from vaccinated mice, suggesting that HO-1 may act as immune modulator in NB.

Methods: We performed western blot, immunohistochemistry and flow cytometry to study HO-1 expression and its immunologic function in a syngeneic NB mouse model. Intratumoral HO-1 inhibition was accomplished i) by i.p. zinc protoporphyrin (ZnPP) injections (5x100 mg/kg) and ii) by s.c. administration of a ZnPP-containing (10 µM) NXS2 suspension. Tumor progression was determined. We performed Cr51 cytotoxicity assay and IFN-gamma ELISA on isolated splenocytes and carried out in vivo depletion of CD4, CD8 or NK cells.

Results: HO-1 expression was high in all murine and human NB cell lines and its dosage-dependent inhibition by ZnPP application correlated with decreased cellular viability. In the first experiments, we were able to suppress s.c. tumor growth and the occurrence of liver metastases by ZnPP application compared to the controls. Splenocytes from ZnPP-treated mice showed a 10-25% higher NXS2 target cells lysis and increased IFN-gamma release than those from control mice. To avoid an inhibition of in vivo tumor cell proliferation due to the pro-oxidant effect of ZnPP, we pre-treated NXS2 cells with non-antiproliferative dosages (10µM) and injected these pre-treated cells into mice. In contrast to the control we found tumor growth suppression. This effect was accompanied by elevated CD8 expression in ZnPP-pretreated tumors. Systemic depletion of CD4+, CD8+ T cells and not of NK cells could abrogate ZnPP-initiated suppression of tumor growth.

Conclusion: HO-1 suppressed anti-NB immune response by blockade of CD4 and CD8 T effector cell function. This suggests HO-1 as an important immune modulator in NB and interesting target for the development of a novel anti-NB immunotherapeutic approach.

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POT096

Regulatory T cells in neuroblastoma (NB): from an animal model to patients.

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Background: Tumors can sustain the proliferation and differentiation of immune suppressive cells, including CD4+CD25+FoxP3+ regulatory T (Treg) and regulatory Type1 (Tr1) cells, by the secretion of factors such as TGF-beta, PGE2 or IL-10. We previously observed that the administration of anti-CD4 cell-depleting antibodies strongly enhances the anti-tumor effects of IL-21 secreted by a genetically engineered NB cell vaccine.

Methods: We studied the mechanism(s) associated with the potent anti-tumor effects of a therapeutic combination of recombinant (r)IL-21 and anti-CD4 antibodies in a syngeneic model of disseminated NB. Moreover, we investigated the presence of immune suppressive Treg cells in peripheral blood samples from mice and from patients with metastatic NB.

Results: Anti-CD4 antibody treatment completely depleted Treg cells and possibly other suppressive CD4+ cell subsets. The co-administration of rIL-21 by-passed the requirement of CD4+ helper cells and allowed the development of an effective anti-tumor CD8+ T cell response. Nonetheless reconstitution of the CD4+ T cell pool after combined immunotherapy was required for an efficient and long-lasting immunity to NB antigens. To gain preliminary information on the role of CD4+ Treg cells in NB patients we studied FoxP3 and IL-10 mRNA expression in peripheral blood cells from patients and healthy controls by RT-qPCR. Both FoxP3 and IL-10 mRNA levels (as ratio with CD45 mRNA) were significantly higher in stage 4 NB patients than in controls ($p=0.0037$ and $p<0.0001$, respectively).

Conclusion: Our data support a role of immune suppressive CD4+ T cells not only in preclinical models, but also in children with NB, opening the possibility of new immunotherapeutic approaches targeting these cells. MC and BC are the recipients of fellowships awarded by Italian Neuroblastoma Foundation

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POT097

Bispecific Antibody Anti-CD3 x Anti-GD2 (3F8Bi) Enhances Cytotoxicity of Activated T-Cells to Neuroblastoma Targets.

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Background: The ganglioside GD2 is a validated target for immunotherapy of neuroblastoma with monoclonal antibodies (mAb). Although therapy with anti-GD2 mAb resulted in improved survival, > 50% of the patients with stage IV neuroblastoma eventually develop recurrent disease. With the goal to enhance GD2-directed cytotoxicity, we combined GD2-targeting with the non-MHC-restricted cytotoxicity by arming anti-CD3 activated T cells (ATC) with anti-CD3 x anti-GD2 bispecific antibody (3F8BiAb).

Methods: 3F8Bi was prepared by chemically conjugating OKT3 mAb that recognizes the CD3 on T cells and 3F8 mAb that recognizes the GD2 expressed on tumor cells. ATC were generated from normal human peripheral blood mononuclear cells (PBMC) by stimulating the PBMC with OKT3 and expanding the T cells in the presence of interleukin 2 (IL-2) for 14 days. ATC were coated (armed) with 3F8BiAb (100 ng/106 cells) prior to use. An irrelevant bispecific antibody anti-CD3 x anti-Her2/neu BiAb was used in control experiments. 3F8BiAb-armed ATC (aATC) were tested ex vivo for their cytotoxicity against neuroblastoma cell lines (LAN1, LAN6, LHN, and KCNR) and their ability to secrete cytokines and chemokines upon binding to targets.

Results: Binding of 3F8BiAb to tumor targets and to ATC was confirmed by FACS analysis. The cytotoxicity exhibited by 3F8BiAb-aATC directed at GD2 positive neuroblastoma cell lines was significantly higher than that mediated by ATC alone ($p < 0.001$). 3F8BiAb-armed ATC secrete significantly higher levels of some Th1 cytokines and chemokines compared to unarmed ATC ($p < 0.001$). FcR blocking experiment demonstrated no ADCC contribution to the cytotoxicity of aATC.

Conclusions: These preclinical findings show that the non-MHC restricted cytotoxicity mediated by ATC can be redirected by 3F8Bi to markedly enhanced cytotoxicity directed at GD2-positive malignancies. This approach can lead to the development of more effective non-toxic strategies for improving overall survival for patients with neuroblastoma.

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POT098

Increased GD2 expression of drug resistant neuroblastoma cell lines facilitates GD2-specific killing by genetically engineered NK cells.

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Background: Drug resistant neuroblastoma remains a major challenge in pediatric oncology. A human NK cell line NK-92tr engineered to express a GD2-specific chimeric receptor may help to address this problem. Thus, we investigated cytotoxicity of NK-92tr in a panel of GD2+ drug resistant neuroblastoma cell lines and report results from early in vivo experiments.

Methods: Pairs of cell lines established at time of diagnosis and after relapse (CHLA-15/CHLA-20, SMS-KAN/SMS-KANR, SMS-KCN/SMS-KCNR, SK-N-BE(1)/SK-N-BE(2)) were analyzed in the presence and absence of GD2-specific NK-92tr cells compared to negative controls in a 51Cr release cytotoxicity assay. The level of GD2 expression on cell line pairs was determined by flow cytometry. Further, the expression level of glucosylceramide synthase (GCS), the first step enzyme of ganglioside synthesis, was investigated by western blot. Homing and anti-tumor activity of GD2-specific NK-92tr towards the drug resistant relapse cell line CHLA-20 were analyzed in a xenograft model.

Results: In all cases, relapse cell lines exhibited a higher GD2 surface expression in contrast to cell lines at diagnosis which correlated with a higher sensitivity towards NK-92tr. The mechanism involved is an increased expression level of GCS in all relapse cell lines used as a ceramide degradation escape mechanism. The GCS mediated effect was confirmed by inhibition of GCS using inhibitor PPPP, which resulted in downregulation of GD2 expression and subsequent abrogation of NK-92tr mediated lysis. Initial in vivo experiments revealed that NK-92tr are able to migrate into tumor tissue and decrease tumor growth in a multidrug resistant xenograft model.

Conclusions: These encouraging results indicate that GD2-directed immunotherapy is an appropriate treatment strategy in relapsed NB that exhibit drug resistance.

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POT099

Functionally active Myeloid Derived Supressor Cells (MDSCs) are found within the blood and tumour of patients with neuroblastoma

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Background: The ability of tumours to evade a patient's immune system is a subject of increasing interest. Myeloid Derived Suppressor Cells (MDSCs), a subset of immature myeloid cells, play a role in the development of an immunosuppressive tumour microenvironment. The exact immunophenotype of human MDSCs has become somewhat controversial although a consensus view is that the suppressive phenotype is associated with expression of CD33, CD11b, and CD66b, and absence of HLA-DR staining. A number of studies in adult cancers have reported an inverse correlation between circulating MDSC numbers and both prognosis and clinical stage. Only a limited number of prior studies have evaluated the role of MDSCs in neuroblastoma. This study aims to determine whether elevated levels of MDSCs are present in neuroblastoma patients.

Methods: Blood samples from patients with neuroblastoma were taken at diagnosis or immediately prior to surgery (as a paired sample with excised tumour). Samples from healthy children were analysed as controls. Blood was lysed and tumour samples disaggregated mechanically prior to staining. Samples were stained with the following multi-fluorochrome panel of antibodies: CD11b, CD33, CD66b, CD14, CD15, HLA-DR. Stained cells were detected using the LSR-II flow cytometer and analysed using FACSDiva. Suppressive function was assayed using thymidine incorporation and CFSE dilution.

Results: Our initial results suggest a population of CD33+CD11b+CD66b+HLA-DR-CD15-CD14+ cells in the blood of all patients with neuroblastoma. In the six samples analysed a functional assay demonstrated suppressive activity in vitro within the mononuclear population of these patients. A phenotypically similar cell population was seen in the disaggregated tumour samples.

Conclusion: Our results support the hypothesis that MDSCs are present in neuroblastoma patients, both circulating in blood and within the tumour. Correlation with tumour stage or treatment outcome is subject to further study. The identification of MDSCs in patients may provide an important therapeutic target.

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POT100

TGFβ1 Receptor I Inhibitor Enhances NK Cell Direct and Antibody Dependent Cellular Cytotoxicity (ADCC) Against Neuroblastoma in vitro and in vivo

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Background: Immunotherapy with anti-GD2 mAb ch14.18, IL2, and GM-CSF improves event-free survival for high-risk neuroblastoma patients who respond to induction and consolidation therapy. The effectiveness of immunotherapy is dependent on NK cell activity. TGFβ1, a NK cell inhibitor, is rich in the tumor microenvironment. We hypothesized that SB431542, a TGFβ1 type I receptor inhibitor, can enhance NK cell anti-tumor activity.

Methods: Purified NK cells from healthy donors were activated with IL-2 for 72 hours in the presence of TGFβ1 +/- SB431542. Direct NK cell cytotoxicity and ADCC with ch14.18 against calcein-AM labeled neuroblastoma cells were quantified using a digital image microscopy system. Cytokines released were quantified with a Luminex® assay. NOD/SCID mice were subcutaneously injected with luciferase labeled CHLA-255 neuroblastoma cells mixed 1:1 with PBMC in Matrigel™, a model in which the TGFβ pathway is activated. Mice (5/group) were untreated or treated with ch14.18 alone, ch14.18 and SB431542, or SB431542 alone. Censored regression analysis was performed for differences in mean survival days (MSD) between groups.

Results: SB431542 prevented TGFβ1-mediated suppression of IL-2 induced NK cell ADCC and cytotoxicity against neuroblastoma cells in vitro. In the presence of TGFβ1, SB431542 increased NK cell secretion of GM-CSF, IFNγ, TNFα, MIP1α, and MIG and production of granzymes A and B and perforin. MSD for untreated mice and mice treated with ch14.18 alone, ch14.18 and SB431542, or SB431542 alone were 29, 49, 89, and 84 days respectively (P value vs. control = 0.27, 0.01, and 0.009). Comparison of MSD for mice treated with ch14.18 alone or ch14.18 and SB431542 suggests improvement with SB431542 (P=0.07).

Conclusions: SB431542 prevented TGFβ1 suppression of NK cells in vitro and enhanced anti-GD2 treatment of tumor cells co-injected with PBMC in NOD/SCID mice. These data support further investigation of TGFβ pathway inhibitors to enhance anti-GD2 based immunotherapy of high-risk neuroblastoma.

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POT101

IRF1 and NF-kB restore MHC-I-restricted tumor antigen processing and presentation to cytotoxic T cells in aggressive neuroblastomas

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Neuroblastoma (NB), the most common solid extracranial cancer of childhood, displays a remarkable low expression of Major Histocompatibility Complex class I (MHC-I) and Antigen Processing Machinery (APM) molecules, including Endoplasmic Reticulum Aminopeptidases, and poorly presents tumor antigens to Cytotoxic T Lymphocytes (CTL). We have previously shown that this is due to low expression of the transcription factor NF-kB p65. Herein, we show that not only NF-kB p65, but also the Interferon Regulatory Factor 1 (IRF1) and certain APM components are low in a subset of NB cell lines with aggressive features. Whereas single transfection with either IRF1, or NF-kB p65 is ineffective, co-transfection results in strong synergy and substantial reversion of the MHC-I/APM-low phenotype in all NB cell lines tested. Accordingly, linked immunohistochemistry expression patterns among nuclear IRF1, p65 and MHC-I were observed in vivo: absence and presence of the three molecules neatly segregating between high-grade and low-grade NB lesions, respectively. Finally, APM reconstitution by double IRF1/p65 transfection rendered a NB cell line susceptible to killing by CTLs anti MAGE-A3 peptide, an endogenous NB antigen, lytic efficiency reaching levels comparable to those seen upon IFN-γ treatment. To our knowledge, this is the first demonstration that a complex immune escape phenotype can be rescued by reconstitution of a limited number of master regulatory genes.

These findings provide molecular insight into defective MHC-I expression in NB cells and indicate feasibility of T cell-based immunotherapy in NB variants refractory to conventional therapy.

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POT102

Binding characteristics of immunocytokine hu14.18-IL2 (APN301) to its nominal antigen GD2 and to anti-idiotypic antibodies 1A7 and ganglidiomab

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Background: Immunotherapy of neuroblastoma with anti-GD2 antibodies in combination with interleukin-2 (IL2) emerges as an important consolidation treatment realized in large cooperative Phase III clinical trials in the MRD setting. Consequently antibody-cytokine fusion proteins (immunocytokines) were developed, which combine the targeting function of an anti-GD2 antibody with the immune stimulation activity of IL-2 (hu14.18-IL2). This concept demonstrated encouraging response rates in Phase I/II trials in relapsed and refractory neuroblastoma. Here, we report the antigen binding characteristics and anti-neuroblastoma activity of a new lyophylized GMP preparation of hu14.18-IL2 (APN301).

Materials: Binding of APN301 to GD2 was analyzed by ELISA and compared to other related antibodies (14G2a, ch14.18/CHO, hu14.18). Affinities of APN301 to GD2 and to anti-GD2 anti-idiotype antibodies (AITAB) ganglidiomab and 1A7 were determined in Biacore analysis. GD2 specific ADCC, CDC and whole blood cytotoxicity was analyzed in calcein- and 51Cr-release assays.

Results: We demonstrate similar binding characteristics of APN301 to GD2 compared to 14G2a, ch14.18/CHO and hu14.18 by ELISA. Importantly, ganglidiomab competitively inhibited binding of APN301 to GD2, and no binding of APN301 to GD1a was detectable, indicating GD2 specificity. The dissociation constants (KDs) of APN301 from GD2 and ganglidiomab as determined in Biacore analyses were in the 10-9M and 10-7M range, respectively, similar to ch14.18/CHO. Vice versa, using "steady state" analysis, the KDs of AITAB 1A7 and ganglidiomab from APN301 were also in the 10-7M range similar to results obtained with 14G2a, ch14.18/CHO and hu14.18. Finally, we demonstrate GD2 specific lysis of neuroblastoma cells in vitro mediated by APN301 in ADCC, CDC and whole blood assays.

Conclusion: In summary, we demonstrate GD2 specific binding of a new lyophylized GMP preparation of hu14.18-IL2 (APN301), describe KDs to GD2 and anti-GD2 anti-idiotype antibodies, and show anti-neuroblastoma activity in vitro. These findings provide an important base line for clinical development of APN301.

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POT103

Distinct metastatic patterns in neuroblastoma are correlated with MYCN amplification

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Neuroblastoma (NBL) is a heterogeneous disorder of complex biology. Clinical-biological factors that may affect neuroblastoma-specific patterns of metastasis have not been well defined. Using a qualitative radiographic scoring method on diagnostic 123I-MIBG scans, we identified distinct metastatic patterns for patients with stage 4 disease and began to correlate these patterns with known clinical-biological parameters.

Pre-treatment 123I-MIBG scans from 253 patients with newly diagnosed, stage 4 NBL were included; 125 from European high-risk studies and 128 from the Children's Oncology Group (COG) A3973 protocol. Two independent observers evaluated 15 anatomic segments (14 skeletal and one soft tissue). Scans were scored for the absolute number of affected segments. Within individual segments, uptake patterns were scored as 'focal' (clear margins distinguishable from the adjacent background) or 'diffuse' (indistinct margins with uptake dispersed throughout the segment).

In both the European and COG cohorts, two distinct patterns of MIBG uptake were noted on pre-treatment 123I-MIBG scans: a 'focal and limited' pattern in which metastases were 'focal' and occurred in a limited number of body segments; and an 'extensive and diffuse' pattern in which metastases showed 'diffuse' invasion of an extensive number of body segments (median no. segments: 2 vs. 11, $p < 0.001$).

From the European cohort, patients with MYCN amplified tumors had significantly fewer affected body segments than patients with MYCN non-amplified tumors (median no. segments: 5 versus 11, $p<0.001$). Patients with diffuse lesions had predominantly MYCN non-amplified tumors in contrast to patients with focal lesions ($p<0.001$) (Table 1). MYCN data from the COG are under analysis.

In stage 4 neuroblastoma, two patterns of metastasis can be discriminated: a 'focal-limited' pattern in MYCN amplified and a 'diffuse-extensive' pattern in MYCN non-amplified cases.

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POT104

Congenital neuroblastoma due to constitutional MYCN gain in a patient with unbalanced translocation t(2;18)(p24.2;q23)

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Background: The most important adverse prognostic factor in neuroblastoma (NB) is MYCN amplification. It has been postulated that MYCN amplicons arise from extra replication rounds of unbroken DNA secondary structures that accumulate at FRA2C – a common fragile site localized on chromosome 2p region. ~25% NB have somatic amplifications at this locus; likewise 15% of patients with constitutional duplications of this region reported in EUCARUCA database developed NB. We report a patient with partial 2p trisomy due to a novel unbalanced translocation t(2;18)(p24.2;q23) involving MYCN region who developed congenital NB.

Methods: MYCN status by FISH was analyzed on peripheral lymphocytes and NB tumor touch imprints. aCGH analysis and qPCR studies were performed on DNA extracted from peripheral blood and tumor cells. As the control, DNA of a child without NB but with another constitutional partial 2p trisomy was used.

Results: Tumor MYCN status was classified as "gain" by FISH (MYCNx3-10) and confirmed by qPCR (4-8n). Similar findings were observed in peripheral blood samples of the patient. Conversely, the control case was found to be trisomic at MYCN locus by both methods. Further analysis of the adjacent genomic regions revealed presence of minute areas of gains and losses within the FRA2C region in both tissues from the NB patient, suggestive for being derived from replication loops. aCGH showed no additional somatic genomic imbalances; no germline ALK gene mutation was detected.

Conclusions: On the contrary to advanced-stage NB, MYCN amplifications are unusual in low-stage tumors. Therefore our case of congenital NB with sole genomic aberration at chromosome 2p seems to be the latent picture of the early events leading to MYCN amplification at later stages of tumor progression. This phenomenon provides new insight on the mechanism of MYCN amplicon rearrangements and its role in the early steps of NB cancerogenesis.

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POT105

MYCN contributes to EZH2 mediated epigenetic dysregulation in Neuroblastoma, which can be reversed by pharmacologic targeting of EZH2

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Background: Recently it was shown that high expression of the epigenetic regulator EZH2 in undifferentiated, poor prognosis NB tumors leads to the repression of tumor suppressor and differentiation genes. What drives EZH2 expression in NB is unknown. We postulate that genetic alterations associated with NB lead to dysregulation of EZH2 and targeting EZH2 may be a novel therapeutic approach.

Methods: SiRNA targeting of MYCN, qPCR and Western blot analysis was utilized to assess changes to histone modification. Six NB cell lines (SKN-AS, SH-SY5Y, NGP, SKN-BE2, SMS-KCNR and LAN5) with different genetic backgrounds were incubated with the EZH2 inhibitor, 3-Deazaneplanocin A (DZNep) (0.1-20uM) for 3 days. Cell growth (MTS assay), cell cycle (FACScan), apoptosis (caspase 3/7 activity) and tumorigenicity (murine xenograft model) were assessed.

Results: Silencing MYCN leads to decreases in EZH2 and its target H3K27me3.. Decreasing EZH2 and H3K27me3 using EZH2 shRNA and DZNep did not decrease MYCN mRNA indicating that EZH2 functions downstream of MYCN. DZNep inhibits NB cell proliferation in all lines tested (mean IC50=1.5uM, range 0.5-5uM). All cell lines had increases in subG1 phase, but in only 2/6 lines was this associated with caspase dependent apoptosis. In these 2 cell lines, DZNep induces a 2-fold increase in caspase3/7 activity and pre-treatment with the caspase inhibitor Z-VAD-FMK blocks DZNep induced cell death. Inhibition of EZH2 leads to differentiation in 4/6 lines with increases in CASZ1 and TrkA mRNA. Treatment of cells with low doses of HDAC inhibitor or retinoids synergizes with DZNep to control cell growth. In animal studies DZNep decreases levels of EZH2 inhibits tumor xenograft growth.

Conclusion: This study indicates the genetic alterations such as MYCN can lead to dysregulation of EZH2 and targeting EZH2 may be an added tool to treat neuroblastoma tumors.

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POT106

β-1,4-galactosyltransferase III expression predicts an unfavorable prognosis in neuroblastoma and enhances malignant cell phenotypes by modifying glycosylation of β1 integrin

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Background: Chromosome 1q gain is associated with an unfavorable prognosis of neuroblastoma (NB). β-1,4-galactosyltransferase III (B4GALT3), which efficiently catalyzes the synthesis of the first N-acetyllactosamine unit and may be involved in neuronal differentiation, locates at chromosome 1q. This study aims at defining the in vivo and in vitro role of B4GALT3 expression in NB.

Methods: B4GALT3 protein expression in 101 NB tumor tissues was evaluated by immunohistochemistry and was compared to other clinicopathological factors and patient survival. The effects of B4GALT3 expression on NB cell behavior and signaling were evaluated by ectopic expression of B4GALT3 in SH-SY-5Y cells.

Results: Positive B4GALT3 immunostaining was demonstrated in 56 NB tumors and correlated with an advanced tumor stage ($P=0.04$) as well as an unfavorable histology ($P<0.001$). Positive B4GALT3 expression predicts an unfavorable prognosis independent of other factors. Overexpression of B4GALT3 enhanced malignant cell phenotypes including migration, invasion, and adhesion as well as resistance to cell differentiation. B4GALT3 transfectants also demonstrated modified β1 integrin glycosylation with subsequent downstream signaling pathway activation including phosphorylation of FAK, paxillin, and Akt.

Conclusions: B4GALT3 expression predicts an unfavorable NB patient prognosis and enhances malignant phenotypes of NB cells possibly through modifying glycosylation of β1 integrin. Targeting of B4GALT3 could potentially provide a novel therapy for NB patients in the future.

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POT107

New epigenetic markers with prognostic value in Neuroblastoma

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Background: The study of epigenetic mechanisms involved in NB progression could contribute to a better understanding of this heterogenic tumor. The aim of the present study was to identify epigenetic biomarkers with prognostic value in NB.

Methods: Illumina's Infinium HumaMethylation27 assay was performed in DNA samples extracted from 48 fresh frozen tumors from NB patients at diagnosis. Chi square and Fisher test methods in combination with multivariate analysis were used as well as Functional Enrichment and Gene Set Enrichment analyses.

Results: We studied the association between the methylation status and clinical and biological parameters (age at diagnosis, stage, group of risk, MYCN status and the onset of relapses or deaths) in the NB patients. The more relevant results were found when comparing the established subgroups with regard to the hypermethylation status than when using the hypomethylation ones. Risk parameter showed the highest number of significant probes/genes in a comparison of the methylation status among the 3 different risk groups. The high risk group with relapses or deaths was the one that produced the most relevant results. In addition, the percentage of hypermethylation in our series was higher in the NB patients who had died (p=0.036). We found 80 genes whose hypermethylated status is significantly associated with the patient's outcome, being of special interest those involved in maturation and maintenance of the overall structure of the nervous system (NNAT), control of the cell cycle (CCND1, JAK2, TP73), cell growth and differentiation (DUSP2, PAX8), tumorigenesis and tumor progression (MAGEA2, RUNX 3, CTSZ, TDGF1, TSPAN32), apoptosis (JAK2, PECAM1, RB1) and DNA repair mechanisms such as MGMT.

Conclusions: We identified a group of hypermethylated genes with prognostic value in NB, some of them not previously reported as such. Studying the epigenetic alterations in the entire genome would help reveal the epigenomic approach of NB.

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POT108

Identification of novel serum protein biomarkers for the early detection of neuroblastoma

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Background: Serum markers of neuroblastoma at an early clinical stage would have great utility to monitor therapeutic response or for test detection. Comparative analysis of the serum proteome for diseased and non-diseased states may reveal novel cancer biomarkers. Homozygote TH-MYCN+/+ transgenic mice develop neuroblastoma, with many of the clinical and molecular features of the human disease at 6 weeks of age.

Methods: Serum from TH-MYCN+/+ and wild type (WT) littermate mice were collected for proteome comparisons at 2, 4 and 6 weeks of age. The proteins within each serum sample were separated into five size fractions using the novel electrophoretic fractionation device, the MF10. The analytical software Progenesis LC/MS was employed to analyse the serum protein differences between TH-MYCN+/+ and WT mice.

Results: We have generated two extensive lists of candidate protein biomarkers for differences at 2 weeks (pre-cancer state) and 6 weeks (cancer state) of age. Two candidate serum proteins with markedly increased levels at 6 weeks of age were chosen for further study: zinc-alpha-2-glycoprotein (ZAG), and, complement component 4-b (C4-b). ZAG has been evaluated as a tumour biomarker for various carcinomas. C4-b forms part of complement pathway C3-convertase. ZAG was present in the serum of 6 week TH-MYCN+/+ mice, at a level 400-fold higher than WT mice. C4-b was only increased 1.42 at 4 weeks but rose to 397-fold higher levels at 6 weeks. Immunoblots of 2D-gels confirmed the differential expression of both biomarkers in pooled serum from 6 mice. Using an ELISA assay we showed that ZAG was present at a concentration of 514.18 ng/ml in TH-MYCN+/+ mice, compared with 6.41 ng/ml in WT mice, an 80 fold difference.

Conclusions: This novel proteomic approach has been able to identify two potential candidates that may serve as accurate serum markers for the sub-clinical detection of neuroblastoma.

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POT109

Toll-like receptor 3 expression as a biomarker that predicts favorable prognosis in patients with neuroblastoma

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Background: Differential expression of Toll-like receptor 3 (TLR3) in human neuroblastoma (NB) tissue has been identified by our group and agonist of TLR3 may trigger apoptotic response of NB cells in vitro. We evaluate the potential of TLR3 expression as a prognostic biomarker for patients with NB.

Methods: Archival NB tissues from 99 patients were available for immunohistochemical staining. TLR3 expression was scored and correlated with biological characteristics of NB and survival rate of the patients. In vitro studies were conducted on three NB cell lines with different levels of TLR3 expression and without or with MYCN amplification to verify the role of TLR3 in differentiation, migration and invasion of NB cells.

Results: Positive TLR3 expression was present in 48 out of 55 tissue (87.3%) samples with favorable histology, but in 22 of 44 samples (50%) with unfavorable histology (P<0.001). TLR3 was positive in 12 of 23 samples (52.2%) with MYCN amplification, but in 58 of 76 (76.3%) without (P=0.036). Multivariate analysis revealed that TLR3 was one of the three variables significantly predicting the survival of the patients. Further in vitro studies in NB cell lines showed that SK-N-AS with high TLR3 expression and without MYCN amplification responded to TLR3 agonist poly(I:C) treatment with significantly increased expression of GAP-43 (a marker of differentiation), but not in SK-N-DZ without TLR3 expression and with MYCN amplification. Other in vitro data are consistent with the in vivo findings.

Conclusions: Our results indicate that TLR3 expression can be a biomarker that predicts a favorable prognosis in patients with NB and TLR3 could potentially serve as a therapeutic target of NB.

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POT110

Identification of new candidate biomarkers in progression of neuroblastoma cells using differential transcriptome and proteome analysis

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Background: Neuroblastoma (NBL) is biologically and genetically heterogeneous and demonstrates both favorable and unfavorable outcomes. Genome-wide genetic aberrations using microarray have already been reported (PSI 2009, BMC Bioinformatics. 2010). In the present study, differential comparison of transcriptome and proteome data was combined to evaluate the mechanism of NBL progression.

Methods: From 200 NBL tumor samples analyzed by Affymetrix SNP and expression arrays, 40 tumor samples were selected, including 20 favorable cases which regressed or matured spontaneously and 20 unfavorable cases who died of tumor progression. The primary cultured cells derived from these tumors and 12 NBL cell lines were examined. The transcriptome using total RNA was examined by Affymetrix U-133B array. LC-MS analysis using cell extracts was performed by mass spectrometers (QSTAR Elite or LTQ Orbitrap XL) with ESI module. MS/MS data of the specific peaks was matched to the data in the MassBank.

Results: About 900 peaks were extracted from the LC-MS data and 1660 genes were listed by tanscriptome analysis. The comparison between these two data sets showed 71 protein groups overlapped. Clustering analysis using these protein data sets identified three different clusters: two unfavorable groups and one favorable one, indicating the existence of two different clusters in progressive NBLs. In these unfavorable groups, MYC-induced and cholinergic pathways are activated. On the other hand, apoptosis pathways, including neuro-differentiation, sphyngomyelin metabolites were upregulated in the favorable group. Moreover, glutathione metabolites including GABA and Taurin were increased in favorable group and glutaminate decarboxylase were depressed in unfavorable groups, suggesting glutaminate metabolism pathway might be correlated with NBL biology.

Conclusions: Global analysis from gene to protein classified different biological clusters in NBL and revealed the main activated pathway in each cluster of NBL. Further pathway analysis provided important candidates of biomarkers for risk assessment and of therapeutic targets for unfavorable NBLs.

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POT111

Can a given presence of acid mucopolysaccharides explain the different prognosis for neuroblastoma patients younger and older than 18 months?

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Background: The quantity of acid mucopolysaccharides (MPS) has been contradictorily related to prognosis in different malignancies. MPS carry certain biofiltering, scaffolding and cell anchoring properties; and their quantity decreases with aging in normal tissue. AIM: to determine if MPS percentage can help to explain the different evolution observed depending on patient age in neuroblastoma (NB).

Methods: We analyzed at least two representative cores of 1mm of primary tumors from 209 patients, included in 12 tissue microarrays (TMA). TMA slices of 3µm were stained with alcian blue at 2.5pH, scanned at 40x with Aperio Scan Scope XT, and analyzed with Aperio positive pixel count algorithm which counts the alcian blue stained pixels per area of analysis. The percentage of MPS per case is taken as the average value of all the cores belonging to the same case.

Results: The percentage of acid MPS ranged from 0.3% to 31.03% (mean=3.42 ±3.49) and was normalized by log transformation. An absence/ scarce presence of MPS was related to histopathology, specifically to INRG poorly differentiated NB and undifferentiated NB group (p=8.37e-11). It was also unexpectedly related to MNA (p=0.02), 17q gain (p=0.03) and 1p deletion (p=0.02) (univariate regression). All variables were interrelated and the most robust in predicting the percentage of MPS was the state of 1p (multivariate regression). The percentage of MPS tended to correlate with age (p=0.057).

Conclusions: In our cohort, the absence/scarce presence of MPS is related to clinical-biological features known to be associated with poor prognosis. Specifically, it is related to a segmental chromosome aberrations tumor profile and unfavorable histopathology. A larger study would explain the different prognosis between both age groups, which would arise due to a change in the percentage and to the different filtering properties and the structural and/or cell anchoring characteristics.

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POT112

Polyamine pathway genes represent powerful prognostic markers in neuroblastoma and important targets for therapeutic suppression

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Background: We have previously shown that the MYC/MYCN target ornithine decarboxylase (ODC1), rate-limiting for polyamine synthesis, is a therapeutic target for neuroblastoma. We have also shown that targeting multiple steps in the polyamine pathway enhances therapeutic efficacy in complementary pre-clinical models (Haber, ANR 2012). An international Phase I trial for refractory neuroblastoma, coordinated by NANT USA, will open shortly using ODC1 inhibition by high-dose DFMO and SAT1 induction by celecoxib. We have now examined the prognostic impact of all PA pathway genes, and a SNP in the ODC1 promoter, in large cohorts of neuroblastoma tumors.

Methods: Gene-expression profiles of 650 primary untreated neuroblastomas were analyzed for all polyamine pathway genes. The G317A promoter SNP was examined by RQ-PCR in three large, and one small, cohorts of primary untreated neuroblastoma from Australia (n=185), USA (n=183), Belgium (n=132) and The Netherlands (n=55), and in an Australian lung cancer cohort (n=161).

Results: Expression of all polyamine pathway genes was highly prognostic of neuroblastoma outcome. High levels of each synthetic gene and low levels of each catabolic gene predicted poor outcome. Multivariate analysis showed 6/11 genes retained independent prognostic significance following adjustment for MYCN, age and stage. Additionally, though previous studies have shown the GG genotype of the G317A SNP to be associated with improved colon cancer outcome, a finding we have now confirmed in lung cancer patients (P=0.017), analysis of three large neuroblastoma cohorts (although not the small cohort), instead suggests an association between improved outcome and the mutant AA genotype. Promoter studies are underway, to analyze tissue-specific effects of the A/G alleles on ODC1 expression.

Conclusions: The results highlight the importance of polyamines in neuroblastoma and identify additional target enzymes for potential therapeutic intervention. Our results also suggest tissue-specificity in the influence of the A317G SNP, which may impact clinical outcome.

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POT113

Influence of Segmental Chromosome Abnormalities on Survival in Children over the Age of 12 Months with Unresectable Localized Neuroblastoma without MYCN Amplification.

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Background: In 2001 SIOPEN launched the European Unresectable Neuroblastoma (EUNB) study for the treatment of children over 1 year of age with localized unresectable tumor as defined by IDRF and without MYCN amplification. Since recent reports indicate that patients with tumors presenting segmental chromosome aberrations (SCA) and MYCN single copy have a poor outcome, we studied the tumor genetic profile of the patients enrolled in the EUNB.

Methods: Between January 2001 and October 2006, 160 patients from 10 different European countries were enrolled in the study. Genetic profile of 103/160 tumors was studied by a multilocus approach (60 samples by Multiplex Ligation-dependent Probe Amplification, 42 by BAC/PAC array-CGH and 1 by SNPs array). All genetic data underwent revision by members of the SIOPEN Biology Group.

Results: Multigenomic analysis showed the presence of one or more SCA in 52 (50%) tumors. Of the 7 recurrent aberrations (gain of 1q, 2p, 17q; loss of 1p, 3p, 4p, 11q), the most frequently observed were 17q gain (33%), 11q loss (23%), and 1p loss (22%). We performed OS and PFS based on age stratification and SCA. In children less than 18 months of age, no significant difference in OS and PFS was observed in presence or absence of SCA. In children over 18 months of age, both OS (in presence of SCA: 62.9%; in absence of SCA 95.8%) and EFS (in presence of SCA: 47.4%; in absence of SCA: 73.1%) were significantly correlated with the presence of SCA (p=0.013 and p=0.035).

Conclusion: We have shown that in patients with not MYCN amplified tumors, the presence of at least one SCA in children >18 months was associated with poor OS and PFS. Our results indicate that both variables, the presence of age over 18 months and the SCA, are significantly associated with patient's poor outcome.

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POT114

Imaging the influence of hypoxia on neuroblastoma cell behaviour in live chick embryos

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Background: Hypoxia occurs in solid tumours such as neuroblastoma and has been suggested to promote reprogramming of neuroblastoma cells leading to the emergence of an 'aggressive' stem cell phenotype able to evade chemotherapy and resulting in poor clinical prognosis. We herein investigate the role of the oxygen microenvironment on the dedifferentiation and aggressiveness of neuroblastoma cells in vivo. Most in vivo findings are based on histological methods, end point measurements and fixed samples which fail to address the spatiotemporal behaviour of tumour cells in patients. Here, we utilise the avian model to study the influence of hypoxia on neuroblastoma invasion and migration over time permitting real time visualisation of cellular dynamic events in the intact living organism.

Methods: Neuroblastoma cells were labelled with fluorescent proteins and grown as adherent cells or tumour spheres under normoxia or hypoxia. Cell proliferation, stem cell and differentiation markers were analysed by immunocytochemistry, histological methods and live whole embryo imaging. To monitor the dynamic events of neuroblastoma cells and their ability to invade tissues and form tumours, single-cell suspensions were injected into chick embryos and imaged with a custom-built high-resolution fluorescent microscope.

Results: Neuroblastoma cells grown as tumour spheres in severe hypoxia form very quickly tumour-like structures in the chick embryo compared to co-injected adherent cells grown in normoxia. Video and image analysis of the first 24 hours post injection permitted the analysis of the dynamic cellular events. We are currently comparing the cells grown in different oxygen conditions in terms of their ability and rate to invade, migrate and form tumours in host tissue.

Conclusions: The ability to study the impact of hypoxia on neuroblastoma cell dynamics in live intact embryos using high-resolution imaging is an important step toward understanding neuroblastoma behaviour and the molecular mechanisms underlying its aggressiveness and unpredictable clinical behaviour.

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POT115

Neuroblastoma express a novel EGFR extracellular deletion mutant that is structurally similar to, but biochemically distinct from EGFRvIII

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Background: High risk neuroblastoma(NB) has a high mortality. Survivors also suffered long term side effects. More precise, less toxic therapy is urgently needed. EGFR has become a popular cancer target. Interestingly, some patients with refractory neuroblastoma showed partial responses to EGFR tyrosine kinase inhibitor(TKI). Since EGFR mutations are now believed to be biomarkers for EGFR-directed therapy response, we hypothesized that EGFR mutations might be present in primary NB.

Methods: 62 snap frozen primary NB were homogenized; the supernatant used for protein and RNA analyses. EGFR expression was analyzed by Western blot. cDNA from NB tumor and cell lines were screened for EGFR mutations using primers to exon 1&8 and exon 17&22. EGFRΔ768 and EGFRvIII were subcloned into a wild type(WT) EGFR-GFP expression vector. GFP, WT-EGFR, EGFRΔ768 and EGFRvIII vectors were transfected into NIH3T3 and SY5Y cells. Their proliferative and migratory potential were analyzed by XTT and Matrigel assays; their phosphorylation status, responses to EGF and EGFR TKI were analyzed by Western blot.

Results: 64% neuroblastic tumors express WT-EGFR; no activating kinase mutations were found. However, 34% express deletion mutants of EGFR. 6 express EGFRvIII; the other 15 express a novel deletion mutation, EGFRΔ768, which has an in-frame deletion of the extracellular domain from nucleotide 102 of exon 2 to nucleotide 869 of exon 7. The resulting transcript has 11 more amino acids than EGFRvIII. EGFRΔ768 expression was also detected in 1 of 5 NB cell lines. Cells overexpressing EGFRΔ768 proliferate and migrate significantly more than controls. While both EGFRΔ768 and EGFRvIII are constitutively active, EGFRΔ768 is autophosphorylated at higher level and significantly more resistant than EGFRvIII to biochemical inhibition by EGFR TKI at the lower concentration.

Conclusion: 24% of neuroblastic tumors express a novel EGFR deletion mutant, EGFRΔ768. This mutant is structurally similar to EGFRvIII, but has distinct biochemical properties.

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POT116

Inhibition of CSNK1e, a MYC-synthetic lethal gene, interferes with SHH and WNT signaling

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Background: Through functional genomics screening, we previously identified and validated CSNK1e as being a gene whose expression is essential for the viability of MYCN-amplified neuroblastomas. As CSNK1e is a key regulator of SHH and WNT signaling, we wished to test the hypothesis that these pathways were differentially activated in the context of MYCN-amplified vs non-amplified neuroblastoma.

Methods: RNAi and small molecule inhibitors against CSNK1e were used to elucidate the signaling pathways perturbed by CSNK1e inhibition in two MYCN-amplified (SKNBE2 and IMR32), and two MYCN non-amplified (SKNAS and SY5Y) NB cell lines.

Results: We found both the SHH- and canonical WNT-mediated transcriptional responses were markedly elevated in MYCN-amplified vs non-amplified neuroblastoma cell lines. However, RT-PCR and western blot analysis suggested that other members of SHH and WNT signaling may be important for non-MYCN amplified cells, a hypothesis confirmed through focused siRNA screening of a large subset of SHH and WNT pathway members. The involvement of CSNK1e in WNT and SHH activity was confirmed by CSNK1e knockdown by RNAi, and inhibition through IC261 treatment. In both cases, the inhibition of CSNK1e reduced canonical WNT and SHH signaling only in MYCN over-expressing cells. We provide evidence that at least part of this effect may be due to a positive correlation between CSNK1e and the expression of Smo, a key receptor for SHH.

Conclusion: These results demonstrate a dichotomy for the requirement of certain SHH/WNT pathway members with respect to MYCN-amplification in neuroblastoma. CSNK1e inhibition at least partially blocks both pathways in MYCN-amplified, but not non-amplified neuroblastoma, which subsequently leads to the selective killing of MYCN-amplified cells, possibly through regulation of Smo levels. Finally, the identification of a distinct subset of WNT/SHH pathway members as specifically important in non-MYCN amplified neuroblastomas, provides a means by which this subset of cancers could be targeted therapeutically.

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POT117

Next generation anti-GD2 Monoclonal Antibody: Fc-Receptor (FcR) Affinity Maturation to Improve Antibody Dependent Cell Mediated Cytotoxicity (ADCC)

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Background: Murine 3F8 (m3F8) mediates efficient ADCC in vitro and shows anti-neuroblastoma activity in phase I/II studies. This murine antibody was humanized to circumvent human anti-mouse antibody (HAMA) response, and its Fc engineered to enhance ADCC properties.

Methods: Chimeric 3F8 (ch3F8), humanized 3F8 (hu3F8-IgG1 and hu3F8-IgG4), as well as enhanced FcR affinity variants hu3F8-IgG1n (with special glycosylation) and hu3F8-IgG1n-FcMut (with Fc mutations) were produced in CHO cells, and purified by protein A affinity chromatography. In vitro comparisons were made with m3F8 and other anti-GD2 antibodies in binding, cytotoxicity, and cross-reactivity assays. Biodistribution and anti-tumor effects of hu3F8 were compared with m3F8 in nude mice bearing established neuroblastoma xenografts.

Results: Docking studies using X-ray crystallographic structure of m3F8 demonstrated its superior affinity when compared to other anti-GD2 antibodies. In GD2-binding studies by surface plasmon resonance, ch3F8 and hu3F8 maintained KD similar to that of m3F8, with ~10 fold slower koff than 14G2a. Similar to m3F8, both ch3F8 and hu3F8 inhibited tumor cell growth in vitro, while cross-reactivity with other gangliosides was comparable to that of m3F8. Peripheral blood mononuclear cell (PBMC)-ADCC and neutrophil (PMN)-ADCC using ch3F8-IgG1, hu3F8-IgG1, hu3F8-IgG1n, and hu3F8-IgG1n-FcMut were more potent (10 to >1000 fold) than m3F8. This superiority was consistently observed in ADCC assays, irrespective of donors or NK92MI-transfected human CD16 or CD32, whereas complement mediated cytotoxicity (CMC) was reduced. As expected, hu3F8-IgG4 had near absent PBMC-ADCC and CMC. When compared to m3F8, hu3F8 had similar tumor to normal tissue ratios in biodistribution studies, but more effective anti-tumor effect against neuroblastoma xenografts.

Conclusions: Humanizing m3F8 plus affinity maturation for enhanced FcR binding can produce next generation anti-GD2 antibodies with substantially more potent ADCC in vitro and anti-tumor activity in vivo. By leveraging ADCC over CMC, they may be clinically more effective, while minimizing pain and HAMA side effects.

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POC01 – POC63

POC01
The Treatment Of Children With Low And Intermedium Risk Neurobastoma
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Purpose: the evaluation of treatment of children with low and intermedium risk neurobastoma.

Methods and Materials: from 2007 to 2011 years we had been observing 86 patients with morphologically confirmed neuroblastoma at the mean age of 2,6 years (from 1 month till 15 years). There were 24 patients with stage I; 20 patients with stage IIA, 22 patients - IIB, 20 patients with stage III, and 4 patients with IVS stage of the disease. All patients received therapy according COG P9641, A3961 protocols after thoroughly evaluation (imaging, MIBG, bone marrow morphology, molecular studies, immunohistochemistry). Surgery treatment was performed before chemotherapy. Stage by INSS and group of the risk were established after histological examination. The general scheme of the treatment included chemotherapy (used doxorubicin, cyclophosphamide, ethoposide, carboplatine) and RT.

Results: 85 (98,6%) patients (from 86) were alive without disease with a followup of 5 to 44 months. One patient at the age of 1 month with multiple liver metastases died from toxicity of chemotherapy.

Conclusion: our data confirm the overall good prognosis of localized NBs. Chemotherapy allows surgical excision and excellent outcome in children with localised and unresectable NB. Less intensive chemotherapy should be investigated in such patients.

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POC02
Phase I Trial of Polyethylene Glycol (PEG) Conjugated SN38 in Pediatric Patients (pts) with Recurrent or Refractory Neuroblastoma (NB) and Other Solid Tumors
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Background: Topoisomerase inhibitors are used for treatment of pts with relapsed NB and other solid tumors. EZN-2208 is a water-soluble PEG conjugate of the topoisomerase inhibitor SN38, the active metabolite of irinotecan (IRN). EZN-2208 has a prolonged half-life, permitting extended exposure to SN38. In preclinical NB models, antitumor activity of EZN-2208 was superior to that of IRN. The maximum tolerated dose (MTD) of EZN-2208 in adults was 16.5 mg/m2/dose administered every 21 days (d); febrile neutropenia was dose-limiting. A phase I trial to define dose limiting toxicities (DLTs) and MTD of EZN-2208 in children was conducted. Recruitment of NB pts was encouraged.

Methods: Escalating doses of EZN-2208 were administered on d1 of a 21-d cycle with PEG-filgrastim support. Five dose levels (12-30 mg/m2) were evaluated using a rolling-six design.

Results: 29 pts [median age 11y, range 2–21y] were treated. 26 pts with neuroblastoma (8), CNS tumors (6) osteosarcoma (4), rhabdomyosarcoma (2), synovial sarcoma (2), or other tumors (4) were eligible and fully evaluable for toxicity. A median of 2 cycles (range 1–19) per pt were delivered. Cycle 1 DLT was observed in 1 pt treated with 16 mg/m2 (diarrhea), 1 pt treated with 24 mg/m2 (dehydration), and 2 pts treated with 30 mg/m2 (thrombocytopenia). The MTD is thus 24 mg/m2. Additional ≥Grade 3 regimen-related toxicities occurring in >1 evaluable pt included neutropenia (11), lymphopenia (5), leukopenia (6), thrombocytopenia (7), anemia (4), nausea/vomiting (4). 2 pts had Grade 3 infusion reactions precluding completion of first dose. 2 pts with NB and bulky soft tissue disease had confirmed partial responses (RECIST) lasting >5 months.

Conclusion: EZN-2208 given every 21d is well tolerated in children with solid tumors. The MTD (24 mg/m2) in children is higher than in adults. Durable PRs were observed in pts with NB. Further development is warranted.

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POC03
Double Scattered Proton Therapy (DSPT) versus Intensity Modulated X-Ray Therapy (IMRT) for Patients (pts) with High-Risk Neuroblastoma (HRNB)
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Introduction: Proton therapy as part of multimodality treatment for HRNB may offer significant sparing of organs at risk (OAR) when compared to IMRT.

Methods: We evaluated nine consecutive pts with HRNB and ≤2 sites requiring RT for DSPT as part of multimodality treatment. DSPT and IMRT plans for 2160 cGy (3600 cGy to residual post-operative disease) were developed for all. We calculated clinical target volume (CTV) and OAR doses in radiobiologic-equivalent-weighted absorbed dose (cGyRBE).

Results: CTV coverage was excellent using both DSPT and IMRT: median % dose delivered to 95% CTV was 99% and 100%, respectively. In 5/6 pts with lateralized disease, DSPT offered significant sparing of the contralateral kidney (CK) both with regard to median dose and dose to 20% (D20%) (median 5.15 v 421, p=0.007; median 97.5 v 703.5, p=0.04, respectively), and was used for treatment. DSPT reduced neither median dose, nor D20%, to the ipsilateral kidney (IK; median 1819 v 1056, p=0.2; median 2121 v 1947, p=0.09, respectively). One pt, for whom DSPT did not provide CK sparing, received IMRT due to improved dosimetry to the IK. Overall, DSPT improved median bowel (median 3.3 v 590, p=0.04), total body (median 0.11 v 27, p=0.002), and liver dose (median 0.13 v 529, p=0.002), and liver D20% (median 19.5 v 980, p<0.001). One pt required RT to the chest; DSPT reduced median heart dose (1.1 v 44) and lung volume receiving 20Gy (9.7% v 5.5%).

Conclusions: For most pts, DSPT reduced radiation exposure to the CK, liver, bowel and total body compared to IMRT; for one, IMRT offered improved bilateral renal dosimetry. Comparison of DSPT and IMRT plans is recommended for HRNB pts with access to DSPT, as is monitoring of local control. In the future, use of scanning-beam PT may increase conformality and further decrease exposure of OAR.

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POC04

MYCN-amplified neuroblastoma presenting with a unique histologic pattern due to focal protein expression: A case report

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Background: The vast majority of MYCN-amplified neuroblastomas express N-myc protein diffusely, presenting with the Unfavorable Histology (UH) feature and leading to a poor prognosis for the patient. While the protein expression can be completely blocked in extremely rare genotype-phenotype discordant peripheral neuroblastic tumors with MYCN amplification (indicating a poor prognosis) and Favorable Histology (FH, indicating a better prognosis), and the patients with those tumors have an excellent prognosis (presented at the 2011 Fall Meeting, Society for Pediatric Pathology).

Material and Methods: We present a neuroblastoma case of right adrenal primary in a patient with stage 4 disease diagnosed at 6 months of age. Unusual characteristics of the tumor were demonstrated histologically (International Neuroblastoma Pathology Classification), molecularly (MYCN FISH), and immunohistochemically (N-myc protein expression).

Results: The tumor was composed of two different histologies: FH (neuroblastoma, differentiating subtype with a low mitosis-karyorrhexis index [MKI]) and UH (neuroblastoma, poorly differentiated subtype with a high MKI). These two histologic areas were well-blended and showed a checker-board pattern under the microscope. After examining a total of 200 cells including ~90% tumor cells from both FH and UH areas, all neuroblasts were determined to have MYCN amplification with more than 20 copies of 2q23-24 gene region compared with 2 control signals for chromosome 2. Immunohistochemically, N-myc protein was expressed only in those UH areas. In contrast, neuroblasts in the FH areas did not express N-myc protein despite of MYCN amplification. The patient was treated according to the COG High-Risk Neuroblastoma Protocol, and will be a candidate for a trial of immunotherapy with Chimeric Antibody 14.18.

Conclusions: This is the first documented case of neuroblastoma with diffuse MYCN amplification associated with focal protein expression causing a mixture of FH and UH. Protein expression rather than DNA amplification seemed to be responsible for histologic changes in this tumor.

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POC05

Use of Multiplex Ligation-Dependent Probe Amplification to Evaluate Genetic Aberrations in Neuroblastoma – A Pilot Study in Singapore

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Background: Neuroblastoma tumor samples in Singapore are routinely tested for MYCN amplification, 1p loss and 17q gain using fluorescent in-situ hybridization (FISH). Multiplex ligation-dependent probe amplification (MLPA) is a variation of multiplex polymerase chain reaction (PCR) which can analyze several target sequences in one single multiplex reaction, and hence is increasingly utilized in a variety of research and diagnostic settings, including tumor characterization. We perform a pilot study to analyze genetic aberrations in our neuroblastoma tumors using MLPA.

Methods: We analyzed 40 neuroblastoma tumor samples. FISH using DNA probes for MYCN (2p24.1), CHD5/p58 (1p36.3) and MPO (17q22) were performed on tumor touch imprints at diagnosis. MLPA using SALSA neuroblastoma kit P251/252/253 was then performed retrospectively on extracted tumor DNA. Selected samples were also analyzed by array-CGH (aCGH) using Agilent SurePrint 3G Human aCGH 4x180k.

Results: There was correlation of MLPA and aCGH results with FISH results for MYCN, 1p and 17q status. In addition, MLPA and aCGH also showed that 10 of our tumor samples had 11q deletion, previously not analyzed by FISH. Seven (70%) of these tumors with 11q deletion had concurrent 1p deletion (n=1), 17q gain (n=4) or both (n=2). Only one (10%) had concurrent MYCN amplification and 17q gain. Two (20%) tumors with 11q deletion did not demonstrate MYCN amplification, 1p deletion or 17q gain.

Discussion: Our pilot study showed good correlation of MLPA results with aCGH and FISH. 11q loss is known to be a poor prognostic marker in neuroblastoma, and also known to be found in subset of tumors that do not demonstrate MYCN amplification. We should include evaluation of 11q status into our routine neuroblastoma diagnostics in Singapore. MLPA has the potential to be a cost-effective tool in neuroblastoma diagnostic workup.

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POC06

INFANTS WITH NEUROBLASTOMA PRESENTING WITH SYMPTOMATIC EPIDURAL COMPRESSION

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Background: Symptomatic epidural compression (EC) in neuroblastoma requires immediate treatment. Chemotherapy, neurosurgery and radiotherapy are all effective, but may cause severe late complications. Infants are conceivably more susceptible to develop these effects, but there are no related publications. We describe 6 such patients observed in a 5-year period.

Patients: Between 2007-2011, a total of 109 children were diagnosed with neuroblastoma at Santobono-Pausilipon, Napoli, or Giannina Gaslini Children's Hospital, Genova, Italy. Eleven of them (10.1%) had EC, of whom 6 (5.5%) where infants. Motor deficit was graded from 0 (none) to 3 (flaccid paraplegia).

Results: M/F ratio was 4:2. Age at diagnosis ranged from 3 to 11 months (median, 10). Interval between EC symptoms and tumor diagnosis was 2-240 days (median, 7). The level of EC was cervico-thoracic in one patient, thoracic in 2, thoraco-lumbar in 2, lumbo-sacral in one. Symptoms included grade 2 motor deficit in 4 patients, grade one in one. One patient had severe back pain and respiratory distress. One patient was treated by chemotherapy alone, two underwent laminectomy and 3 laminotomy first. All patients recovered neurologically in 0.5-6 months (median, 2). Primary tumor was resected completely in 5 patients, incompletely in one. The latter patient presented tumor regrowth requiring additional chemotherapy. All patients are alive and well from 8 to 62 months (median, 20). One patient presents equinism of right foot.

Conclusions: Angelini et al reported that infants with neuroblastoma and EC developed more late complications than older children, especially when EC was treated by neurosurgery. Despite recommendation to limit neurosurgery when EC symptoms evolve rapidly, the tendency to operate is gaining supporters, in part because neurosurgeons claim that modern approaches are significantly safer. In our series, five patients underwent neurosurgery (3/5 were laminotomies) with excellent outcome, although follow-up is short. A larger study is in progress.

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POC07

A phase I/II study of 131I-Meta-Iodobenzylguanine (MIBG), hyperbaric oxygen (HBO) and Vitamin C in patients with recurrent neuroblastoma (NBL).

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Purpose: Addition of radio-sensitizing agents can improve targeted-radiotherapy with 131I-MIBG in NBL. Hyperbaric oxygen (HBO) with 131I-MIBG was shown to improve quality of life and survival in relapsed NBL patients. We investigated the potency of additional Vitamin C to HBO. Vitamin C is cytotoxic in vitro to NBL cells and potentially increases the formation of radicals in combination with HBO.

Materials and Methods: Relapsed patients with MIBG accumulation expected to sustain minimally two courses of 131I-MIBG + HBO/ Vitamin C were eligible. Vitamin C starting dose (100 mg/kg) was escalated (50 mg/kg) for each intra-patient course and the start dose was increased for each consecutive cohort of 3 patients. At 4 weeks interval, courses of combined therapy were given after 4 weeks followed by an evaluation. Primary objectives were maximum tolerated dose (MTD) of vitamin C and the overall response of treatment. The excretion pattern of catecholamines (CME) metabolites (i.e. HMM and HVA) ratio and O-methyl derivatives (metanephrines) were studied. Response was scored according to the International Neuroblastoma response Criteria (INRC). If patients were not eligible for response evaluation, the best clinical response after the last treatment was registered, using CME, imaging and clinical condition.

Results: Twenty-two patients (16 males) were included. The MTD of vitamin C was 250 mg/kg/day, because of intake problems. The overall response rate in 22 patients was 32% (7/22). Ten evaluable-patients showed 3 very good partial responses (VGPR), 2 partial remissions (PR) and 5 stable diseases (SD). Twelve non-evaluable patients showed; 1 VGPR, 1 PR, 1 lost to follow up, 9 progressive diseases (PD). Although the CME increased, the pattern of CME corresponded with a more mature pattern. Haematological toxicity was comparable to treatment without vitamin C.

Conclusion: 131I-MIBG with HBO and vitamin therapy is feasible with an overall response rate of 32%.

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POC08

Treatment with Long-term Topotecan Plus Cyclophosphamide in Children with Recurrent or Refractory Neuroblastoma: Hospital for Sick Children Experience

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Background: Reports of the response and toxicities for salvage therapies for relapsed neuroblastoma are rare and often confounded by effects of preceding and subsequent treatments. Our objective was to describe the outcomes, toxicities, and quality of life for children treated with an outpatient regimen of topotecan and cyclophosphamide (TOPO/CTX) for first relapse of neuroblastoma.

Methods: We retrospectively reviewed charts of relapsed and refractory neuroblastoma patients treated between 1999 and 2009 with our standard of care outpatient TOPO/CTX (0.75mg/m2/day and 250mg/m2/day x 5 days q 3-4 weeks) for up to 2 years.

Results: Twenty-eight patients received 349 cycles of TOPO/CTX (median= 10 cycles per patient, range =1 to 32). The majority of patients (n=25) had undergone autologous stem cell transplantation. Seventeen (61%) patients had an objective response (CR + PR + MR). The 3-year progression-free survival (PFS) after relapse was 16% ± 9% and the 3-year overall survival (OS) after relapse was 34% ± 10%. The median PFS was 1.3 years and the median OS was 2.4 years. Eight patients are alive with follow-up of 1.7 to 4.4 years. The majority of patients experienced chemotherapy delays, transfusions, and febrile neutropenia (n= 8 bacterial infections). The mean number of hospitalized days, measured as a surrogate for quality of life, was less than one day per cycle. Shorter time from diagnosis to first relapse (6 to 18 months) was significantly associated with shorter OS (p=0.0019).

Conclusions: Long term TOPO/CTX in patients with first relapse following stem cell transplant was well tolerated. The point-estimates of PFS and OS were higher than reported by POG 9462, in which patients received up to 12 cycles. Our study will provide additional historical endpoints, toxicity and time to progression data against which new agents and combination therapies using TOPO/CTX as a backbone can be measured.

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POC09

The expression of Retinoic Acid Related Genes in Neuroblastoma can predict the Patients' Prognosis.

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Background: The Retinoic Acid (RA) signaling pathway is made up of several RA related factors such as ZNF423, NF1, and another downstream factor, HOX cluster. This study found that the mRNA expression levels of these RA related genes were useful prognostic factors in neuroblastoma patients.

Method: Neuroblastoma cell lines, SK-N-SHSY5Y, IMR32, GOTO, KP-N-RTBM1, and SK-N-AS were cultured in with all-trans RA. The expression levels of NF1 and HOXC9 were examined in those cell lines as well as 42 tumor samples by RQ - PCR and expression ratios were calculated. The correlation of the expression ratios of those genes with the clinical stages, age and prognosis by was evaluated using the Mann-Whitney U-test, log rank test, and Cox proportional hazard model.

Result: The expression of NF1 and HOXC9 was lower in SK-N-AS than in the other cell lines before RA stimulation. HOXC9 was upregulated in SK-N-SHSY5Y 72 hours after RA stimulation, but not in SK-N-AS. NF1 and HOXC9 expression was significantly lower in the tumors from stage 4 patients than from the stage 1 patients ($p<0.05$, Mann-Whitney U-test). HOXC9 were significantly higher in the patients < 18 months old than in those 18 months old ($p<0.05$, Mann-Whitney U-test). The five-year over-all survival rates were significantly lower in the NF1 low/HOXC9 low group than the NF1 high/HOXC9 high group (25%, and 96.6%, respectively. $P<0.01$, log rank test). The Cox proposal hazard model showed that the hazard ratios of NF1 and HOXC9 expression were 7.872 and 3.328, respectively.

Conclusion: These experiments suggested that NF1 controlled the expression of HOXC9 through the RA signaling pathway in neuroblastoma cell lines. The analysis of tumor samples showed the observed low expression ratios of both NF1 and HOXC9 to correlate with a poor prognosis and these ratios can predict the prognosis in neuroblastoma patients.

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POC10

High Risk Neuroblastoma treated with the MSKCC guidelines at Hospital Sant Joan de Déu, Barcelona. Intensive Minimal residual Disease monitoring and Outcome.

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Background: We report our institutional experience following the Memorial Sloan Kettering Cancer Center (MSKCC) guidelines for the management of High-Risk (HR) Neuroblastoma (NB). Primary goal was to achieve the MSKCC N7 response rates after induction as a necessary condition to improve outcome. Secondary aim was the early and continued monitoring of bone marrow (BM) minimal residual disease (MRD) to predict outcome.

Methods: Thirty consecutive HR-NB patients (pts) (22 stage 4 and 8 non-stage 4) were prospectively (2004-2011) enrolled. Treatment included: modified MSKCC N7 induction regimen; surgical resection after 3 courses; only after complete remission (CR) and negative MRD attainment, consolidation followed with two cyclophosphamide-topotecan based cycles or one topotecan based ABMT; hyperfractionated RT (21Gy); and isotretinoin plus m3F8 and GM-CSF for stage 4 cases. BM MRD analysis included the analysis of GD2 synthase, PHOX2B and cyclin D1 mRNAs.

Results: After 3 cycles and before surgery, all patients showed BM histological response and 10 (34%) of 29 were in CR by MRD analysis (early responders). At the end of induction, 123I-MIBG was normal in 23 (76%) cases, 15 (50%) showed MRD and 29 (96%) immunocytology negative BM. CR was documented in 20 (66%), VGPR in 2, PR in 7 and progressive disease (PD) in one. CR or VGPR was achieved in 22 (73%) patients. Disease progression during treatment occurred in 5 (16%) and relapse (two in the CNS) in 12 (40%) patients. Nineteen (63%) patients remain alive, 15 in continued CR, median follow-up 32 (12-90) months. All 10 early BM MRD responders are alive (median follow-up, 32 months), 7 of these with
[3] 5-year overall survival, (P=0.001). Conversely, 11 of 20 MRD positive patients have died (median follow-up, 21 months).

Conclusions: This dose-intensive, short induction regimen achieved our primary goal with 73% CR. Early negative BM MRD predicts long-term survival.

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POC11

Development of an open-source, flexible framework for interinstitutional data sharing and collaboration

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Background: Clinical information, "-omic" datasets, and tissue samples are difficult to harmonize and manage for advanced data mining. We believe that clinical research data can be centralized and provide direct access to sample availability and associated data from a variety of information stores.

Methods: We obtained a standardized set of patient data from the International Neuroblastoma Risk Group, consisting of more than 11,000 children diagnosed worldwide between 1974 and 2002. The data consist of 34 metrics, including age and stage of tumor at diagnosis and other clinical and biological markers. We instantiated the dataset into a Postgres database, and using the Django web framework, created a data model for rapid development of tools and views and built a front-end interfwace for generating complex queries. To test the feasibility of accessing information on disparate and geographically distinct data samples, we have a formal agreement with the Children's Oncology Group Tumor Bank at The Research Institute at Nationwide Children's Hospital. Based on query results, we consume the Tumor Bank tissue inventory data through a web-facing application programming interface. The end-user is presented only with the number of patients who match their query search terms and for whom tissue samples are available.

Results: Since our initial implementation, we have collaborative agreements with other consortium groups. We have developed a paradigm for statisticians to securely update and add data with internal validity checking. Our system can initiate queries and accept results in a variety of standards-compliant formats and will be available in demonstration form by May 2012.

Conclusions: Querying patient data while interrogating external sources allows researchers to seamlessly observe, request, and download available data and samples. While designed around a neuroblastoma dataset, our system can be applied to a variety of clinical scenarios and will be made available through an open-source license.

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POC12a

Impact of post-induction Curie scores as prognostic marker in high risk neuroblastoma. A Children's Oncology Group report.

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Post-induction Curie scores (CS) have been reported as predictive for outcome in patients with high risk, MIBG avid neuroblastoma. We now examine post-induction CS in the context of other reported clinical and biologic factors.

Study Design: 237 patients with stage 4, MIBG avid neuroblastoma treated on COGA3973 were examined. MIBG scans were evaluated at 10 anatomic regions, with each site scored 0-3 based upon the extent of disease in each region. Cox proportional hazards models were used to determine the prognostic strength for survival in the presence of other prognostic factors.

Results: A post-induction CS=2 had the greatest sensitivity and specificity in predicting outcome, compared to any other cut-point. Patients with a post-induction CS≤2 (n=185) had superior outcomes than those with CS>2 (n=52) [3-yr EFS: 44.9±3.9% vs 15.4±5.3%,p<0.001]. In patients with MYCN amplified disease, the difference in 3-yr EFS was even more significant [3-yr EFS: 44.2±6.9% (CS≤2) vs 0.0%(CS>2),p<0.001]. Using the Cox model for predicting EFS, a post-induction CS>2 corresponded to a 2.473 (n=187;p<0.001) increased risk of an event, with CS>2 carrying greater significance in predicting outcome than (in order) age, histologic grade, MKI indices, and MYCN status. If extra-osseous disease was excluded from the analysis, a post-induction CS>3 was associated with markedly inferior outcomes [3-yr EFS: 9.5±5.2% (CS>3) vs 42.1±3.5% (CS≤3),p<0.0001], with a 5-yr EFS=0.0% if CS>3. Post-induction, the presence of residual MIBG-avid osseous disease in any site other than the cranial/facial region was associated with a 3-yr EFS <20.0%. In patients with a CS≤2 post-induction, 3-yr EFS was not impacted if chimeric antibody (ch14.18) was given (n=39) or not given (n=146) during maintenance [48.7±8.0% vs 43.9±4.5%,p=0.53]

Conclusion: Post-induction Curie scores (CS>2) identify a cohort of high risk neuroblastoma patients who are at greater risk for an event independent of other standard predictive measures.

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POC12b

MIBG scoring as a predictor of early relapse or disease related death in high risk neuroblastoma. A Children's Oncology Group report.

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A semiquantitative MIBG scoring method (Curie scoring) has been developed as a prognostic indicator in high risk neuroblastoma. The use of the Curie scoring (CS) to predict patients at greatest risk for early relapse or disease related death is now examined. **Design:** Newly diagnosed patients with stage 4, MIBG avid neuroblastoma enrolled on COGA3973 were examined(n=280). MIBG scans were evaluated at 10 anatomic sites, with sites scored 0-3 based upon the extent of disease per site. Differences in CS between patients with early events (relapse or disease related deaths <12 months from diagnosis or post-induction) versus no early events were examined. Logistic regression models were fit to determine if CS was predictive of the timing of an event. Patients with non-disease related deaths were censored.

Results: At diagnosis, the median CS for patients with an early event was 13(n=62) vs a median CS=13 for those(n=207) without an early event, p=0.92. Post-induction, the median for patients(n=72) who experienced an early event was 13 vs a median CS=0 for patients(n=157) in which no early event occurred. Logistic regression modeling indicated that CS were predictive of early post-induction events, with each 1 unit increase in Curie score associated with a 5.2% increase in the odds of an event occurring <12 months from the end of induction. For patients with a CS=0 post-induction, the odds of an early event occurring were 0.398, increasing to 0.419 for CS=1, 0.514 for CS=5, and 0.661 for CS=10. Thus, patients with a CS=10 post-induction had a 66% greater risk of an event compared to patients with a CS=0 post-induction.

Conclusion: Curie scores were not predictive of early events from diagnosis. However, CS were highly predictive of events occurring from the completion of induction, with each incremental increase in CS associated with a 5.2% increased risk of an event.

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POC13

Clinical features of neuroblastoma patients after discontinuation of mass screening

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Background: The mass screening (MS) of neuroblastoma (NB) in Japan was discontinued in 2004 because the patients detected by MS usually showed favorable prognosis. In this study, we analyzed the patients' profiles and outcomes with NB after discontinuation of MS (101 cases, from 1988 to 2003).

Methods: We retrospectively reviewed the charts of 24 patients with neuroblastoma who were treated from 2004 to 2011 at our institution. We investigated their clinical and biological features, and compared the outcome between before and after discontinuation of MS.

Results: The age ranged from 1 to 156 months (median age, 30 months). There were 6 patients with International Neuroblastoma staging system stage 1 or 2 disease, 3 patients with stage 3 disease, 15 patients with stage 4 disease. Eleven patients were detected by the symptoms caused by the primary tumors. Eleven patients were detected by the symptoms caused by the metastatic tumors. Two patients were detected by chest X-ray or MRI conducted because of nonrelated symptoms. The primary tumor sites were the adrenal grand region in 14 patients and the nonadrenal grand region in 10 patients. Urinary levels of vanillymandelic acid (VMA) and homovanillic acid (HVA) were elevated in all patients. MYCN was amplified in 6 patients. DNA content was aneuploid in 5 patients and diploid in 13 patients. Overall survival rate tended to be better in the patients after discontinuation of MS (91.7%) than the patients before discontinuation of MS (86.2%) (p=0.40).

Conclusion: The survival rate of the patients with NB after discontinuation of MS tended to be better than the patients before discontinuation of MS although the number of early stage NB was reduced after MS was discontinued. This study suggested that the progress of treatment against advanced stage NB provided improvement of the survival rate of NB.

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POC14

Comparison of PCR and flow cytometry results of bone marrow involvement assessment

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Background: Bone marrow (BM) involvement detection in neuroblastoma is a useful tool for patients stratification, prognosis defining and risk-adapted treatment performing. Real-time quantitative PCR (RQ-PCR) of tumor-specific gene transcripts and multicolor flow cytometry (FC) are commonly applied for this purpose.

Aim: To evaluate qualitative concordance between RQ-PCR and FC data of BM involvement detection in neuroblastoma patients at various treatment stages.

Methods: PHOX2B gene expression detection and tumor cells percentage calculation were performed by RQ-PCR and FC respectively in 326 BM samples from 52 neuroblastoma patients. 108 samples were obtained at the time of primary diagnostics, 168 during treatment and 51 at the time of relapse.

Results: Sensitivity of RQ-PCR PHOX2B expression detection achieved 1E-06 while sensitivity of FC ranged from 1E-03 to 1E-05. In 193 (59.2%) samples BM involvement was not detected by both methods, 38 (11.7%) samples were negative by FC but positive for PHOX2B expression while 31 (9.5%) samples were positive by FC but negative for PHOX2B and 64 (19.6%) samples were positive by both techniques. Thus overall qualitative concordance between RQ-PCR and FC data achieved 78.8%. Concordance between two methods was 75.0% in samples taken at the time of diagnosis, 80.8% – obtained during treatment and 80.4% - at the time of relapse diagnostics (p=0.490). In group of localized (stages I-III) and disseminated neuroblastoma (stage IV) concordance values were 80.7% and 79.9% respectively (p=0.981). FC and RQ-PCR data concordance also didn't depend on FC sensitivity (higher or lower than 10-4): 81.1% and 75.6% correspondingly, p=0.292.

Conclusions: Qualitative concordance between PCR-based PHOX2B expression detection and FC for BM involvement detection in neuroblastoma patients achieved 78.8% and did not depend on time of BM sampling, stage of the disease or FC sensitivity.

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POC15

Biological analysis of the first nation-wide clinical trial for high-risk neuroblastoma by Japan Neuroblastoma Study Group (JNBSG)

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Background: JNBSG prospectively enrolled patients with high-risk neuroblastoma according to COG risk group (2008) on the phase II clinical trial. This was the first nation-wide clinical trial by JNBSG, which consist of 5 courses of induction chemotherapy comprised vincristine, cyclophosphamide, cisplatin and pirarubicin, and surgery, followed by high-dose chemotherapy (HDC) comprised melphalan, etoposide and carboplatin with autologous PBSCT, and radiation therapy.

Patients and Method: Between March 2007 and February 2009, 50 patients were enrolled. Median age at diagnosis was 36 months (range 13-174). Forty-five patients were diagnosed pathologically according to the central review, as neuroblastoma (n=40) or ganglioneuroblastoma, nodular type (n=4). Array CGH was used for genomic grouping in 33 patients.

Result: The 3-year OS and PFS for 50 patients were 69.3+/-6.6% and 30.4+/-8.0% respectively. MYCN amplification was observed in 20 patients, whose OS and PFS were 60.0+/-11.0% and 36.0+/-12.0%, respectively, while the OS and PFS of MYCN non-amplified cases were 75.6+/-8.1% (p=0.24) and 29.9+/-8.7% (p=0.57), respectively. Twenty-six patients could complete the whole protocol therapy; 3 experienced disease progression during the protocol therapy. Treatment-related death occurred in 3 patients after HDC. Thirty-two tumors were in partial chromosomal gain/losses (GGP) and only one is in whole chromosomal gain/losses (GGW). In GGP, 11 tumors were P1a (1p loss, MYCN amp) and 11 were P3s (11q loss, MYCN single). The 3-year OS for P1s and P3s were 63.6+/-14.5% and 100+/-0%, respectively; the PFS for them were 36.4+/-17.9% and 40.9+/-15.6%, respectively. Discontinuation of the protocol therapy because of tumor progression or insufficient tumor response occurred in six patients analyzed, and 4 of them were categorized in P3s but none in P1a.

Conclusion: This is the first report of the clinical trial by JNBSG and molecular profiling including genome aberrations will be useful to compare with our future studies and also with other groups' studies.

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POC16

ALARA: "As Low As Reasonably Achievable" radiation exposure to parents during molecular radiotherapy for neuroblastoma.

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Purpose: Paediatric molecular radiotherapy is perceived as challenging and unkind to children because of strict limits on the time parents may spend caring for their children. In the United Kingdom, adults who have been trained in basic radiation hygiene precautions and given informed consent, may be legally designated as comforters and carers (C&C). They may then spend as long as is necessary to care for and comfort their child during treatment without any time limit providing their radiation exposure is kept as low as reasonably achievable (ALARA). The English Health Protection Agency recommends a dose constraint of 5mSv to an individual C&C for a treatment course. This study audits radiation exposure to C&C during the delivery of two different therapies.

Patients and Method: Parents of children receiving molecular radiotherapy with 131I-mIBG or 177Lu-DOTATATE for neuroblastoma were trained in radiation protection precautions, advised of the risks, and gave consent to be C&C. They monitored their radiation exposure during therapy with a personal dose-meter.

Results: Between November 2002 and March 2011, there were 113 administrations of 131I-mIBG, and 22 of 177Lu-DOTATATE. The median age was 6 years (range 1-18 years). The median administered activity of 131I-mIBG was 8.83GBq (range 2.35-35.9GBq); and of 177Lu-DOTATATE was 7.27GBq (range 2.5-7.5GBq). For 131I mIBG the median exposure per comforter and carer episode was 160.5μSv (range1-3104μSv); for 177Lu-DOTATATE the median exposure was 8μSv (range 1-79μSv). The total accumulative exposure within 1 year was in the majority of cases <1mSv. In 18 comforter and carers the total accumulative exposure in one year was >1mSv but <3mSv. Only one comforter and carer received a cumulative exposure above the recommended limit of 5mSv.

Conclusion: Strict time limits on parental contact are not necessary during paediatric molecular radiotherapy, providing C&C are trained to keep their personal radiation exposure ALARA.

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POC17

Evaluation of intensity modulated arc radiotherapy for dose escalation to tumour in high-risk neuroblastoma and reduction of high doses to uninvolved normal organs.

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Purpose: Radiotherapy reduces local recurrence rates in high-risk neuroblastoma. It is conventionally delivered by parallel opposed fields, where tolerance of uninvolved normal organs may compromise delivery of the protocol dose to the Planning Target Volume (PTV). This study evaluates one of several available intensity modulated arc radiotherapy (IMAT) techniques, (RapidArcTM, Varian Medical Systems), for its potential to improve the dose distribution and protocol compliance in neuroblastoma.

Patients and Methods: Twenty patients previously treated with conventional radiotherapy for abdominal neuroblastoma were studied. Ten patients had received the full prescribed dose with conventional planning (protocol compliant group) and IMAT was used to see if the dose distribution could be improved. In the other 10 patients (protocol non-compliant group) it had not been possible to deliver the full protocol dose to the PTV and we examined whether this would have been possible with IMAT.

Results: No difference in PTV volume between the protocol compliant and non-compliant groups ($p = 0.5$) was seen. PTV coverage by D98, homogeneity and conformity indices were all improved with IMAT ($p < 0.001$). In patients with lateralized tumors in the protocol compliant group, there was no significant difference in kidney V15's and ipsilateral mean doses between conventional and IMAT plans but an increase in contralateral mean kidney dose was seen with IMAT. There was a significant reduction in V19 to the liver for all lateralized tumors in the protocol compliant group. In the protocol non-compliant group IMAT improved the dose deliverable to the PTV in all cases, enabling delivery of the full protocol dose in 8 out of 10 cases.

Conclusions: This study has shown that IMAT has potential for improving outcomes in patients with neuroblastoma through improved dose distributions. We plan to evaluate this in a prospective clinical trial.

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POC18

Comparison of Circulating and Bone Marrow Neuroblastoma Cells with a Highly Sensitive Five-Gene TaqMan® Low Density Array Assay

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Background: We developed a TaqMan® Low Density Array (TLDA) assay that quantifies expression of five genes (chromogranin A (CHGA), doublecortin (DCX), dopadecarboxylase (DDC), paired-like homeobox 2B (PHOX2B), and tyrosine hydroxylase (TH) that are highly expressed by NBL cell lines and tumors and are rarely expressed by normal blood cells. This assay has a 6-log dynamic range and a detection sensitivity of one tumor cell per million normal cells. Data are reported as positive or negative for tumor cells and as the geometric mean Cycle Threshold (Ct) of the expression of the five genes (detection gene score=DG).

Methods: 44 paired bone marrow (BM) and blood samples were obtained from patients on COG ANBL0532/ANBL00B1 (diagnosis n=8), COG ANBL0032/ANBL0931 (pre-study n=9, post-study n=25, relapse n=2). Higher DG indicates lower tumor content, with each unit increase equaling ~0.3 log lower tumor content. A DG score of 40 indicated a negative result.

Results: The overall frequency of positivity in BM was 33 (75%), while positivity in blood was 17 (38.6%). BM DG was correlated with blood DG (rank correlation 0.46, p=0.002). Blood signals were less than BM signals (4.7 Ct difference in DGs), but the difference ranged from 8.3 in patients with the BM DG <30 to 1.8 in those with BM DG>35. Only 3 patients (6.8%) whose blood was positive (mean DG 39.4) had undetectable tumor in BM, while in 19 patients (43.1%) BM was positive (mean DG 38.8) but blood was negative.

Conclusions: Tumor is more often detectable in BM than in blood, and the DG is higher in BM, although the 2 are correlated. Future studies will determine if this assay is more sensitive than standard clinical evaluations for quantifying disease and if the DG score in BM and/or BLD is an early surrogate biomarker for clinical outcome.

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POC19

Comparison of a Sensitive Five-Gene Taqman® Low Density Array (TLDA) Assay for Tumor Cells in Bone Marrow and Blood with Histologic Bone Marrow Examination and Imaging for Disease Assessment in Patients with Recurrent/Refractory Neuroblastoma: A New Appro

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Background: Accurate quantification of tumor burden in neuroblastoma patients is needed to define homogenous populations for therapy and establish response criteria that predict outcome. The 5-gene TLDA assay was developed for sensitive quantification of neuroblastoma cells in bone marrow (BM) and blood.

Methods: Expression of CHGA, DCX, DDC, PHOX2B, and TH (neuroblastoma genes) and of B2M, GAPDH, HPRT1, and SDHA (housekeeping genes) was quantified with TLDA. Results are reported as positive/negative for tumor and as the geometric mean Cycle Threshold for the five genes (DG=detection gene score, which is inversely related to tumor content). TLDA was performed on 16 BM and 7 blood samples from 17 patients with recurrent/refractory neuroblastoma. The number of 123I-MIBG avid sites, the longest tumor dimension (LD) by CT/MRI, and BM tumor cells by morphology (positive/negative, percentage) were scored by central review of radiology and pathology reports.

Results: The TLDA assay detected tumor cells in 14/16 (87.5%) BM and 4/7 (57%) blood specimens. For 6 BM/blood samples obtained <6 days apart, the average DG was 30.9 in BM and 37.3 in blood. For all patients, TLDA detected tumor in 11/12 MIBG+ and 3/4 MIBG-, 8/9 CT/MRI+ and 5/7 CT/MRI-, and 9/9 BM+ and 5/7 BM- by morphology. TLDA and morphologic detection of tumor cells in BM (n=16) were correlated when BM was positive (p=.028) and with percent BM tumor cells (p=.0074). There was no correlation with numbers of MIBG sites or of tumor LD.

Conclusions: This TLDA assay detects neuroblastoma cells in both BM and blood in patients with recurrent/refractory neuroblastoma at high rates, and it frequently detects tumor cells when BM morphology and imaging evaluations do not. Further studies are needed to determine if the DG score can be used to quantify response in BM and/or blood and predict outcome.

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POC20

Population Based incidence and improved survival of Patients with Recurrent Neuroblastoma in Ontario, Canada

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Background: Despite substantial improvements in therapy over the past 2 decades, outcomes for neuroblastoma remain suboptimal, particularly in the setting of relapse. We sought to examine trends in survival for newly diagnosed and relapsed neuroblastoma in Canada by reviewing data in the province of Ontario, representing approximately 1/3 of all pediatric cancer cases in Canada.

Methods: Since 1985, the Pediatric Oncology Group of Ontario (POGO) has collected data pertaining to initial diagnosis, treatment and outcome of pediatric oncology patients in all 5 pediatric centers in Ontario. Since 1995, sufficient data has been collected to calculate survival for relapsed neuroblastoma patients. We determined survival and relapse rates using period analyses for 1995-1999 and 2000-2004 for patients with newly diagnosed and relapsed neuroblastoma.

Results: 219 cases of neuroblastoma were diagnosed in Ontario between 1995 and 2004. The cohort had a 5-year Overall Survival (OS) and Event Free Survival (EFS) of 68% and 63%, respectively. 55 of 219 patients had at least one recurrence; 38 were diagnosed between 1995 and 1999 and 17 between 2000 and 2004. 92% of recurrences occurred in stage 4 (non-4S) patients. The 1, 3 and 5-year OS for all relapsed patients were 93, 53 and 11%. The 1, 3, and 5 year OS increased from 89, 47 and 16% for patients diagnosed between 1995 and 1999 to 100, 65 and 29% for patients diagnosed between 2000 and 2004.

Conclusions: Despite recent advances in therapy, outcomes in relapsed neuroblastoma remain poor. Similar to other published registries our data for Ontario patients suggests a trend towards both reduced recurrences and prolonged post-relapse survival in patients more recently diagnosed. This likely reflects both improvements related to up-front therapy and more effective second-line and palliative therapies that prolong survival in recurrent neuroblastoma.

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POC21

Infant Metastatic (4+ 4S) MYCN Non-amplified Neuroblastoma has a very high survival rate with moderate dose chemotherapy

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Background: Infants <12 months of age with widespread neuroblastoma in an INSS Stage 4S or 4 metastatic pattern without MYCN amplification have had favorable outcomes with either observation (4S patients only) or moderate dose chemotherapy regimens. Direct comparisons between these patient groups have been lacking.

Methods: An analysis of 298 patients treated on the recently analyzed Children's Oncology Group Protocols P9641 and A3961 was completed comparing INSS Stage 4 vs. 4S for event free survival (EFS) and overall survival (OS), by biologic, genomic and treatment groupings. To identify factors prognostic of (EFS) and (OS) in the combined 4 and 4S cohort, the following clinical, biologic, genomic and treatment grouping variables, were tested via log-rank tests: Treatment assigned (per risk factors), treatment received, INSS stage, histology, ploidy, 1pLOH, 11qLOH, primary and metastatic site.

Results: Five-year EFS and OS were (78.9±2.8%) and (92.1±1.8%) respectively. EFS was significantly better for stage 4S than 4 patients: a) within those assigned to receive 8 cycles of therapy (93±5.8% [n=29] vs. 68.2±6.2% [n=73] at 5 years; p=0.0096) and b) within those with diploid tumors (89.9±6.7% [n=30] vs. 65.8±6.8% [n=62] at 5-years; p=0.0162). In the combined 4+4S cohort, neither histology nor ploidy were significantly prognostic. Only 26% of tumors were tested for 1p and 11q abnormalities; neither were significantly prognostic. The only significant factors identified were initial treatment assignment (5-year EFS: observation 60.9±8.7% [n=44] vs. 4 cycles 87.7±3.1% [n=148] vs. 8 cycles (75.3±5.0% [n=102]; p<0.0001) and actual treatment received (5-year OS: observation 80.9±7.9% [n=35] vs. 4 cycles 90±3.2% [n=110] vs. 8 cycles 96±1.9% [n=152]; p=0.0149).

Conclusion: Infants < 12 months of age with INSS Stage 4 or 4S MYCN- non-amplified neuroblastoma have excellent outcomes however patients with INSS Stage 4S disease initially observed and/or similar patients who never received chemotherapy had a significantly reduced EFS and OS.

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POC22

A Trial Treatment Of Long-Term Maintenance Chemotherapy For Refractory Neuroblastoma: A Single Institution Experience In 7 Patients

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Objectives:

To evaluate the possibility of long-term maintenance chemotherapy (LTMCT) without high-dose chemotherapy (HDCT) for refractory neuroblastoma (RNB).

Background: Neuroblastoma is the most common malignant solid tumor of childhood. Even though HDCT could be performed, the disease free survival rates of high-risk neuroblastoma would be approximately 40% in recent reports. If HDCT could not be performed, the prognosis would be very poor. In this retrospective case series study, we have evaluated the possibility of LTMCT without HDCT for RNB.

Methods: Retrospective medical charts analysis of 7 children with refractory neuroblastoma admitted in Nihon University Itabashi Hospital between 1991 and 2011 was performed.

Results: Seven RNB children were treated with LTMCT without HDCT. One was male and 6 were female. The median age at diagnosis was 3 years 8 months old (range, 1y5m to 12y4m). Original sites of tumor were 2 posterior mediastinal, 3 adrenal gland, and 2 posterior peritoneal. All children were stage 4 and 6 had unfavorable histology. No one had MYCN amplification. After 3 or 4 courses of induction chemotherapy, all children were diagnosed as RNB. Four children were received tumor resection, but 3 were not received. Six children were received local irradiation therapy. All children were treated with 13-cis retinoic acid during LTMCT. LTMCT consisted of A3 (cisplatin, pirarubicin, vincristine, and cyclophosphamide), IE (ifosfamide and etoposide), ICE (ifosfamide, etoposide, and carboplatin), irinotecan, topotecan, TC (topotecan and cyclophosphamide), TI (ifosfamide and topotecan), or VDC (vincristine, doxorubicin and cyclophosphamide). Median duration of LTMCT was 2y4m (range, 1y2m to 7y6m). All children were alive and median age at the time of evaluation was 11y7m (range, 2y2m to 25y 1m) and Karnofsky performance status were 80 to 100%.

Conclusion: These results suggest that LTMCT without HDCT would become one of a treatment option for RNB especially in terms of keeping the quality of life and long-term survival.

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POC23

A novel high-risk neuroblastoma subset with abdominal primaries mimicking Wilms spreading to lungs but not bone marrow is defined by amplification of two distinct regions on chromosome 12

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Background: Neuroblastoma (NB) is a clinically heterogeneous disease. A proper biological characterization of different clinical subsets may allow for optimal treatment.

Methods: In a national clinical material we identified a subset of individuals with primary abdominal NB mimicking Wilms tumors. None of these metastasized to bone marrow, but at least two to the lungs. After identifying amplifications of chromosome 12 we extended the study to children (n=7) and cell lines (n=2; LS and NGP) with similar amplifications. Using high-density SNP arrays we characterized two distinct regions of amplification at 12q13.3-14.1 and 12q15, resp., in all tumours/cell lines. These amplifications were accompanied by amplification at 11q13 in some cases. Taqman low density array cards were used to analyze the mRNA expression of the genes within these amplified regions, and siRNA targeted knockdown of a subset of these genes was performed in order to evaluate their tumorigenic potential.

Results: All nine patients with neuroblastoma diagnosis showed similar features with aggressive abdominal primaries, often renal mimicking Wilms, four obtaining preop nephroblastoma therapy. None had bone marrow infiltration whereas all 4 with metastatic stage had lung metastases and two had extension to vena Cava. The commonly amplified regions included several genes involved in cell cycle regulation and cell proliferation. A majority of the genes in the amplified regions were over-expressed in the tumors with amplification compared to other NB, thus indicating a close correlation between gene copy number and gene-expression for most of these genes. siRNA knockdown of either CDK4 or CCND1 was further shown to result in decreased cell proliferation in cell lines carrying these amplifications.

Conclusions: We identified a novel poor prognostic subset of neuroblastoma with primaries mimicking Wilms' tumor and metastatic spread to lungs but not bone marrow, that were characterized by amplification of two distinct regions on chromosome 12. These amplifications result in over-expression of oncogenic cell cycle de-regulation genes including CDK4, CCND1 as well as MDM2. We believe that this poor prognosis group would benefit from alternative treatment protocols, involving cell cycle targeting drugs and/or MDM2 targeting drugs.

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POC24

Ethnic differences in neuroblastoma indicated by the pattern of frequent ALK mutations in Vietnamese tumors; Eight novel tyrosine kinase domain mutations identified.

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Background: Neuroblastoma (NB) is a heterogeneous tumor of global prevalence with great impact on rich and poor societies. However, ethnic differences in incidence and biology have rarely been investigated. We embarked on a study investigating NB from the National Hospital of Pediatrics, Hanoi, Vietnam and comparing those to NB from Sweden and elsewhere.

Method: A national material of NB was collected and sections of FFPE blocks were used for DNA extraction and DNA samples were subjected to analysis by (i) 250K SNP array (Affymetrix/ CNAG3.0 software) and (ii) mutation screening of the tyrosine kinase domain (TKD) of the ALK gene with Sanger sequencing.

Results: SNP array profiles have been performed for a first set of 40 NB; twenty-four of which gave clear results: 9/24 had MYCN amplification (MNA), 10/24 were 11q deleted, two of these harbored both MNA and 11q-del, one had 17q gain without 11q-del or MNA, 2/24 had numerical aberrations only, 4/24 showed no aberrations.

Sequencing of the ALK TKD was performed in 53 samples. Sequence covering the whole TKD, exons 21-25, was obtained in 47 samples. The screening revealed mutations in the described hot spot codon 1174 in two NB (F1174L and F1174I). In addition, we found eight novel missense mutations not previously reported (D1163N, I1170V, G1201R, R1212C, P1213S, E1241K, E1242, S1251T).

Conclusion: 21% (10/47) of Vietnamese NB showed an ALK mutation; significant more common (p<0.01-0.001) than any previously described subset of NB including tumor collections from Sweden (5%), USA (~7%), Japan (6%) or Europe (6-7%). Eight of ten mutations were not previously published and outside the described hot spots 1174, 1245 and 1275 significant different from previously studied tumors (p<0.05-0.01) and cell lines (p<0.001). Also, the SNP array aberration pattern was different from our Scandinavian experience with higher frequency of 11q- vs. MNA cases.

This ongoing project aims at describing the clinical and genetic profile of NB in a country with different ethnicity and living conditions than usually investigated. Data obtained may contribute to essential future understanding of mechanisms behind NB development including the significance of ALK aberrations, as well as provide a basis for future adapted clinical trials including the introduction of tailored targeted therapies.

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POC25

Successful liver transplantation in an infant with stage 4S(M) neuroblastoma: Case report clinical and ethical issues

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Stage 4S(M) neuroblastoma, multifocal or bilateral, is extremely rare, with high rates of spontaneous regression reported in these babies, except in cases showing MYC-N amplification.

We report a case of a one-day old infant who presented with abdominal distension, hepatomegaly and respiratory distress. A diagnosis of stage 4S(M) neuroblastoma was made based on an abdominal ultrasound and CT scan demonstrating bilateral enlarged adrenal glands and infiltrative liver metastases, as well as an elevated urinary VMA and HVA. On day four of life, emergency chemotherapy was started based on the COG protocol ANBL 0531 (cycle 1 Carboplatinum 18.6mg/kg Etoposide 4mg/kg). Due to deteriorating respiratory status, local radiotherapy was subsequently administered as three doses of 180cGy per fraction for five days on the liver field.

Life-threatening liver failure in the context of veno-occlusive disease occurred on day 40. In view of life-threatening liver failure and absence of progressive disease, the decision was to proceed with a live donor liver transplantation at 10 weeks of age. Ethical issue were raised, as the MYC-N status of the child was unknown.

This patient's post-operative course was uncomplicated. No further chemotherapy was given after the liver transplantation. Biopsy of the explanted liver demonstrated changes of end-stage micronodular cirrhosis, severe cholestasis, extensive bridging fibrosis, significant VOD and absence of viable neuroblastoma. Biopsy of the right adrenal gland demonstrated only in-situ viable neuroblasts. No lesions or co-factors suggesting an underlying metabolic disease were detected.

This patient remains in complete remission seven months post-diagnosis and five month post liver transplant.

Cirrhosis has not been reported early after chemo-radiation therapy in this patient population.

Conclusion: The role of this multimodal therapy played in the etiology of the cirrhosis and VOD is unclear. Liver transplantation should be considered in this patient population when life-threatening cirrhosis occurs.

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POC26

Comparison of sphingosine 1-phosphate receptor 4 gene expression between patients with neuroblastoma and healthy children

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Background: To compare the expression level of sphingosine 1-phosphate 4 gene in patients with neuroblastoma to healthy children.

Methods: 37 neuroblastoma patients were investigated. After RNA isolation and cDNA were performed, S1P4 receptor gene expression levels of the patients were measured.

Results: Clinical features of patients were listed in Table 1. The relationship between neuroblastoma patients and healthy children was statistically different for S1P4 receptor gene expression levels (p=0,028) (Figure 1). The difference was not statistically significant between the patients followed up without treatment and those who continued to receive chemotherapy. However, S1P4 receptor gene expression levels were statistically different between the patients receiving maintenance therapy and patients followed up without chemotherapy (p=0.023).

Conclusions: Higher S1P4 receptor gene expression levels were seen in the patient group. The suppression of sphingozin 1-phosphate 4 gene expression level during consolidation phase and the increasing of expression in following up without chemotherapy meant that the chemotherapy caused to the decreasing of cell migration and/or induction of apoptozis.

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POC27

Targeting the PI3K/Akt pathway: Perifosine monotherapy for resistant neuroblastoma (NB) in a phase I/Ib study

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Background: Perifosine is a synthetic alkylphospholipid that inhibits Akt (which is aberrantly activated in NB) and is cytotoxic in mM concentrations to NB cell lines. Phase I trials in children and adults with various solid tumors showed modest toxicity and steady state serum levels of ~32mM with dosages 50-75mg/m2/day using 50mg tablets after a loading dose of 100-200mg/m2 on day 1.

Methods: Patients with evidence of NB by 123I-metaiodobenzylguanidine (MIBG) ± other imaging studies receive a loading dose of oral perifosine on day 1, followed by daily maintenance until progressive disease (PD) or excessive toxicity (http://www.clinicaltrials.gov NCT00776867).Disease evaluation is every 8 weeks.

Results: When started on perifosine, the 22 patients treated to date were 4.7-33.5 (median 8.7) years old and 2.5-8.0 (median 4.6) years from diagnosis. Three patients were treated for primary refractory NB, and 19 were treated for NB resistant to salvage therapy after 1-5 (median 2) prior relapses. Sites of resistant NB at study entry were osteomedullary by 123I-MIBG scan alone (n=13) or plus bone marrow (BM) histology (n=6); osteomedullary plus soft tissue (n=2); or soft tissue alone (n=1). Prior therapy included high-dose conventional induction and 2nd-line chemotherapy (all patients); 3F8 anti-GD2 antibody (n=18); autologous stem-cell transplantation (n=9); and/or targeted radiotherapy with 131I-MIBG (n=9) or 131I-3F8 (n=2). Anti-NB activity was evident as follows: complete response (CR) in 1 patient (123I-MIBG scan normalized) and prolonged progression-free survival at 9+-33+ (median 11+) months of 9 patients, including 3 with CR in BM by histology. Eight patients had early PD (<1-3 months), other patients are progression-free with short follow-up (<4+ months). From day 1 of perifosine, progression-free survival is 56% (SE+11%) at 12 months. No significant toxicity was seen.

Conclusions: Perifosine shows activity against chemoresistant and radioresistant NB while allowing excellent quality of life and sparing vital organs.

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POC28

5-Day/5-Drug (5D5D) Myeloablative Outpatient Regimen for Resistant Neuroblastoma (NB)

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Background: 5D5D is a novel regimen for NB resistant to standard chemotherapy which now includes topotecan in induction or salvage therapy. 5D5D was designed to maximize antitumor activity, including in the central nervous system (CNS), while minimizing acute extramedullary toxicity and morbidity in patients with resistant NB and poor hematologic reserve due to extensive prior therapy.

Methods: 5D5D comprises: carboplatin 500 mg/m2/d on days 1 and 2; irinotecan 50 mg/m2/d on days 1-3; temozolomide 250 mg/m2/d on days 1-3; etoposide 200 mg/m2/d on days 3-5; and cyclophosphamide 70 mg/kg/d on days 4 and 5. Autologous peripheral blood stem cells are infused on day 8.

Results: 15 patients (including 13 with >2 prior relapses) received 19 courses administered 1.5-8.9 (median 2.7) years from diagnosis. Patients were treated for progressive disease (PD) occurring despite retrieval chemotherapy (n=7), relapse off therapy (n=1), or PD (n=4) or stable disease (SD) (n=3) post-131I-metaiodobenzylguanidine (MIBG) therapy. Treatment was outpatient but febrile neutropenia necessitated admission with 18/19 courses. Toxicities included transient grade 3 elevations of liver enzymes in 3 courses, mild mucositis in 4 courses, and bacteremia in 1 course. Engraftment was uncomplicated. Responses were: 3 complete, 1 partial, 6 minor, 4 SD, 1 PD. After treatment with 5D5D, 9 patients were formally enrolled on clinical trials.

Conclusions: 5D5D shows activity against chemoresistant and radioresistant NB in heavily prior-treated patients, with acceptable toxicities. Especially appealing is administration immediately before stem-cell rescue post-MIBG therapy and use in patients who relapsed in the CNS. 5D5D can halt PD or reduce substantial tumor burden - and thereby improve prospects of benefit from novel investigational therapies.

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POC29

Intensity Modulated Radiation Therapy Provides Excellent Local Control in High Risk Abdominal Neuroblastoma

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Background: Locoregional failure persists with current high-risk neuroblastoma therapy, and reported 2 year local failure rates following single-transplant treatment regimens range from 10-30%. We document enhancement of local control using International Commission on Radiation Units and Measurements (ICRU) report 62 volumetric conventions for radiation planning, which account for physiologic motion and more accurate targeting of volumes at risk.

Methods: We evaluated the locoregional outcomes for 20 children with high risk abdominal neuroblastoma treated between 2007-2010. Twelve patients had complete resection of their abdominal disease and received 23.4Gy locoregional RT while eight patients had postoperative residual disease requiring dose escalation; 2 received 36Gy and 6 received 30.6Gy. All patients were treated using identical immobilization techniques, 4DCT assessment of physiologic motion, and cone-beam CT localization. Due to minimal organ motion in the infant abdomen, the setup was made more reproducible by raising the legs and flattening the natural lordotic position of the spine against the table. ICRU-62 volumetric conventions were employed based on previously published data for pediatric target volume and organ motion.

Results: With median follow-up of 2.1 years (range: 1.1-3.7 years), no child has developed primary site infield or locoregional failure. The estimated 2-year event-free survival was 57.4%±14.2% while the cumulative incidence of local and distant failure were 0% and 42.6%±12.2%, respectively. Distant failure occurred in 8 patients, leading to death from disease in 5. Asymptomatic loose stool during RT was the most common acute side-effect and occurred in all cases although it required no intervention.

Conclusions: Intensity modulated radiotherapy is feasible, and in the short term is safe and yields excellent local control. Despite subtotal resection in 40%, local control was enhanced with ICRU-62-compliant volumes. As most patients were treated with 30.6Gy, dose escalation beyond 30.6Gy may not be necessary with improved target volume coverage.

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POC30

Surgical Outcomes of Extra-Abdominal Neuroblastoma: A Single Centre Experience

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Background: The prognosis for children with extra-abdominal neuroblastoma is more favorable than for those with abdominal disease. However, the morbidity associated with the resection of these sites is high, and this may influence the quality of life of survivors. The aims of this study were to report our experience in the management of extra-abdominal neuroblastoma and to evaluate the incidence and severity of complications.

Methods: Between November 2004 and January 2012, 30 consecutive patients (18 boys and 12 girls) with extra-abdominal neuroblastoma (mediastinum: 19, cervical: 6 and pelvis: 5) were included in this analysis.

Results: The median age at diagnosis was 32.5 months (range 4-108). Twenty-four (80%) tumors were localized and 6 (20%) were disseminated. Eleven patients had radiological evidence of intraspinal extension (thoracic 9, pelvic 2) of disease; 3 of these patients required decompressive surgery. The number of low, intermediate and high-risk patients were 9 (30%), 17 (57%), and 4 (13%), respectively. Six patients underwent upfront tumor resection, the remaining received chemotherapy prior to surgery. Gross total resection was achieved in 19 patients, more than 95% in 8 and biopsy alone in 3 patients. There were three postoperative complications (leg weakness. transient impairment of deglutition and intestinal obstruction).

After initial control, 3 patients relapsed. The relapses were local only, both local and distant and distant only in 1 each patients. After a median follow-up of 31 months, 24 patients are alive and 4 are alive with disease. All except one patient with incomplete excision survived. The probability of 5-yr OS and EFS rate for all 30 patients was 91% and 81%, respectively.

Conclusions: Our data confirms the excellent survival for extra-abdominal neuroblastoma. Complete excision is feasible in majority with acceptable morbidity and incomplete excision does not result in poor outcome.

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POC31

The progressive risk can stratified in various neuroblastoma groups: Statistical analyses on the data over more than 3 decades in Japan

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Background: The aim of this study is to prove a significance of risk stratification in neuroblsastomas (NBs) with biological diversity.

Methods: From anonymous 582 NBs data, following information were extracted ; "age", "era" and "stage" at diagnosis, "treatment", "period of event-free survival (EFS) ", "clinical outcome", three risk factors ["MYCN status", "INPC finding" and "Ha-ras/Trk A expression"]. Kaplan-Meier method and Cox proportional hazard model were used for statistic analyses.

Results: (1)Independence for EFS: The multivariate analysis showed that each of MYCN amplification, INPC unfavorable histology and Ha-ras/trk A low expression associated independently with the EFS in 582 NBs. The significant independence of the each in relation to "stages"," era" or "age" was also proved not only in 264 clinically diagnosed (non-mass) NB groups, but also in other non-mass groups such as 196 advanced NBs, 135 childhood (>=12mo) NBs, and 129 infant(<12mo)NBs, respectively. (2) Stratification of NBs: Kaplan-Meier analysis showed significant difference of EFS in 462 NBs when patients were stratified with number of the risk factors (p<0.0001). It was proved in other groups, such as in194 non-mass NBs (p<0.0001), 148 not-localized non-mass NBs (p<0.0001), 37 stage III non-mass NBs (p=0.0011), 96 stage IV non-mass NBs (p=0.0028), 107 childhood (>=12m) non-mass NBs (p=0.0046), 87 infant (<12m) non-mass-NBs (p<0.0001) and even in 244 infant mass-screening diagnosed NBs (p=0.0009), respectively. Furthermore, these stratifications elucidated not only "therapy-resistant" NBs but also "therapy-sensitive" NBs in the respective groups. A part of this study has been reported [Cancer letters 306 (2011) 27-33] .

Conclusion; Therapeutic intensity according to the risk prediction might improve the efficacy of the therapies in high risk NBs and decrease therapy-related sequelae in the lesser risk NBs.

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POC32

Changes in clinical feature of neuroblastoma after the cessation of the mass screening in Japan
-Analysis of the Neuroblastoma registry data of the Tumor committee of the Japanese Society of Pediatric Surgeons-

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Background: MS in Japan have been stopped since 2003. In order to know the efficacy of MS, we compared clinical features in neuroblastoma before and after the cassation of MS in Japan.

Method: 424 neuroblastoma patients who were born between 2004 and 2009 were conducted from the Neuroblastoma registry data of the tumor committee of the Japanese Society of Pediatric Surgeons between 2005 and 2010 (group A). 1852 patients who were born between 1990 and 2000 were conducted from the data of the Japanese MS study group (group B).We compared these groups in terms of clinical features.

Results: Significant decrease in number of the patients under one year of age after the cessation of MS was observed (192 patients in group A (45.2%) and 1586 patients in group B (86.6%)). On the other hand, significant increase in number of the patients of two year of age after the cessation of MS was observed (69 patients in group A (16.3%), 44 patients in group B (2.3%)). Among 2-year-old patient units in both groups, a significant increase in ratio of the stage 4 patients was observed after the cessation of MS (47 / 69 in group A (68.1%), 24 / 44 in group B (54.5%)).

Conclusion: A significant increase in number of the patients of two year of age, especially stage 4 patients, was observed after the cessation of MS and it may produce poor prognosis of this age of the patients in Japan.

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POC33

Clinical and Imaging Diagnosis of Neuroblastoma

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Objective: To study the clinical and imaging characteristics of neuroblastoma in the cases referred into our hospital.

Materials and Method: From 2005-2012,altogether 100 cases of neuroblastoma were recruited into this study. All the cases were confirmed by operation, pathology or biopsy of the primary lesion. Bone marrow examination performed in all cases. Conventional and enhanced CT, conventional and/or enhanced MRI, and bone Scan were performed. Result of treatment of the patients in this group were discussed.

Results: In the 100 cases, male to female ratio was 4:32. Age ranged from 4d-41yrs, ≤3y 63 cases (63%), 29 cases died (29%). Primary lesion site: adrenal n=47 (47% left 29, right18) post-peritoneal n=25, mediastinum n=25, other sites n=3. Metastases: Bone marrow metastases 49cases, bone mets 30cases, metastases to liver 13 cases, lymph nodes metastases to post-peritoneal 43cases, mediastinal lymph nodes mets. 25 cases, pleura mets. 16,lung mets 6, intracranial mets. 9 cases. Surgical therapy was performed in 86 cases (post-chemo n=45, direct operation before chemo n=41). In this group according to the clinical material and imaging characteristics, 82cases(82%) belong to INSS stage 3 and stage 4. Fifty-seven cases had serum NSE(neuron-specific-enolase) level detected, among them 8 cases died NSE >150-370ng/ml in 7 cases, recurrent 11cases, in whom NSE>50-370ng/ml, disease stable in 27 cases, NSE<20-30ng/ml. In patients with a favorable or a poor outcome, the difference in NSE serum levels was statistically significant.

Conclusion: Clinical manifestations and imaging characteristics of 100 cases were presented in the study. The imaging features correlated well with the INSS staging and NSE is valuable in the management of the patients to monitor the effect of treatment and detect recurrence of disease.

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POC34

Genomic profiling in low risk neuroblastoma to refine treatment stratification and improve patient outcome – LINES: a SIOPEN Trial

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Background: In low risk neuroblastoma (NB), new strategies are required to further stratify therapy in order to minimize treatment burden while maintaining excellent survival. Recent studies have shown that genomic profiles with segmental chromosome alterations are associated with a poorer event free survival, requiring more intensive salvage treatment.

Methods: SIOPEN has launched a European trial, LINES (Low and Intermediate Neuroblastoma European Study), with the intention, in low risk NB patients, to maintain or improve the excellent outcome, while decreasing the overall treatment burden. In addition to clinical parameters, treatment is stratified according to the tumor genomic.

Results: NB samples that contain >60% of tumor cells are analyzed in real-time by a multi-locus or pangenomic technique such as MLPA, array-CGH or SNP-arrays. The genomic profile results are centrally reviewed by two independent reviewers using the expanded SIOPEN-R-NET database, and the conclusion is returned to the treating center within 3 weeks. Genomic profiles are classified into two large classes: those harboring numerical chromosome alterations (NCA) only, versus those harboring segmental chromosome alterations (SCA) known to frequently occur in NB, with or without NCA. For low risk NB patients without MYCN amplification, with stage L2, age <18 months, or stage Ms, age <12 months, treatment is then stratified into 6 different groups according to stage, the presence or absence of life threatening symptoms and the genomic profile. In infants with L2 disease without symptoms, absence of SCA identifies a group of patients for whom the hypothesis that observation only might be sufficient treatment is tested in a randomized phase III trial. When SCAs are present, upfront chemotherapy is proposed.

Conclusion: SIOPEN has launched a prospective trial, LINES, in which, to stratify treatment, a genomic profile defined by the presence or absence of typical SCAs is used in addition to the standard clinical parameters.

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POC35

Multifocal Metastatic Neuroblastoma (NB) to the Central Nervous System (CNS)

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Background: We reported on the addition of intrathecal radioimmunotherapy (RIT) using 131I-3F8 or 8H9 targeting GD2 or B7-H3, respectively, for CNS NB (J NeuroOncology 97: 409-18, 2010). We noted a high survival (80%, 6.7-68 months, median 40.5) compared to historical controls (median 6 months). The prognostic impact of multifocal disease (>2 parenchymal lesions and/or leptomeningeal disease (LM) was uncertain.

Methods: Subjects had radiographic +/- pathologic multifocal CNS NB diagnosed at MSKCC since 2000. Patients with single metastases or those diagnosed elsewhere but referred to MSKCC for RIT were not included.

Results: 21 patients (11 LM; 10 parenchymal lesions; 13 MYCN amplified) had multifocal CNS NB. Six patients (29%) had disease discovered by routine CT (3) or MR brain (3), 4 missed on MIBG. The most common symptom prompting diagnosis was headache (5). 20 patients (95%) had disease detected while on treatment for systemic NB, median 23.1 months (4.4-104 months) from initial diagnosis. In 14 patients (66%) relapse was isolated to CNS. Treatments included complete surgical excision (n=3), biopsy/subtotal resection (n=8), radiation plus multiagent chemotherapy (n=16) (craniospinal irradiation [CSI] n=11; focal or whole brain n=5; CSI with chemotherapy and RIT n= 9) or chemotherapy only (n=3). Median survival was 21.6 months (0.4-102 months.) 6 patients treated with RIT (29%) remained alive (median 41.6 months, 8-102 months); 3 in complete remission (3+, 4+ and 8.5+ years). Patterns of subsequent disease progression were CNS alone (8), systemic (3) or CNS plus systemic (6).

Conclusions: Time to CNS, symptomatology, and confinement to CNS were similar for patients with multifocal disease comparable to published data for patients with unifocal disease. Although overall survival has improved, prognosis remains guarded; >50% progress further in the CNS; <30% achieve long term remission. Early detection before multifocal spread may improve survival.

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POC36

The amount of GD2 positive tumor cells in bone marrow does not predict the outcome from disease in high risk neuroblastoma patients

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Background: The amount of tumor cells in bone marrow may serve as a pars pro toto compartment for risk evaluation in stage 4 neuroblastoma patioents. This study evaluates the prognostic relevance by GD2-immunocytology at diagnosis, during and after induction chemotherapy.

Methods: Inclusion criteria were neuroblastoma stage INSS 4, age > 18 months at diagnosis, availability of centrally referenced GD2-immunocytology at diagnosis and at least one follow-up.

Results: Between 01.11.2002 and 31.12.2010, 249 patients met the inclusion criteria.

At diagnosis, 5.6 % of patients were free of tumor cells in the bone marrow, 22.1% had <1%, 13.7% 1-<10%, 7.6% 10-<30% and 51.0% 30-100% tumor cells. Patients with ultra high risk (death from tumor progression within 1 year after diagnosis, n= 33) demonstrated amounts of tumor cells that were not different, neither at diagnosis (chi2= 0.938) nor after 2,4,6 chemotherapy cycles (chi2= 0.375/0.723/0.513 respectively).

During treatment, the bone marrow became free of tumor cells in 30.9% after 2 cycles, 64.9% after 4 cycles, and 81.6% after 6 cycles. In ultra high risk patients 42.9%, 61.1% and 92.9% showed clearance after 2, 4, and 6 cycles.

EFS and OS curves and 5 year EFS and OS rates by the amount of GD2 positive tumor cells at diagnosis or after 2,4, and 6 cycles of chemotherapy showed prognostic information for subgroups only (e.g. for the combined groups with >10% vs. <10% vs. no tumor cells, 5 year EFS 18%/36%/41% respectively, p=0.046) and if discriminative, then only with marginal statistical significance.

Conclusions: This large series of immunocytological bone marrow investigations does not support the hypothesis that bone marrow might serve a pars pro toto compartment for risk estimation in stage 4 neuroblastoma. This applies for the high risk and ultra high risk group evaluated at diagnosis and at response and for EFS and OS.

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POC37

Pilot feasibility and toxicity of 2 cycles of upfront 131I-MIBG therapy followed by the standard arm of high-risk GPOH NB 2004 protocol.

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Purpose: Meta-iodobenzylguanidine (MIBG) has been shown to have a significant efficacy against NBL, with response rates between 20- 60%. In a pilot study we have evaluated the feasibility, efficacy and toxicity of upfront 131I-MIBG, followed by HR NBL treatment.

Methods and Materials: HR NBL patients from the AMC and SKZ have been included from 1/1/2005 till 1/1/2011. The NBL DCOG 2009 HR protocol consisted of 2 cycles of upfront 131I-MIBG therapy (7400MBq/ 5500MBq) for patients with adequate MIBG uptake in all tumor sites followed by the standard arm of HR GPOH 2004 protocol (3x N5 and 3x N6 chemotherapy courses, surgery, mega therapy with autologous stem cell rescue, radiotherapy and retinoic acid). We tested the feasibility of giving 131I-MIBG within 2 weeks after diagnosis and analysed the dose intensity and toxicity of chemotherapy after MIBG pre-treatment compared to non-MIBG treated patients. Response analysis was based on the International Neuroblastoma Response Criteria (INRC), response is defined as complete-remission, very good partial response, partial response and mixed response.

Results: Of the 33 evaluable patients, 16 (48%) received 2 cycles of upfront 131I-MIBG therapy (group A), 17 (51%) patients were treated without upfront 131I-MIBG therapy (group B; insufficient MIBG uptake, weak clinical condition and hypertension) and 2 patients were excluded (they received prior chemotherapy). 131I-MIBG therapy within 2 weeks from diagnosis was feasible in all patients eligible for 131I-MIBG therapy. Interval between subsequent N5/ N6 chemotherapy courses was similar in both groups (22- 30 days). There was no serious haematological toxicity. Stem cell harvest after 131I-MIBG therapy was undisturbed and did not compromise HD chemotherapy with ASCT treatment. Response analysis (FU 1/1/2005- 1/1/2012) showed an effect of 2 131I-MIBG courses in 10/15 (67%) patients (1 missing) after 3 times N5/N6 of 15/16 (94%) and at FU of 13/16 (81%).

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POC38

PHOX2B immunolabeling: a novel tool for the diagnosis of undifferentiated neuroblastomas among childhood small round blue cell tumours

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Although a reliable diagnostic is achievable in a majority of cases of peripheral neuroblastic tumours, a minority of cases of peripheral neuroblastic tumours (especially undifferentiated neuroblastoma) remains a difficult diagnostic versus the other pediatric small round blue cell tumours. A panel of immunohistochemical markers and fusion transcripts is available for the diagnosis of such tumours but the immunohistochemical markers for neuroblastoma lack of specificity or sensitivity. The transcription factor PHOX2B is highly specific of the peripheral autonomic nervous system from which peripheral neuroblastic tumours derive. We observed PHOX2B expression in all peripheral neuroblastic tumours, paraganglioma and pheochromocytoma tested but in no other pediatric tumours among the 388 cases studied by microarray and the 102 cases studied by immunohistochemistry. We then assessed the interest of PHOX2B immunochemistry in 12 cases of undifferentiated pediatric neoplasms with difficult diagnosis and for which the hypothesis of neuroblastoma was clinically plausible: PHOX2B was expressed in 6/6 undifferentiated neuroblastomas and no other small round blue cell tumours. Finally, we showed that PHOX2B immunochemistry improves the diagnosis of undifferentiated neuroblastoma with high specificity and sensitivity.

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POC39

Striking dichotomy in outcome of MYCN-amplified neuroblastoma (NB) in the contemporary era

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Background: We exploited a large database to investigate the outcome of high-risk NB.

Methods: Our hospital's registry lists 1053 NB patients (2000-2010); we studied the 230 patients <12 years old at diagnosis treated during induction for high-risk NB (MYCN-amplified(+) stage 2, 3, 4S, or 4; >18 months old MYCN-non-amplified(-) stage 4).

Results: MYCN(+) and MYCN(-) patients had similar rates of complete/very good partial responses (CR/VGPR) to induction (p=.3661), but only MYCN(+) patients had progressive disease (PD) response (p=<.0001) and their early PD (<12 months post-diagnosis) was significantly more common (p=<.0001).

Patients achieving CR/VGPR had significantly superior event-free (EFS) and overall survival (OS), with equivalence between MYCN(+) and MYCN(-) patients. In contrast, among patients with <VGPR, MYCN(+) was associated with a significantly inferior outcome, which accounted for the significantly worse outcome of the entire MYCN(+) cohort versus MYCN(-) cohort.

Conclusions: MYCN(+) patients have either an excellent response to induction and good long-term outcome, or early PD with poor outcome. This extreme dichotomy in clinical course is not seen with MYCN(-) high-risk NB patients who, in contrast, display a continuous spectrum of response and outcome. The results point to underlying biological differences with MYCN(+), the elucidation of which may impact risk classification.

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POC40

Treatment Results Of Neuroblastoma Patients With Stage IV: Single Center Experience

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Background: The stage IV is one of the most important risk factors in patients with neuroblastoma. We report treatment results in stage IV neuroblastoma patients treated in the Regional Children's Hospital, Ekaterinburg, Russia.

Aim: The retrospective study was conducted in order to determine the treatment efficacy of German Cooperative Group NB protocols in patients with stage IV of neuroblastoma.

Methods: Among 154 children with primary neuroblastoma admitted to our clinic since October 1991 till November 2011 - 54(35%) patients (29 males and 25 females) were diagnosed with stage IV of disease aged from 10 days to 11 years (median 27 months). 49(90,7%) children were eligible for analysis. MYCN status was evaluated in 35(71,4%) cases. Treatment was performed according to NB90, NB97 and NB2004 protocols: 20(40,8%), 18(36,7%) and 11(22,4%) correspondingly. Only 8(16,3%) children underwent high-dose chemotherapy and autologous peripheral blood stem cells transplantation (PBSCT). Median of follow up is 54 months.

Results: The majority of primary tumors (81,6%) were of retroperitoneal and/or adrenal origin. MYCN amplification was detected in 11of 35 evaluated patients (31,4%). Complete remission and very good partial remission were achieved in 23(46,9%); partial remission in 19(38,8%) children. Unfavorable events (relapses, stable disease, progressive disease and therapy-related deaths) were registered in 39(79,6%) cases. At present time 14(28,6%) patients are alive; 10(20,4%) are alive without progression; 1(2%) patient was lost to follow up. 16-years event free survival (EFS) is 15%±6% and overall survival is 25%±6%. EFS in patients with MYCN amplification (9%±8%) differed significantly from other patients (17%±10%)(p=0,02). Among 8 children who are after high-dose chemotherapy and PBSCT - 6 patients developed progressive disease and relapses, 1 patient died from progressive disease and 2 patients are alive in complete remission.

Conclusion: Treatment results of stage IV neuroblastoma still remain unsatisfactory and new treatment approaches should be developed for these patients.

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POC41

Prognostic value of the MRD in the peripheral blood apheresis product in high risk group patients with neuroblastoma.

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The purpose is to assess the impact of the MRD in the peripheral blood stem cell (PBSC) detected with both molecular biological method and immunophenotyping on the disease prognosis in high risk group with neuroblastoma.

Methods: 22 patients (2 with 1st stage, 3 with 2nd stage, 17 with 4th stage) who received treatment according to NB2004m protocol from 2008 till 2011 were assessed. All patients underwent autologous PBSC transplantation. MMD and MRD were detected in the bone marrow (BM) and PBSC, with multiparameter flow cytometry and quantitative PCR (expression of tyrosine hydroxilase (TH)).

Results: Thirteen out of 17 patients with the 4th stage (76%) had morphologically verified BM involvement at diagnosis. According to immunophenotyping data, BM involvement was found in 15 out of 20 (75%) patients (13 with 4th stage, 2 with 3rd stage). PCR method revealed BM lesion in 15/20 (75%) patients (1 with 3rd stage, 14 with 4th stage, including 2 patients without morphological involvement). Spearmen correlation coefficient between two methods comprised r=0,5468 (p<0.02).

MRD in the PBSC was detected in 2 out 21 (9%) patients using immunophenotyping. Trace quantities of TH in the PSC were revealed in 11 out of 21 (52%) patients. However, those levels were below positivity threshold. Our preliminary results demonstrate that MRD detected in the PSC with flow cytometry significantly decreased overall 0.62+0,11 vs. 0.00 (p=0.005) and event-free survival 0.41+0.11 vs 0.00 (0.045). MRD detected with the quantity PCR did not have prognostic value for the overall and event-free survival.

Conclusion: Both flow cytometry and quantity PCR methods assessing TH expression in the bone marrow can be used to detect metastatic disease in children with neuroblastoma. Because of small follow –up group prognostic impact MRD in PBSC detected by immunophenotyping is not clear, while TH expression in PBSC did not correlate with outcome.

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POC42

Infants and children with stage 4 neuroblastoma express significantly different levels of specific molecular markers

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Background: Age at diagnosis is a strong prognostic factor in neuroblastoma (NB). Hereby, we evaluated whether patients with metastatic NB below 1 year of age (infants) at diagnosis expressed lower levels of NB-specific molecular markers compared to patients over 1 year of age (children).

Methods: Bone marrow (BM) and peripheral blood (PB) samples collected at diagnosis between January 2001 and June 2008 from 54 Italian patients with metastatic NB were analysed by RT-qPCR for TH, PHOX2B and DCX mRNA expression. The diagnostic performance was evaluated by ROC analysis, correlations among different markers and between samples' types were estimated by Spearman's coefficient, while univariate analysis was performed to verify association with other known prognostic factors.

Results: In spite of similar levels of morphological BM infiltration, TH, PHOX2B and DCX expression levels were significantly lower in stage 4 infants than in stage 4 children, in both BM and PB samples. No significant differences were found between stage 4 and stage 4S infants, with the exception of higher TH levels in BM samples from the former patients. TH expression levels showed the highest accuracy in both BM and PB samples and, most importantly, BM and PB TH values significantly associated not only with the presence of BM metastasis, but also with the presence of skeletal metastasis.

Conclusions: RT-qPCR analysis of BM and PB samples showed potential clinical significance that should be evaluated in future multicentre prospective studies for infants with metastatic NB in comparison with conventional morphological evaluation and other prognostic factors evaluable in the primary tumors, such as segmental and numerical aberrations. The use of a quantitative method to evaluate BM infiltration may also contribute to a more uniform staging of infants among different countries.

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POC43

Busulfan pharmacokinetics following intravenous and oral dosing regimens in high-risk neuroblastoma patients treated on the HR-NBL-1/SIOPEN trial

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Background: Busulfan is widely used in high-dose myeloablative chemotherapy regimens in neuroblastoma, with several studies reporting marked inter-patient variability in busulfan pharmacokinetics and pharmacodynamics. The current study reports on the pharmacokinetics of oral versus intravenous (IV) busulfan in high-risk neuroblastoma patients, including those treated on the European HR-NBL-1/SIOPEN study.

Methods: Busulfan was administered four times daily for 4 days, either orally at a dose of 1.45-1.55 mg/kg or by the IV route, at doses of 0.8-1.2 mg/kg according to body weight strata, without dose adaptation. Blood samples were obtained prior to administration, 2.5 and 6h after the start of infusion or oral administration for dose 1. Busulfan analysis was carried out by gas chromatography-mass spectrometry (GC-MS) and data analysed using a NONMEM population pharmacokinetic approach.

Results: Busulfan plasma concentrations obtained from 38 patients receiving IV busulfan and 25 patients receiving oral busulfan, were fitted simultaneously using a one-compartment pharmacokinetic model. Lower variability in drug exposure was observed for patients receiving IV busulfan, with a mean busulfan AUC value of 1,146±187μM.min (range 838-1622), as compared to 953±290μM.min (range 434-1427) following oral busulfan. A total of 87% of children treated with IV busulfan achieved AUC values within the defined target of 900-1,500μM.min, versus 56% of patients following oral busulfan. Busulfan AUC values in HR-NBL-1/SIOPEN trial patients who experienced hepatic toxicity or VOD were significantly higher than in patients with no hepatic toxicity (1,177±189μM.min vs 913±256μM.min; p = 0.0086). Further stratification based on route of administration suggested that the incidence of hepatic toxicity observed was related to both high busulfan AUC and oral drug administration.

Conclusions: The preliminary results obtained indicate that the clinical use of IV busulfan in a high dose myeloablative setting results in reduced pharmacokinetic variability, providing better control of busulfan AUC and reduced toxicity as compared to oral dosing.

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POC44

Prognostic value of ferritin, neuron-specific enolase, lactate-dehydrogenase, urinary and plasmatic catecholamine metabolites in children with neuroblastoma

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Background: Different plasma/serum and/or urinary parameters have been tested as valuable prognostic markers for children with neuroblastoma (NB), but conclusive results are still lacking due to the limited number of studies in which multivariate analyses have been performed.

Methods: Five hundred and five children diagnosed in Italy between June 1994 and November 2010 were included in the study. All analysis were performed at the time of diagnosis at the Italian reference laboratory according to standard methodologies. Patient clinical data were retrieved from the Italian NB Registry. For statistical analysis patients were grouped according to different variables: stage (INSS 1,2, 3, 4 and 4s), age at diagnosis (≤18 months, >18 months), MYCN status and outcome. Odds Ratio (OR) with 95 confidence interval (95%CI) was calculated. Multivariate analysis was performed by the Cox regression model by considering only the significant variables.

Results: When the entire cohort of patients was considered none of the different parameters had an independent prognostic value. However, in patients with localized disease without MYCN amplification the significant associations between urinary and plasmatic HVA/VMA ratio (OR = 3.80, 95%CI 1.39-10.42; OR = 6.11 95%CI 1.33-28.03 p<0.01, below 0.5 and 0.1, respectively) and worse prognosis remained significant, as well as that between LDH values and prognosis (>1300 IU/mL OR = 7.35, 95%CI 3.90-13.86). Moreover, in stage 4 patients without MYCN amplification NSE levels above 200 ng/mL and LDH above 2500 IU/mL maintained their significant association with a worse outcome.

Conclusions: In NB patients of all stages without MYCN amplification, LDH has an independent prognostic value. In addition, in patients with localized disease also urinary and plasmatic HVA/VMA ratio are independently associated with outcome. Since at diagnosis LDH and catecholamine metabolites are always measured in all patients, these findings may be helpful for a easy and cost effective patient risk stratification.

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POC45

Efficacy of Treosulfan as a single agent in newly diagnosed neuroblastoma stage IV pts

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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 2011, 13 pts (M/F 8/5) 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). Eleven 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 morphologically clean after 2 courses in 8 out of 11 pts (in 1 pt with relapse). Seven pts achieved PR and 6 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. Ten pts have received transplantation BM. Five pts have relapsed diseases, two died. EFS is hole group was 31%. EFS in transplant group 40%

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POC46

Induction of transforming neuroblastic cells into ganglionic cells with tumor regression in neuroblastoma by using low-dose chemotherapy plus traditional Chinese medicine: A report of 8 cases

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Objective: According to the bionomic characteristics, neuroblastoma (NB) has the possibilities of self-regression and self-healing, the aim of this study is to explore the pathways of which neuroblastic cells are transformed and differentiated into ganglion cells.

Materials and Methods: Use low-dose chemotherapy associated with traditional Chinese medicine L-3 in treating patients of NB with huge tumors that cannot be operated on or with postoperative recurrence. CT and/or MRI, B-mode ultrasonography, pathology, blood biochemical detection were performed to observe tumor size and clinical changes.

Results: From 1988 to 2011, 8 cases of NB were successively treated in our department by using low dose of chemotherapy with traditional Chinese medicine, all were survived with disease free. Among them 4 cases were mediastinal NB, they could not accept operations at the time of discovery due to massive tumor, after treatment for 1-2 years the tumor completely disappeared, now two of them had been graduated from universities, 3 cases were retroperitoneal NB , they had partial resection of the tumor for the first operation, the second operations for them were preformed after 1-2.5 years of medication, and the pathology of tumors turned out to be ganglioma; 1 case was NB of the left adrenal gland, the turmor disappeared after chemotherapy for one year.

Conclusion: Low-dose chemotherapy plus traditional Chinese medicine, Zhong Luo NO.3, were effectively used to transform NB into the ganglioma or self regress in 8 cases. This treatment will become a feasible way to treat NB.

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POC47

Plasma and serum levels of potential prognostic markers in neuroblastoma patients and healthy children

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Background: Patient risk stratification is required to increase the rate of clinical responses and to decrease unnecessary toxicity. In this view, prognostic markers detectable in plasma or serum samples may be preferable to those detectable in tumor specimens and/or bone marrow samples.

Methods: Plasma and serum collected at onset from children diagnosed with NB according to INSS, and from age and sex-matched healthy children, were tested by ELISA for the presence of B7H3, cyt-MAA/LGALS3BP, I-CAM1, HLA-E, HLA-F and calprotectin molecules. Correlations between markers' levels and outcome, as well as with other known prognostic factors, were then evaluated.

Results: All markers' levels, but B7H3, were higher in NB patients than in healthy children. However, their levels were not significantly different among different stages with the exception of calprotectin. In patients with localized NB, calprotectin serum levels were similar to those found in healthy children, whereas significantly higher levels were found in children with stage 4 NB.

Conclusions: Calprotectin, a protein selectively expressed by the BM-infiltrating metastatic NB cells, may deserve further evaluation as potential prognostic marker to stratify high risk patients with metastatic NB.

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POC48

Neuroblastoma in the adult. The Italian experience.

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Supported by Italian Neuroblastoma Foundation

Background: The incidence of neuroblastoma in the adult should be around 0.1 case/million/year (SEER data) translating into 4-5 new cases/year in Italy. Only few come to the attention of pediatric oncologists and are included into National Registries. No studies have investigated the disease at this age to detect specific characteristics. We report herewith the experience with 23 patients.

Patients: Between 1986-2011, twenty-three adults with newly-diagnosed neuroblastoma were referred for consultation. Age at diagnosis ranged from 220 to 440 mos (median, 283). Ten were male and 13 female. The primary was abdominal in 10 patients, adrenal in 5, thoracic in 5 and pelvic in 3. Nine patients had disease stage 1 or 2, six had stage 3, and 8 had stage 4.

Results: Stage 1-2. Of 9 patients, four were treated by surgery alone, of whom 2 died at 25 and 48 mos, and 2 are alive at 12 and 100 mos; and 5 were treated by surgery and chemotherapy +/- radiotherapy, of whom 2 are alive at 6 and 147 mos, and 3 had multiple recurrences and eventually developed metastases of whom one died at 130 mos and 2 survive with stable disease at 73 and 108 mos. Stage 3. All 5 patients received chemotherapy and 4/5 underwent tumor resection and radiotherapy. One is alive with disease at 6 mos and 4 died at 23, 32, 43 and 96 mos. Stage 4. All 7 patients were treated intensively without achieving complete response and died at 4-80 mos (median, 24).

Conclusions: Survival data of our 23 patients indicate that outcome is even worse than in adolescents. For stage 1-2, the clinical course was independent of therapy administered. Of 12 stage 3-4 patients, 11 died and one survives with disease. Biological understanding of the tumor at this age is mandatory.

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POC49

Early response on 18F-FDOPA PET in stage 3 and 4 neuroblastoma

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Background: Recently we proved that 18F-FDOPA positron emission tomography (PET) was more sensitive than 123I-MIBG scintigraphy and 18F-FDG PET in neuroblastoma (NB). Whether 18F-FDOPA PET can evaluate treatment response remains to be determined.

Methods: During 2008-2011, patients with stage 3 or 4 NB were treated at National Taiwan University Hospital with TPOG-N2002, a risk-directed multimodal regimen. Patients who have undertaken 18F-FDOPA PET and 18F-FDG PET before and after induction chemotherapy were included for analysis. The change of tumor-to-liver standard uptake value ratio (T/L) was used as an indicator of treatment response.

Results: Nine of 16 patients with stage 3 or 4 NB were eligible, with male:female 2:1 and median age 3.5 (range 0.3-6.9) years. Three patients (33%) demonstrated early response on 18F-FDOPA PET (decrease T/L after chemotherapy), while 6 patients (67%) demonstrated that on 18F-FDG PET. Astonishingly, in stage 4 NB (n=7), 18F-FDOPA uptake increased significantly after 2-3 cycles of chemotherapy (mean T/L, 2.050 +/- 0.604 vs. 3.297 +/- 1.032; P=0.0477 by paired t test). In contrast, 18F-FDG uptake did not change significantly (mean T/L, 3.637 +/- 1.413 vs. 2.288 +/- 1.563; P=0.1688). The change in 18F-FDOPA uptake in stage 4 NB was significantly higher than that in stage 3 NB (mean difference in T/L, +1.437 +/- 2.049 vs. -3.869 +/- 2.572, P=0.0045 by Student's t test), while their uptake of 18F-FDG was similar (-1.348 +/- 2.280 vs. -0.141 +/- 2.172, P=0.53). Four of the 9 patients had follow-up 18F-FDOPA PET after 4-6 cycles of chemotherapy and all showed subsequent decrease in 18F-FDOPA uptake (P=0.16).

Conclusions: In comparison to stage 3 NBs, most stage 4 NBs showed an initial raise and subsequent drop in 18F-FDOPA uptake during chemotherapy. The prognostic implication of early response on 18F-FDOPA PET requires further investigations.

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POC50

Clinical analysis of Pulmonary Metastases at Diagnosis of Neuroblastoma in Pediatric Patients

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Objective: To analyze the clinical characteristics, treatment and prognosis of the rare occurrence of pulmonary metastases among children presenting with neuroblastoma.

Methods: Analyze the clinical manifestations, imaging characteristics, treatment and prognosis of the 5 patients of neuroblastoma with pulmonary metastases who were treated by the Fourth Affiliated Hospital China Medical University Hospital from 2009 to 2011.

Results: There are 2 males and 3 females of the 5 cases, range 32.2 months. Of the 102 cases which were confirmed neuroblastoma in the same period, 5 patients were diagnosed with pulmonary metastases, the incidence is 4.9%. All the primary tumor of the 5 cases were located in the abdomen. The 2 cases with metastases to the back of peritoneum is prone to happen spinal canal and surrounding soft tissue infiltration. There are non-specific signs and symptoms of pulmonary metastatic.The chest CT showed the lesions could present as a solitary or multiple nodules with smooth or pointed margins, which are noncalcified and solid of ground-glass density or mixing density ,often accompanied by pleural metastasis. The histopathology of tumor revealed four of the five cases were neuroblastoma. And one of the five cases is ganglioneuroblastoma. All the patients of this group were given preoperative and / or postoperative chemotherapy, primary tumor resection, one case was given the lung and pleural metastases resection, two cases's pulmonary metastases were disappeared after chemotherapy, and one of the two was recurrence ,and death. Two cases showed progressive deterioration and dead.

Conclusion: There are no specific clinical manifestations of pulmonary metastases, chest CT has some characteristic features, routine chest CT examinations were helpful for the early detection of lung metastases, chemotherapy is effective for pulmonary metastases, but relapse may happen,and the prognosis of NB children with pulmonary metastases is poor

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POC51

Neuroblastoma with isolated 11q13 (CCND1) amplification: Report of a case

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Background: Neuroblastoma (NB) with chromosome 11q13 amplification encompassing the CCND1 (cyclin D1) gene is a rare molecular subtype that is associated with older age at diagnosis, more advanced stage, poor prognosis, and other structural or numerical copy number aberrations (CNAs) (Michels, 2007; Molenaar, 2012). We report a case of NB with isolated 11q13 amplification that has been treated as high-risk disease.

Methods: The patient's clinical history and relevant study results were reviewed. Tumor sample was obtained during surgery, immediately frozen in liquid nitrogen, and extracted for DNA. Bacterial artificial chromosome (BAC)-based array comparative genomic hybridization (array-CGH) of was performed using CMDX BAC-based aCGH CA3000 chips (CMDX, Irvine, CA), with a mean resolution of 1 Mb across the entire genome.

Results: A 3-year-3-month-old boy developed fever and unsteady gait and was diagnosed with stage 4 NB with a left retroperitoneal primary tumor and metastasis to the spine. With 6 cycles of chemotherapy under the TPOG-N2002-HR regimen and radiation therapy to the spine, his symptoms have resolved and the tumor volume has markedly decreased. Gross total resection of the main tumor and left adrenal gland was then performed smoothly. Histopathology showed a differentiating neuroblastoma with extensive necrosis and an adjacent lymph node positive for NB, while the adrenal gland was unremarkable. Array-CGH of the tumor revealed a 1.63-Mb amplification at 11q13.2-q13.3 (1.943-2.409 folds on 6 consecutive probes, RP11-149G19–>RP11-368I20) as the only CNA. Three months later, the patient underwent high-dose chemotherapy plus autologous stem cell transplantation, followed by maintenance therapy with retinoic acid. He has remained in progression-free survival for 25 months after diagnosis.

Conclusions: A young Asian NB patient with CCND1 amplification has shown complete response to multimodal therapy and has remained disease-free for more than 2 years.

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POC52

High-dose 3F8 anti-GD2 monoclonal antibody (MoAb) plus granulocyte-macrophage colony-stimulating factor (GM-CSF) for high-risk neuroblastoma (NB)

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Background: 3F8/GM-CSF is active against NB in bone marrow (BM) (J Clin Oncol 21:1087). Based on the dose-responsiveness of NB to 3F8-mediated cytotoxicity in vitro (Blood 73:1936), the prognostic importance of early BM response to 3F8/GM-CSF (J Clin Oncol 21:3853), and the safety of high-dose intravenous (iv) 3F8 after premedication with 2 mg/m2 of iv heat modified 3F8 (J Clin Oncol 29:1168), we initiated trials to test the efficacy of high dose 3F8.

Methods: NB patients in 1st complete/very good partial remission (CR/VGPR) without prior myeloablative therapy (http://www.clinicaltrials.gov NCT01183429) or with primary refractory BM disease (http://www.clinicaltrials.gov NCT01183897) receive 2 cycles (5 days/cycle) of high-dose 3F8 (80 mg/m2/day), followed by cycles of standard-dose 3F8 (20 mg/m2/day) in the absence of human anti-mouse antibody, x2 years from study entry. GM-CSF is injected subcutaneously x5 days before, and on each day of, 3F8 treatment. Consolidative primary site radiotherapy follows cycle 1, and 13-cis-retinoic acid starts after cycle 2. BM minimal residual disease (MRD) is measured by qRT-PCR.

Results: 12 patients (8 MYCN-amplified) in first CR/VGPR were enrolled 5.5-15 (median 6.5) months from diagnosis; 7 had required 2nd-line therapy to achieve CR/VGPR. One patient relapsed quickly (brain, 1.5 months), 11 patients remained relapse-free at 1+-to-13+ (median 6+) months. 10 patients treated for resistant NB, as evidenced by BM histology (n=5), 123I-metaiodobenzylguanidine (MIBG) scan (n=4), or both (n=1), were 5.5-24 (median 10) months from diagnosis and remained progression-free at 1.5+-13+ (median 8+) months. After 1 cycle of 3F8/GM-CSF, BM histology showed CR in 5 patients (including 1/1 previously treated with the anti-GD2 MoAb ch14.18) and PR in one patient. 123I-MIBG scans were unchanged (n=3) or improved (n=2). 67% of patients cleared MRD after cycle 2.

Conclusions: Two-cycle high-dose 3F8 approach was tolerable. Radiographic and histologic responses were seen. BM MRD response was favorable.

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POC53

Phase II study of bevacizumab plus irinotecan and temozolomide for refractory or high-risk relapsed neuroblastoma: preliminary results

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Background: The rationale for the combination of bevacizumab plus irinotecan and temozolomide (BIT) is based on: (a) Association of vascular endothelial growth factor (VEGF) expression with aggressive phenotype in neuroblastoma. (b) Anti-VEGF antibody bevacizumab enhances irinotecan-mediated suppression of neuroblastoma xenografts. (c) Effectiveness of Irinotecan+temozolomide as salvage chemotherapy for neuroblastoma. (d) Safety profile of bevacizumab in pediatric phase I studies (J ClinOncol 26:399).

Methods: We initiated a phase II study to evaluate tumor responses to BIT in children with refractory or relapsed stage 4 neuroblastoma (www.clinicaltrials.gov NCT01114555). Each cycle consisted of bevacizumab (15mg/kg intravenously) (days 1 and 15) plus irinotecan (50mg/m2/day intravenously) and temozolomide (150mg/m2/day orally) (days 4-8). Patients were monitored for toxicity and response assessed after every two cycles; cycles were repeated every four weeks after resolution of toxicities, if any in the absence of progressive disease (PD). Early stopping rules for toxicity and efficacy were incorporated so that study could continue only if >5 of the first 27 evaluable patients achieved partial (PR) or complete response (CR) after 4 cycles of BIT. Secondary objectives included evaluation of angiogenic profile before and after BIT.

Results: 25 heavily-pretreated patients have thus far received 1 (n=3), 2 (n=5), 3 (n=1), 4 (n=9), 5 (n=2), 6 (n=4) or 8 (n=1) cycles respectively. Grade 4 toxicities were myelosuppression (36%) and proteinuria (8%). Grade 3 toxicities included hepatic transaminitis (36%), epistaxis (28%), diarrhea (24%) and vomiting (4%). All toxicities were expected and transient. Overall responses in 24 evaluable patients were: 1CR, 6PD and 17 stable disease. 1/17 patients assessable for early stopping rule regarding efficacy achieved CR/PR.

Conclusion: Preliminary results from this ongoing study indicate that although BIT is well tolerated, addition of bevacizumab did not appear to significantly increase response rates in resistant neuroblastoma when compared to historical data for irinotecan+temozolomide (J ClinOncol 24:5271).

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POC54

Analysis of pangenomic profiles in localized neuroblastoma without MYCN amplification – a preliminary report from SIOP Europe neuroblastoma (SIOPEN) group on the LNSEG2 Trial

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Background: The second European study on localized resectable neuroblastoma (LNESG2) aimed at a prospective complete pangenomic analysis of tumor samples and its impact on prognosis.

Methods: LNSEG2 diagnostic tumor samples, without MYCN amplification and >60% tumor cells were analyzed by pan/multigenomic assays (Array comparative genomic hybridization or multiplex ligation–dependent probe amplification). All genetic data were quality controlled by the SIOPEN Biology Group.

Results: Complete pan/multigenomic data were available for 135 tumors, 94 INSS stage 1 and 41 stage 2. Segmental chromosomal aberrations (SCAs) were observed in 58 tumors (43%). Fifty of them showed typical SCAs (TS) on chromosome arms 1p, 1q, 2p, 3p, 4p 11q and 17q, and 8 showed atypical SCAs (AS). Full numerical profiles (FN) were found in 77 samples (57%). SCAs were more frequent in patients > 18 months of age (p = 0.0015) but did not correlate with the disease stage (p=0.78).

Follow-up (FU) was known for 121 patients with a relapse-free survival (RFS) at 18 months of 90.3%. Eleven relapses occurred, 5/68 FN (7% of FN) (all concerning patients < 18 months) and 6/ 46 TS profiles (13% of TS). Of 6 relapses in the TS group, 1 occurred in a patient < 18 months and 5 in patients > 18 months at diagnosis, all but 1 in the first 12 months of FU. Thus, only in patients > 18 months at diagnosis, a significant association with TS could be observed with a RFS at 18 months of 72% (p = 0.0381).

Conclusion: These preliminary results indicate that pangenomic analyses appear particularly pertinent for patients >18 months. However, on-going analysis of the complete LNESG2 patient cohort will allow drawing definite conclusions. Further studies will focus on early relapses observed in patients below 18 months with a FN profile.

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POC55

Pediatric reference ranges for plasma free and total metanephrines and their relevance in diagnosis of neuroblastoma

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Background: Plasma total and free metanephrines are the gold standard for diagnosis of pheochromocytoma in adults and children with higher sensitivity and specificity than urinary catecholamines and their metabolites. We postulated that plasma metanephrines should be more sensitive markers also in neuroblastoma because directly produced by the tumor. The aim of our study was to establish complete reference values for plasma total and free metanephrines in healthy children and to provide preliminary results on its diagnostic value in patients diagnosed with neuroblastoma.

Methods: Blood samples (1ml) were drawn prospectively from healthy children aged 0- 18 years needing venous puncture for other reasons and put in lithium heparinized tube, immediately stocked at -70° C and sent to laboratory for analysis.

Total and free plasma normetanephrine (NMN) and methoxytyramine (MT) was measured by HPLC with electrochemical detection. Patients with suspected neuroblastoma underwent the usual diagnostic procedures.

Results: 191 healthy children (age 0-17 years, 122 boys) constituted the reference population. The following upper reference limits of total and free plasma NMN and MT were established in nmol/l (95% CI): tNMN 9.95 (9.63-35.43), tMT 6.97 (5.67-14.93), fNMN 0.79 (0.72-1.01), fMT 0.07 (0.04-0.09). The following cut-off values with sensitivity/specificity/PPV/NPV for ten neuroblastoma patients were determined: tNMN 19.64 - 100%/99.48%/90.01%/100%, tMT 9.43 - 100%/98.95%/83.33%/100%, fNMN 3.16 - 100%/100%/100%/100%, fMT 0.11 - 88.90%/100%/100%/99.50%. There was neither a significant difference between gender nor significant dependence on age.

Conclusion: These are the first complete pediatric reference limits for total and free plasma metanephrines established in healthy children. Their diagnostic utility, high sensitivity and specificity have been shown in ten patients with neuroblastoma and need to be confirmed in a larger patient group.

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POC57

Clinical and Imaging Diagnosis of Neuroblastoma

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Objective: To study the clinical and imaging characteristics of neuroblastoma in the patients referred into our hospital.

Materials and Method: From 2009-2011 100 cases of neuroblastoma were recruited into this study. All the cases were confirmed by operation, pathology or biopsy of the primary lesion. Bone marrow examination performed in all cases. Conventional and enhanced CT, conventional and/or enhanced MRI, and bone Scan were performed according to the site of involvement. Treatment of the patients in this group were discussed.

Results: In the 100 cases, male to female ratio was 3:2. Age: ranged from 4d-24yrs, ≤3y 60 cases (60%), >3y 40 cases (40%), 26 cases died (26%). Primary lesion site: adrenal n=44 (44% left 26, right18) post-peritoneal n=28, mediastinum n=22, other sites n=6. Metastases : Bone marrow metastases in 78cases, bone mets in 24cases, metastases to liver in 9 cases, lymph nodes metastases in 7cases, mediastinal lymph nodes mets. in 6 cases, pleura mets. in 5, lung mets in 5, intracranial mets. in 4 cases. Surgical therapy was performed in 89 cases (post-chemo n=49, direct operation before chemo n=36) In this group according to the clinical material and imaging characteristics correlated to INSS stage 1 in 5 cases, stage 2 in 5 cases, stage 3 in 25cases, stage 4 in 62cases, stage 4S in 3 cases. There were 87 cases belong to stage 3 and stage 4 (87%), so most of the cases in our group belong to stage 3 and stage 4. Imaging characteristics of this group were discussed.

Conclusion: Clinical manifestations and imaging characteristics of 100 cases were presented in the study. The imaging features correlated well with the INSS staging.

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POC58

Radiotherapy Quality Assurance Review in the International Society of Paediatric Oncology (Europe) Neuroblastoma Group's High Risk Neuroblastoma Trial: a SIOPEN Study.

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Purpose: Radiotherapy is important for local control in neuroblastoma. This study reviewed the compliance of plans with the radiotherapy guidelines of the International Society of Paediatric Oncology (Europe) Neuroblastoma Group (SIOPEN) high-risk trial protocol.

Methods and materials: The SIOPEN trial central electronic database has sections to record diagnostic imaging and radiotherapy planning data. Individual centres may upload data remotely, but not all centres involved in the trial chose to use this system. A quality scoring system was devised based on how well the radiotherapy plan matched the protocol guidelines, to what extent deviations were justified and whether adverse effects may result. Central review of radiotherapy planning was undertaken retrospectively in that proportion of patients where complete diagnostic and treatment sets were available. Data were reviewed and compared against protocol guidelines by an international team of radiation oncologists and radiologists. For each patient in the sample the central review team assigned a quality assurance score.

Results: Data on a total of 100 patients were reviewed. It was found that in 48 cases, (48%) there was full compliance with protocol requirements. In 29 cases (29%), there were deviations for justifiable reasons with no likely long term adverse effects resulting. In five cases, (5%), deviations had occurred for justifiable reasons, but which might result in adverse effects. In one case, (1%), there was a deviation with no discernable justification, and which would not lead to long term adverse events. In 17 cases (17%), unjustified deviations were noted with a risk of an adverse outcome resulting.

Conclusions: Because of concern over the proportion of patients in which unjustified deviations were observed, a protocol amendment has been issued. This offers the opportunity for central review of radiotherapy plans prior to the start of treatment and the treating clinician a chance to modify plans.

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POC59

Advanced Metastatic Neuroblastoma Treatment: A Single Institution Experience And Analysis Of Rare Cases

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Background: More than a half of patients with neuroblastoma present with disseminated disease, which confers a poor prognosis and is associated with a high mortality rate. The choice of treatment strategy in the case of unusual widespread advanced metastatic disease or multifocal neuroblastoma remains controversial.

Methods: 23 children mostly from western part of Ukraine with INSS stage 4 neuroblastoma were observed and treated in our clinic during the period of 1995-2011. The age was from 0 to 15 years. The primary tumour was localised in the adrenal gland in 5 patients, retroperitoneum in 7 pts, mediastinum in 3 pts , neck region in 2, presacral region in 2, CNS in 1 case and multifocal primary tumor was observed in 3 cases. The metastatic sites were mostly the lymph nodes (72%) and bone marrow (86%) with nearly 30% of skeletal bone involvement and 26 % of liver metastatic lesions; 3 cases of rare widespread metastatic lesions of the head with CNS involvement in children 2 year of age and 1 case of congenital diffuse multifocal neuroblastoma with total soft tissues and parenchymatous organs affection in newborn child are described. 78 % (mostly since 2000) of patients received aggressive multimodal treatment (chemotherapy with primary tumor surgery, distant beam radiotherapy if necessary) followed by ASCT (if possible) or retinoic acid treatment. 22% received only palliative treatment (low dose chemotherapy regimen).

Results: At the end of 2011, with a median follow-up of 36 months, the 3-year OS is 43%.

The 2-year EFS is 26%.

Conclusions: Aggressive treatment of advanced stage 4 neuroblastoma increased and prolonged the OS. Still in the case of potentially incurable disease or progression during treatment palliative therapy should be considered to provide higher quality of life.

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POC60

Low GFR correlates with poor survival in high risk neuroblastoma patients

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Background: The prognosis for high risk neuroblastoma (HR-NB) remains poor despite aggressive multimodal therapy. All up-front chemotherapeutic regimens contain platinum-based, nephrotoxic compounds whose renal clearance is closely related to glomerular filtration rate (GFR). Deviations in GFR from normative values can result in altered drug half-life, and therefore potential anti-tumor efficacy. As such, we hypothesize that abnormal GFR values at the time of diagnosis are associated with worse survival outcomes in children with HR-NB.

Methods: We performed a retrospective chart review of patients with HR-NB treated at our institution from Jan 2001 to May 2011. 73 patients were included in the study. Data collected included age, stage, mycN status, initial GFR value, bone marrow involvement, tumor size, number of vessels encased by tumor at presentation, and time to relapse and/or death. Patients were categorized into groups (low, normal, and high GFRs) based on established normative values by age.

Results: In our cohort, 69% patients had normal, 18% had low, and 13% had high GFRs at presentation. Children with abnormal GFRs (either low or high) were more likely to relapse than those with normal GFR values (p=0.0497). Further, those patients with low GFRs had a worse OS (p=0.04). With multivariate analysis, both the number of vessels encased by the tumor at presentation and a low GFR were significant predictors of decreased OS (HR 1.21, 95% CI 1.02-1.45, p=0.03; HR 2.28, 95% CI 1.01-5.15, p=0.048, respectively).

Conclusions: Children with HR-NB that had an abnormal GFR at the time of diagnosis were more likely to relapse, and those with a low GFR had a decreased overall survival. Further investigation is required to determine if deviations in GFR, both high and low, necessitate chemotherapy dose modifications in order to improve rates of response and survival.

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POC61

Morbidity and mortality risks in infants with stage 4S neuroblastoma: A report from the Children's Oncology Group study ANBL0531, "Response- and Biology-Based Therapy for Intermediate-risk Neuroblastoma"

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Background: Infants with stage 4S neuroblastoma (NB) generally have excellent outcomes; however, some are at increased risk of death due to rapidly progressive disease and complications from massive hepatomegaly. Infants with stage 4 NB with liver metastases may be at similar risk.

Methods: Between 10/8/2007 and 4/1/2011, 44 patients with stage 4S NB were enrolled on study (28 [64%] <3 months at diagnosis). Eligible patients were <365 days with newly diagnosed INSS stage 4S MYCN-not amplified (NA), unfavorable INPC histology and/or diploid NB. Clinically symptomatic 4S patients, regardless of histology/ploidy status, or ability to undergo biopsy (N=4), were also enrolled to receive emergent chemotherapy. Assignment to receive 2, 4, or 8 cycles of chemotherapy was based on histology and tumor biology. Baseline assessment of organ dysfunction was determined for 4S patients. One-hundred fourteen patients with INSS stage 4 NB <365 days (18 [16%] <3 months at diagnosis), MYCN-NA with any histology/ploidy were also enrolled and assigned 4 or 8 cycles of chemotherapy.

Results: Baseline toxicities (grade ≥3) reported in 4S patients included respiratory compromise (12 [27%]), coagulopathy (6 [14%]), failure to thrive (2 [5%]), and liver dysfunction (8 [18%]). Grade 4/5 toxicity reported in 4S included 28 [64%] overall (19 [69%] <3 months), and in stage 4 included 70 [61%] overall (12 [67%] <3 months). There were 7 deaths (5 in stage 4S, 2 in stage 4) related to complications of hepatomegaly. Age at death ranged from 4-67 days. In 2 patients the primary cause of death was infection but in all patients massive hepatomegaly was a contributing factor.

Conclusions: Infants with stage 4S or stage 4 NB < 3 months of age with rapidly evolving hepatomegaly appear to have a disproportionately high rate of early mortality (7/46 [15%]). Preemptive intervention with chemotherapy is warranted in this population.

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POC62

The complementary role of 18F-FDOPA and 18F-FDG PET scans in the follow up of neuroblastoma.

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Background: MIBG is used for the diagnosis and follow up of neuroblastoma (NB). For those MIBG is not available, 18F-FDOPA (FDOPA) and 18F-FDG (FDG) PET scans are alternative for the diagnosis and staging of NB. We tried to use these tools in the follow up of disease progression.

Methods: From 2006 to 2011, FDOPA and FDG PET were used in the staging and follow up of patients with NB in National Taiwan University Hospital. All expired cases were enrolled in this study for retrospective analysis. FDOPA and FDG PETs were done every 3-6 months during the follow up period.

Results: Thirteen patients (male:female 9:4; mean age of onset 3.7 [range 1.0-6.9] years; mean survival time 32 [range 9-60] months; MYCN amplification n=5; all are stage 4 except one stage 3) were analyzed. In 12 cases with bone mets, 10 FDOPA and 11 FDG detect bony lesions. However, 6 of them had skull and 3 had mandible lesions were missed by FDG PET. None of the FDOPA or FDG PET detect brain lesions. In 8 cases with soft tissue lesions, 6 FDOPA and all 8 FDG PET were positive. However, in two of them the FDG and FDOPA showed different uptake of the agent.

Conclusions: FDOPA and FDG PET scans are complementary in the follow up of recurrence and/or metastatic status of the NBs. The skull and facial bone lesions are better defined by FDOPA because of FDG uptake in the brain. And for brain mets, MRI is still the test of choice.

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POC63

KIR ligand incompatible cord blood transplantation for high risk neuroblastoma as allogeneic NK cell based immunotherapy

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Background: Allogeneic stem cell transplantation(SCT) for acute myeloid leukemia from KIR ligand incompatible donor has been reported significantly better survival than from KIR lignad compatible donor in both HLA haploidentical SCT and cord blood transplantation (CBT) due to NK cell based graft versus leukemia effects. Venstrom et al. reported that superior survival was strongly associated with "missing KIR ligand" even in patients with neuroblatoma (Clinical Cancer Research 2009). We hypothesized that alloreactive NK cells from KIR ligand incompatible donor can target minimal residual disease and reduce bone marrow relapse rate in patients with high risk neuroblastoma.

Methods: We conducted tandem transplantation using high dose chemotherapy followed by autologus SCT and allogeneic CBT with reduced intensity conditioning regimens from KIR incompatible donor based on "missing KIR ligand model". Interval of autologous SCT and CBT was 2-3 months. Reduced intensity conditioning regimen for CBT were used either BU, Flu and L-PAM or Flu, L-PAM and TBI 2Gy. Tacrolimus and methotrexate were used for GVHD prophylaxis. Eligibility criteria were Stage IV neuroblastoma patients with 1) 2nd CR after relapse, 2) 1st CR with MYCN amplification, 3) over 10 years old or 4) MIBG or PET positive metastases remained before SCT. Single KIR2DL1 positive NK cells were monitored after CBT from HLA-C2 ligand missing donor to access alloreactive NK cells by flowcytometry.

Results: Seventeen patients received the tandem transplantation (9 were MYCN amplified, 2 were over 10 years old, 3 were relapsed disease and seven were with MIBG or PET positive tumors before SCT). All patients achieved engraftment. Three patients died of non-infectious pulmonary disease and all of them received BU containing regimen prior to CBT. Three patients developed grade II acute GVHD and were well controlled by steroid. No patients developed chronic GVHD. Two patients relapsed with 2 years and 6 months of median follow up after CBT. Of note, both relapse sites were local without bone marrow invasion. Single KIR positive NK cells were significantly increased after CBT suggesting alloreactive NK cell activity (0.045%±0.027 vs 2.14±1.59%, p=0.007).

Conclusions: Tandem transplantation with autologous SCT and allogeneic CBT from KIR ligand incompatible donor for neuroblastoma was feasible. Further observation is necessary to access whether this strategy improves the prognosis of high risk neuroblastoma.

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