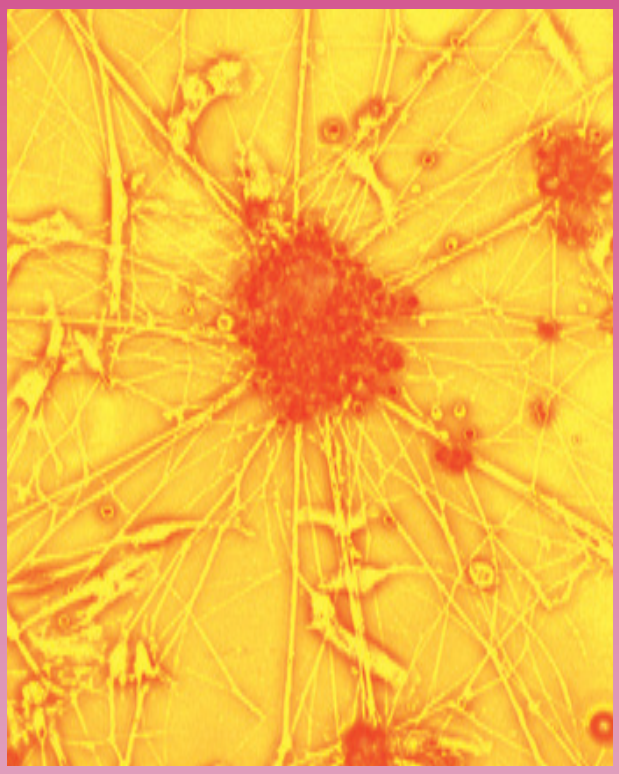
Advances in Neuroblastoma Research 2008

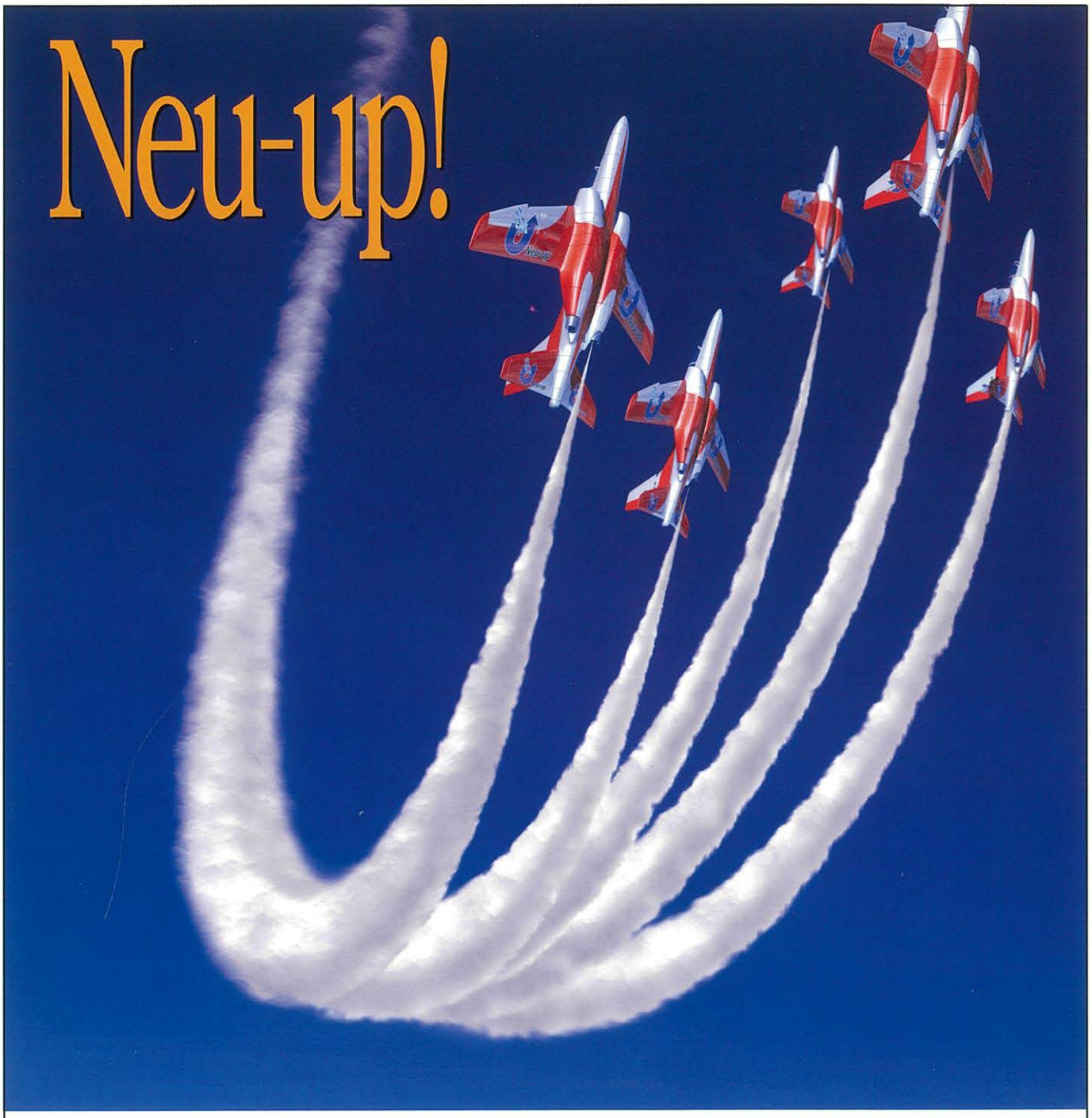
Program & Abstracts



Place: Makuhari Messe International Conference Hall Chiba, Japan

Dates: May 21-24, 2008

ANR 2008



遺伝子組換えヒトG-CSF誘導体製剤

指定医薬品／処方せん医薬品*　〈薬価基準収載〉

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Neu-up for Injection

注射用ナルトグラスチム（遺伝子組換え）
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*注意－医師等の処方せんにより使用すること

■「効能・効果」、「用法・用量」、「使用上の注意事項」は製品添付文書をご参照ください。

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http://iyaku.kyowa.co.jp/
KYOWA

06.06.

Advances in Neuroblastoma Research 2008

Program & Abstracts

http://www.anr2008.com/
http://www.anrmeeting.org/

Contents

Welcome Message

Dear colleagues:

Welcome to Chiba!

Advances in Neuroblastoma Research (ANR) was founded in 1975 in Philadelphia by Drs. Audrey E. Evans, Everet Koop and Guilio D'Angio, starting with the attendance of less than 20 people. Subsequent series of ANR conferences were held in Philadelphia, Heidelberg, Bath, Paris and Genova, succeeded by the meeting in Los Angeles in 2006. The aim of this meeting is to find ways in moving forward in treating patients with neuroblastoma through the advancement of laboratory and clinical research.

Now, the ANR conference is rapidly growing in both its scientific levels and the number of attendee. The By-laws of ANR association (ANRA) has been formalized at the ANR 2006 meeting in Los Angeles. ANR is not solely a society but is also linked to the International Neuroblastoma Risk Group (INRG), the International Neuroblastoma Pathology Committee (INPC) and some other newly founded organizations including International Neuroblastoma Tissue Bank (INTB) and Nurses group.

The ANR2008 is now held in Chiba, Japan, the first time in Asia. More than 390 abstracts have been applied from 35 countries in the world. All of them were reviewed and scored by about 7 authorities, and the categories were divided into three: basic, translational and clinical researches. Their scientific levels are extremely high and truly competitive. The genome-wide approaches in neuroblastoma have rapidly progressed and now reached identification of many new candidate genes and proteins which can be proper molecular targets for screening novel therapeutic small compounds as well as antibodies.

In the afternoon on May 24, the Japanese volunteer groups of childhood cancers hold an international open symposium on childhood cancer survivorship by linking to ANR2008. This may encourage our challenge conquering aggressive neuroblastomas in world-wide scale.

Now Japan is really a beautiful season. Chiba is very convenient place to embark on any sightseeing as many beautiful and exotic places in central Japan are easily accessible. It is also a main gateway of Japan opening to the pan-pacific countries including those in Asia.

I truly hope that all of us have a wonderful and fruitful time together at the ANR 2008 meeting for the future development of better cures for patients suffering from neuroblastoma.

Best wishes.

Akira Nakagawara, MD, PhD
President, ANR 2008
Director, Chiba Cancer Center Research Institute
Chiba, Japan

Organization

Steering Committee (elected on May 19th, 2006)

Present president:	Frank Berthold
Past president:	Akira Nakagawara
Incoming president:	Susan L. Cohn
Representatives North and South Americas:	Garrett M. Brodeur, Carol J. Thiele
Representatives Europe including Russia:	Manfred Schwab, Frank Spelemann
Representative Asia, Australia, Africa:	Akira Nakagawara, Michelle Haber
Secretary:	Audrey E. Evans
Homepage Webmaster:	Patrick C. Reynolds (adopted)

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Akira Nakagawara
Barbara Hero
Murray D. Norris
Per Kogner
Robert C. Seeger
John M. Maris
Rogier Versteeg

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Nai-Kong V. Cheung
Susan L. Cohn
Javed Khan
John M. Maris
Katherine Matthay
Vincente Odone
Julie Park
Patrick C. Reynolds
Robert C. Seeger
Carol J. Thiele

Europe including Russia
Huib Caron
Victoria Castel
Bruno de Bernardi
Angelika Eggert
Ruth Ladenstein
Tommy Martinsson
Jean Michon
Andrew D.J. Pearson
Manfred Schwab (Chair)
Gian Paolo Tonini
Dominique Valteau-Couanet
Rogier Versteeg

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Michio Kaneko
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Nili Peylan-Ramu
Jin Hua Zhang

Local Organizing Committee

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Ohira, Miki
Nakamura, Yohko

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General Affairs/Public Relations

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Local Organizing Committee Members

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Bessho, Fumio
Fujimoto, Junichiro
Fukuzawa, Masahiro
Hara, Junichi
Hara, Toshiro
Hareyama, Masato
Hayabuchi, Naofumi
Hayashi, Yutaka
Hemmi, Hiromichi
Hiyama, Eiso
Horie, Hiroshi
Hosoya, Ryouta
Iehara, Tomoko
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Ishii, Shin
Ishimoto, Koichi
Ito, Etsuro
Iwanaka, Tadashi
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Kawakami, Kiyoshi
Kawano, Yoshifumi
Kojima, Seiji
Komada, Yoshihiro
Kosaka, Yoshiyuki
Kubota, Masayuki
Kurosawa, Hidemitsu
Kusafuka, Takeshi
Masaki, Hidekazu
Matsunaga, Tadashi
Mizutani, Shuki
Morikawa, Yasuhide
Nagase, Hiroki
Nakahata, Tatsutoshi
Nozaki, Miwako
Ohta, Shigeru
Okamura, Jun
Okimoto, Yuri
Ryu, Munemasa
Sasaki, Fumiaki
Taguchi, Tomoaki
Takamatsu, Hideo
Tanaka, Takeo
Todo, Satoru
Tsuchida, Masahiro
Tsuchiya, Shigeru
Tsuneyoshi, Masazumi
Yokomori, Kinji
Zaizen, Yoshio

Scientific Program Committee

Kadomatsu, Kenji (Chair)
Arakawa, Hirofumi
Hayashi, Yasuhide
Hishiki, Tomoro
Ichikawa, Hitoshi
Ikeda, Hitoshi
Kaneko, Yasuhiko
Kumagai, Masaaki
Makimoto, Atsushi
Okita, Hajime
Sakai, Ryuichi
Tajiri, Tatsuro
Ushijima, Toshikazu

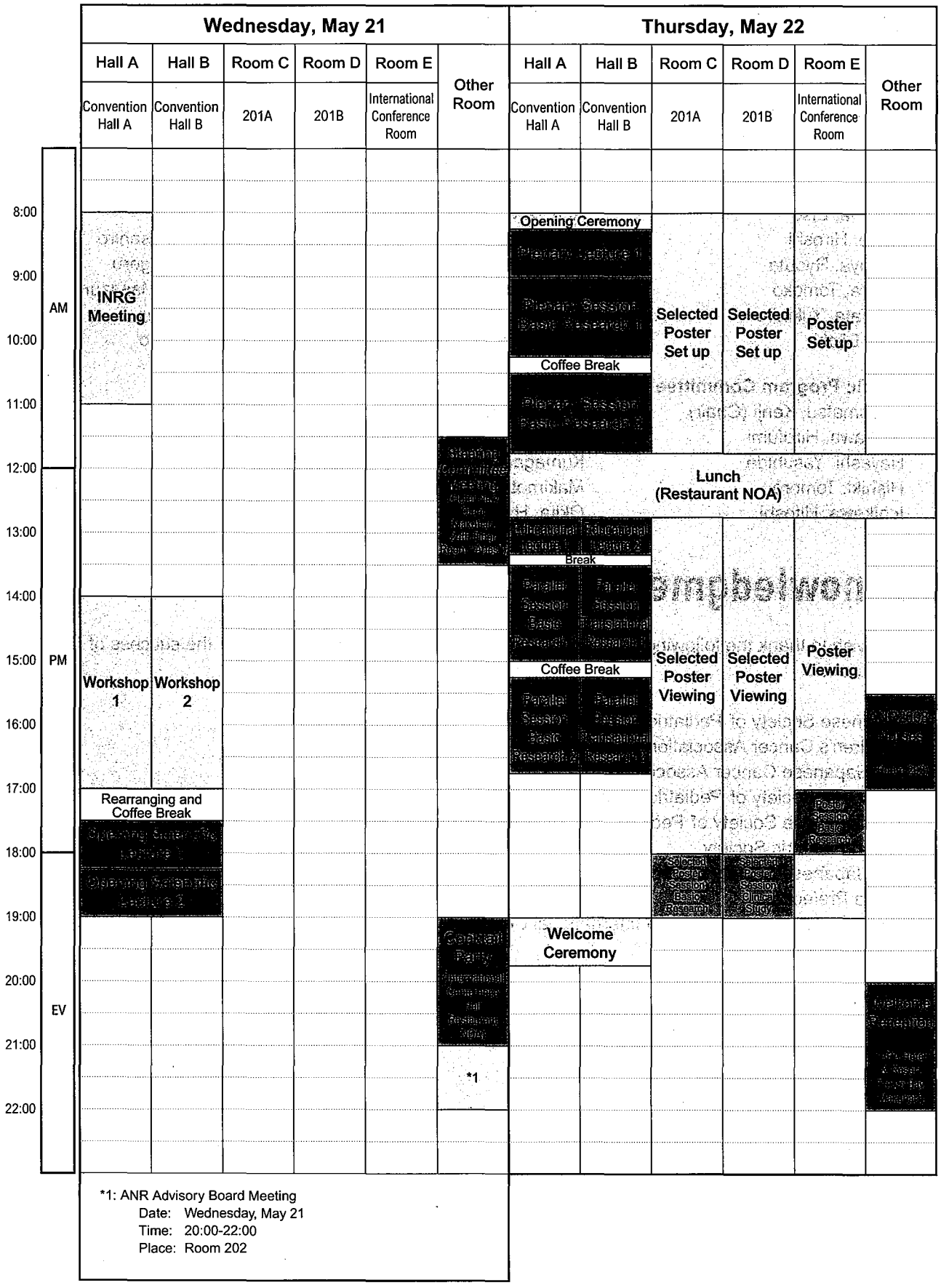
Acknowledgment

We wish to thank the following companies and organizations for their support for the success of this conference:

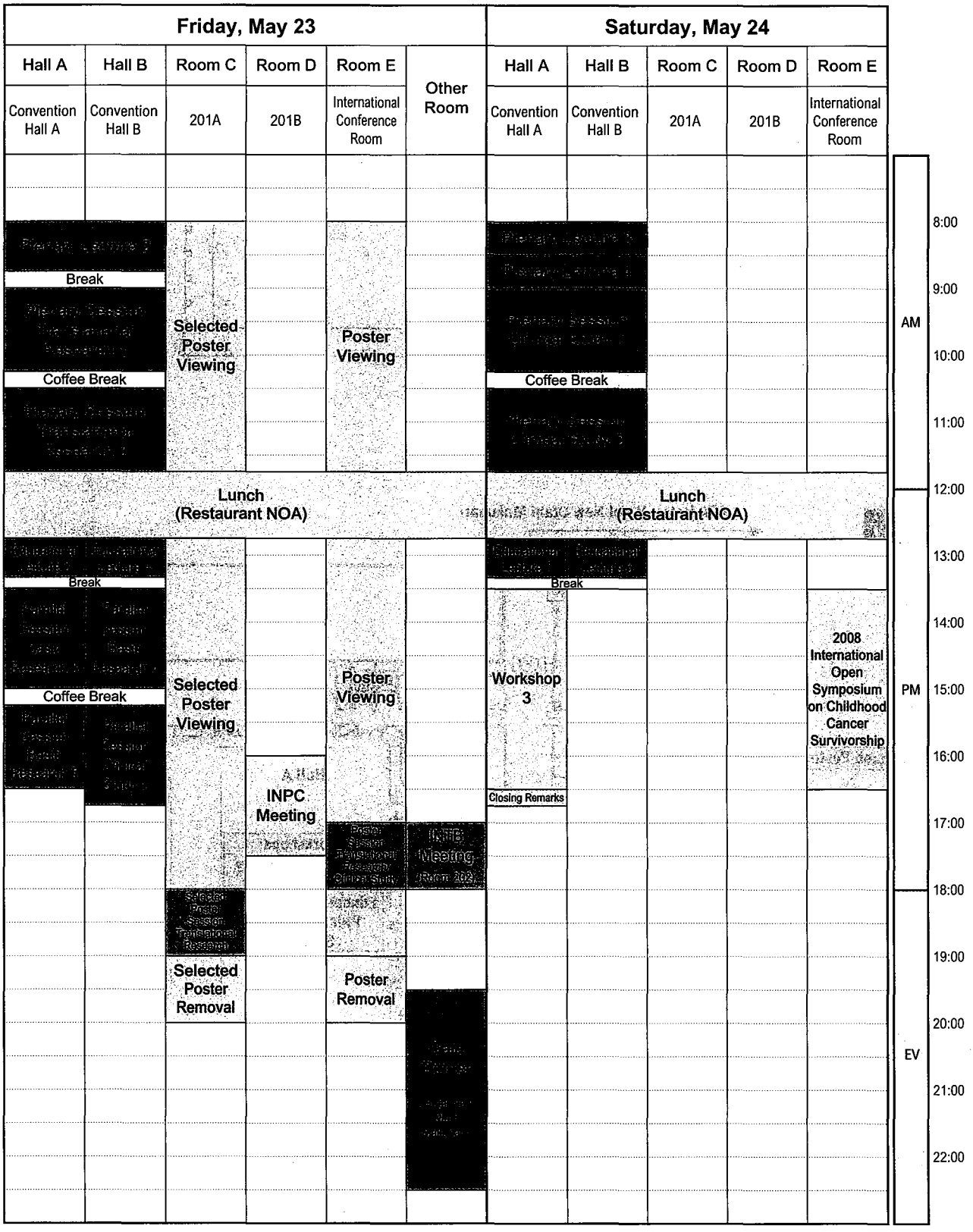
Japanese Society of Pediatric Oncology
Children's Cancer Association of Japan
The Japanese Cancer Association
Japanese Society of Pediatric Oncology Nursing
The Japanese Society of Pediatric Hematology
Japan Pediatric Society
The Japanese Society of Pediatric Surgeons
Chiba Prefecture
Chiba Convention Bureau and International Center
Chiba Cancer Center

Commemorative Organization for the Japan World Exposition '70
The Kao Foundation for Arts and Sciences
The Uehara Memorial Foundation
Terumo Lifecience Foundation
Life Science Foundation of Japan
Sankyo Foundation of Life Science
Setsuro Fujii. Memorial: The Osaka Foundation for Promotion of Fundamental Medical Research
Francebed Medical Home Care Research Subsidy Foundation

Program at a Glance

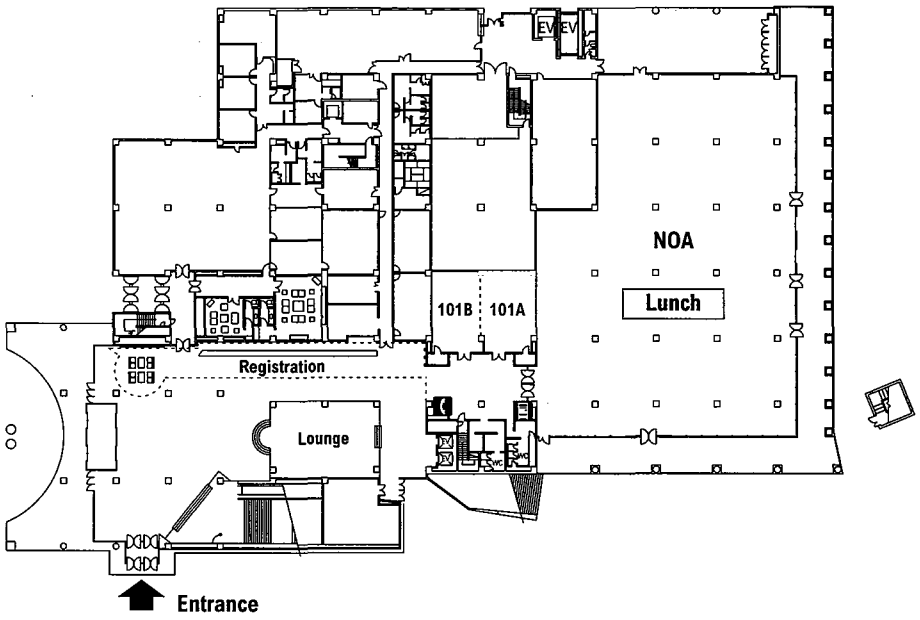


*1: ANR Advisory Board Meeting
Date: Wednesday, May 21
Time: 20:00-22:00
Place: Room 202

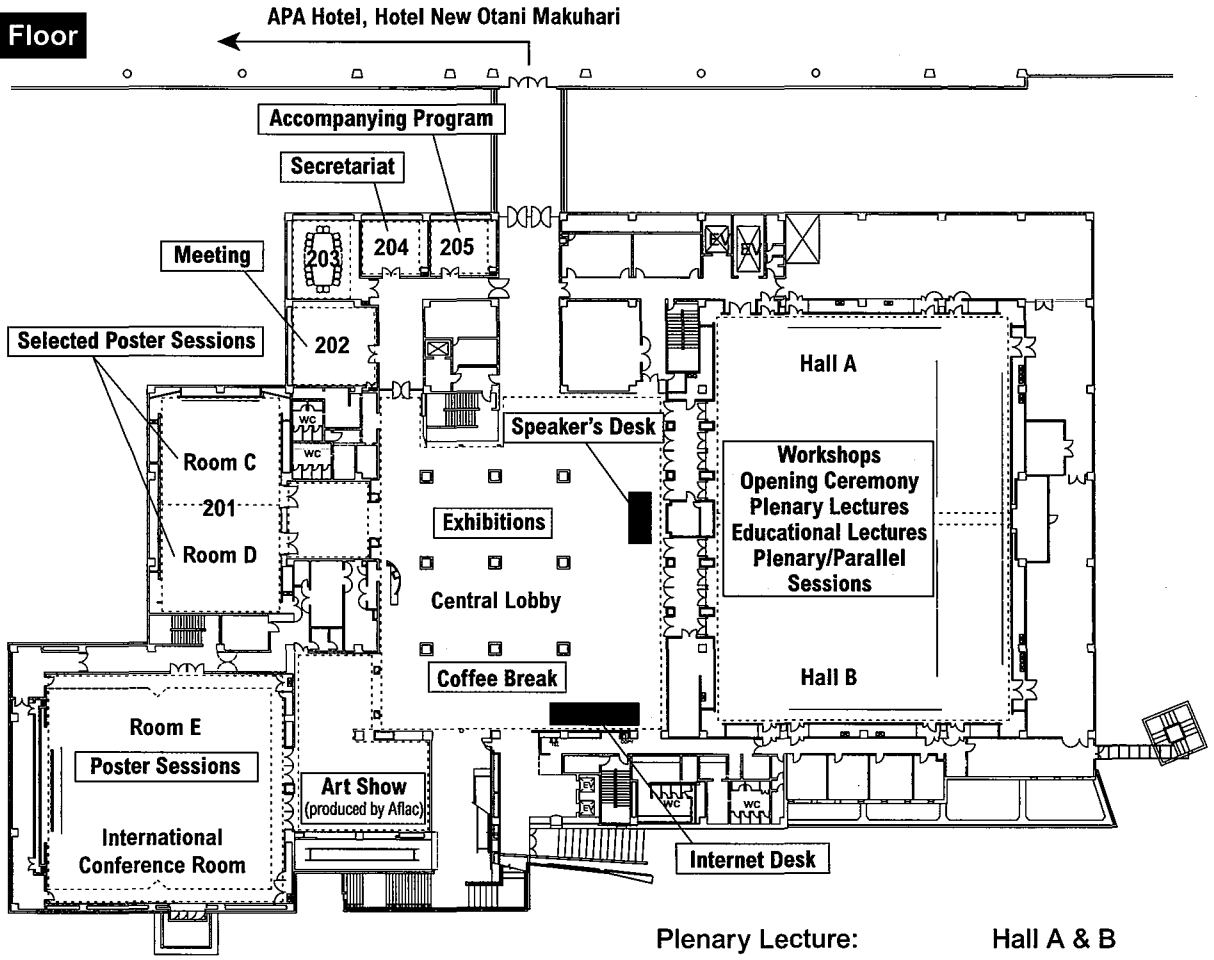

Friday, May 23
Hall A
Convention Hall A
Hall B
Convention Hall B
Room C
201A
Room D
201B
Room E
International Conference Room
Other Room
Break
Selected Poster Viewing
Poster Viewing
Coffee Break
Lunch (Restaurant NOA)
Break
Coffee Break
Selected Poster Viewing
INPC Meeting
Poster Viewing
Selected Poster Removal
Poster Removal
Saturday, May 24
Hall A
Convention Hall A
Hall B
Convention Hall B
Room C
201A
Room D
201B
Room E
International Conference Room
Coffee Break
Lunch (Restaurant NOA)
Break
Workshop 3
Closing Remarks
2008 International Open Symposium on Childhood Cancer Survivorship
AM
PM
EV
8:00
9:00
10:00
11:00
12:00
13:00
14:00
15:00
16:00
17:00
18:00
19:00
20:00
21:00
22:00

Map of the Venue

1st Floor

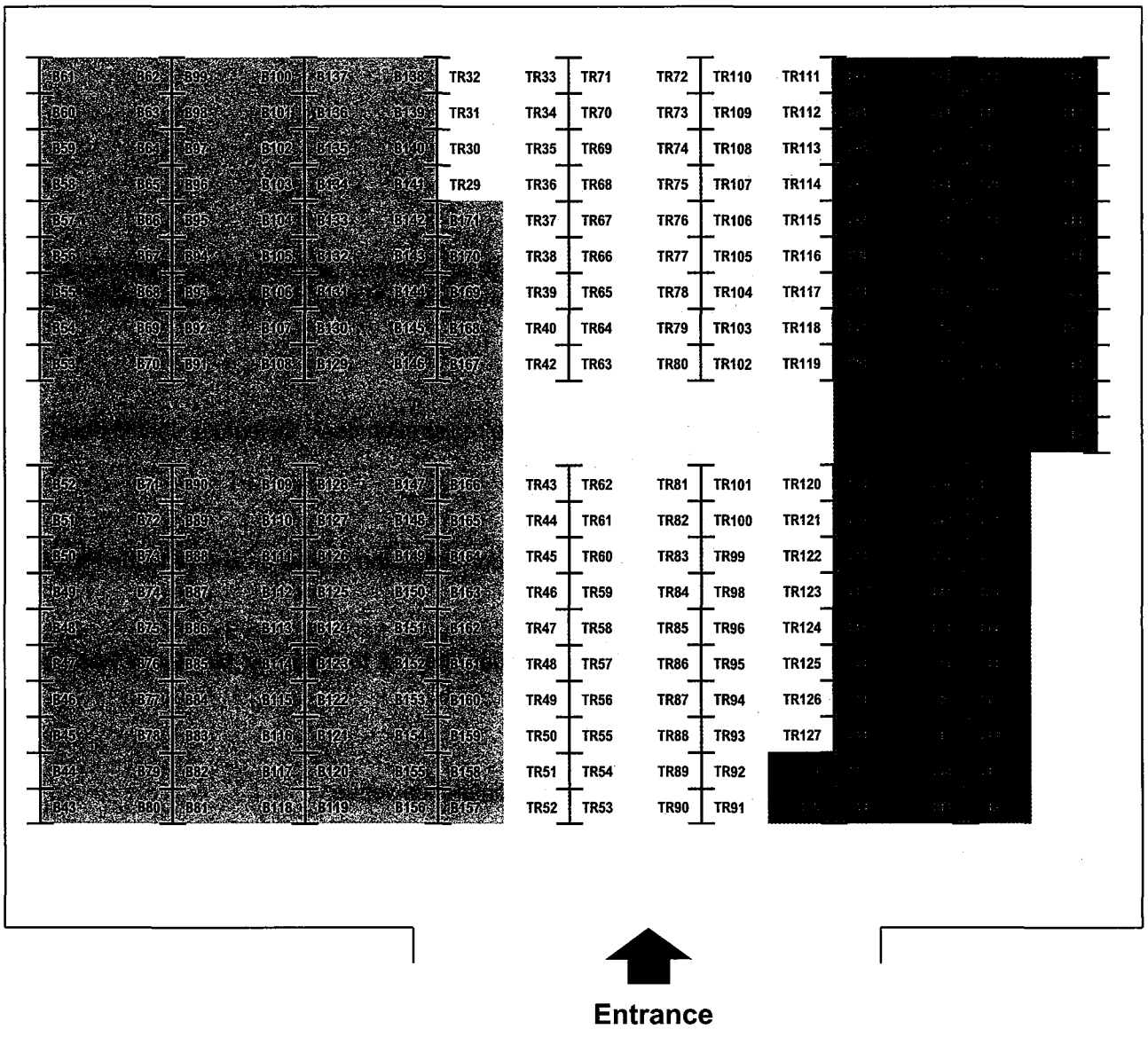


2nd Floor



Plenary Lecture:	Hall A & B
Plenary Session:	Hall A & B
Parallel Session:	Hall A, Hall B
Selected Poster Session:	Room C, Room D
Poster:	Room E

Poster Layout



Congress Information

Registration

Registration desk at Entrance Hall on the 1st floor, Conference venue is open during the following hours:

Wednesday, May 21	10:00-19:00
Thursday, May 22	7:30-17:30
Friday, May 23	7:30-17:30
Saturday, May 24	7:30-12:00

Name Badge

All delegates are required to wear the official name badge at all times while in the Conference venue. This badge will serve as your admission to all scientific sessions and official functions included in your registration fee.

Message Board

A message board for the delegates will be located near the registration desk. Delegates should check the message board regularly for any personal messages.

Refreshments

Refreshments are served at the central lobby on the 2nd floor during the breaks as shown in the Program.

Lunch

Lunch will be available during the conference from Thursday, May 22 to Saturday, May 24 at Restaurant NOA on the 1st floor, Conference venue.

Internet Access

Wireless network connections are available in 2nd floor, Conference venue.

Internet Desk

Common Use PCs are available at the Lounge on the 2nd floor, Conference venue.

Secretariat Office

Secretariat Office is located at Room 204 on the 2nd floor, Conference venue.

Accompanying Persons' Programs

AC-1 Flower Arrangement *Japanese Culture Experience Program

Date: **Thursday, May 22**
Time: 13:30-16:00
Place: Room 205

AC-2 Tea Ceremony *Japanese Culture Experience Program

Date: **Thursday, May 22**
Time: 13:30-16:00
Place: Room 205

Official and Social Programs

Cocktail Party

Date: **Wednesday, May 21**
Time: 19:00-21:00
Place: Restaurant NOA
(on the 1st floor, conference venue)

Welcome Ceremony

Date: **Thursday, May 22**
Time: 19:00-19:45
Place: Hall A & B
(on the 2nd floor, conference venue)

Welcome Reception

Date: **Thursday, May 22**
Time: 20:00-22:00
Place: APA Hotel & Resort Tokyo Bay Makuhari (located within walking distance)
Room: (on the 2nd floor, Makuhari Hall)

Gala Dinner

Date: **Friday, May 23**
Time: 19:30-22:30
Place: Hotel New Otani Makuhari (located within walking distance)
Room: (on the 2nd floor, Tsuru)

Other Meetings

ANR Advisory Board Meeting

Date: **Wednesday, May 21**
Time: 20:00-22:00
Place: Room 202

International Neuroblastoma Risk Groups (INRG)

Date: **Wednesday, May 21**
Time: 8:00-11:00
Place: Hall A

ANR2008-Nurses

Date: **Thursday, May 22**
Time: 15:30-17:00
Place: Room 202

International Neuroblastoma Tissue Banks (INTB)

Date: **Friday, May 23**
Time: 17:00-18:00
Place: Room 202

International Neuroblastoma Pathological Classification (INPC)

Date: **Friday, May 23**
Time: 16:00-17:30
Place: Room D

Instruction for Chairpersons and Speakers

The time allocated for each presentation is as follows:
Opening Lecture: 45 min.
Plenary Lecture: 45 min.
Educational Lecture: 35 min.
Plenary Session: 10 min. + 5 min. (for short question/short answer)
Parallel Session: 10 min. + 5 min. (for short question/short answer)
Selected Poster Presentation: 5 min. + 3 min. (for short question/short answer)

Instruction for Chairpersons

Session chairs are responsible for
- Introducing the speakers
- Moderating the question period
- Enforcing time limitations

* In order to ensure the smooth operation of the sessions, please keep the time allocation as stated above.
* You are kindly requested to be seated at the "Next Chairpersons' Seat" which will be reserved in the front row.

Instruction for Speakers

All speakers are requested to bring the data of their presentation on a CD-ROM or a USB memory stick to the speakers' desk and upload their presentation at least 30 minutes before your session(or earlier).

The speakers' desk will be open during the following hours:

Place:	2nd floor, in front of Hall A&B	
Time:	Wednesday, May 21:	13:00-17:30
	Thursday, May 22:	7:30-17:30
	Friday, May 23:	7:30-17:30
	Saturday, May 24:	7:30-13:00

* If you have a presentation in the morning, it is suggested that you finish reception on the day before.
* Please come near the Operator's desk located in the front row 30 minutes before your session.
* All speakers are requested to operate the slide by themselves.

Instruction for Selected Posters

1. Poster presentation

   Please prepare posters for display on the poster board.

Set-up:	Thursday, May 22:	8:00-12:00
Removal:	Friday, May 23:	19:00-20:00

   You are also responsible for setting up and removing your poster. The Secretariat will not be responsible for the loss of any remaining posters after removal time.

2. You are requested to make the selected poster oral presentation by PC at the same room as displaying your poster.

   Presentation and Q&A: 5 min. + 3 min. (for short question/short answer)

Basic Research (B38-B42):	Thursday, May 22:	18:00-19:00
Clinical Study (C19-C23):	Thursday, May 22:	18:00-19:00
Translational Research (TR23-TR28):	Friday, May 23:	18:00-19:00

Instruction for Posters

All posters will be displayed at the Poster Session Area. Poster presenters are expected to attend the sessions to answer questions in front of their posters. Please be ready 20 minutes before the session in front of your poster.

Set-up:	Wednesday, May 21:	17:00-19:00
	Thursday, May 22:	8:00-12:00
Presentation and Q&A:		
Basic Research (B43-B171):	Thursday, May 22:	17:00-18:00
Translational Research (TR35-TR127):	Friday, May 23:	17:00-18:00
Clinical Study and Nursing (C29-C94):	Friday, May 23:	17:00-18:00
Removal:	Friday, May 23:	19:00-20:00

You are also responsible for setting up and removing your poster. The Secretariat will not be responsible for loss of the poster after removal time.

Opening Scientific Lecture 1

Wednesday, May 21 17:30-18:15 Hall A & B

Chairperson: Akira Nakagawara
Sponsored by Dainippon Sumitomo Pharma Co., Ltd.

Opening Scientific Lecture 2

Wednesday, May 21 18:15-19:00 Hall A & B

Chairperson: Yasuhiko Kaneko

Plenary Lecture 1

Thursday, May 22 8:15-9:00 Hall A & B

Chairperson: Garrett M. Brodeur
Sponsored by Daiichi Sankyo Co., Ltd.

Plenary Lecture 2

Friday, May 23 8:00-8:45 Hall A & B

Chairperson: Tohru Sugimoto
Sponsored by Chugai Pharmaceutical Co., Ltd.

Plenary Lecture 3

Saturday, May 24 8:00-8:30 Hall A & B

Chairperson: Hirofumi Arakawa

Plenary Lecture 4

Saturday, May 24 8:30-9:00 Hall A & B

Chairperson: Hirofumi Arakawa

Educational Lecture 1 — Thursday, May 22 12:45-13:20 Hall A

Chairperson: Michelle Haber

Educational Lecture 2 — Thursday, May 22 12:45-13:20 Hall B

Chairperson: Toshihisa Ishikawa

Educational Lecture 3 — Friday, May 23 12:45-13:20 Hall A

Chairperson: Patrick C. Reynolds

Educational Lecture 4 — Friday, May 23 12:45-13:20 Hall B

Chairperson: Takeo Tanaka

Educational Lecture 5 — Saturday, May 24 12:45-13:20 Hall A

Chairperson: Eiichi Ishii

Educational Lecture 6 — Saturday, May 24 12:45-13:20 Hall B

Chairperson: Shuki Mizutani

Workshop 1

Wednesday, May 21 14:00-17:00 Hall A

Chairpersons: Javed Kahn, Miki Ohira, Peter Ambros
Sponsored by NGK Insulators, Ltd.

		Page
14:00-17:00	**Translational OMICS in Neuroblastoma**	83

Workshop 2

Wednesday, May 21 14:00-17:00 Hall B

Chairpersons: David Kaplan, Frank Spelemann, Takehiko Kamijo

		Page
14:00-17:00	**Neuroblastoma Stem Cell**	84

Workshop 3

Saturday, May 24 14:00-17:00 Hall A

Chairpersons: Simone Fulda, Patrick C. Reynolds, Murray D. Norris

		Page
14:00-17:00	**Translational Targeting Therapies Against High-Risk Neuroblastoma**	85

International Neuroblastoma Risk Group (INRG)

May 21; 8:00-11:00: Hall A

Organizers: Andrew D. J. Peason, Susan L. Cohn

Agenda:

Time		
8:00	Review of Progress to date	Cohn/Pearson Group Discussion
8:20	INRG Histology	Shimada Group Discussion
8:40	Update of INRG MIBG score	Matthay Group Discussion
9:00	INRG Response Definitions	Pearson/Cohn Group Discussion
9:30	Future Biology Studies	Ambros/Maris Group Discussion
10:00	Expansion of INRG Database	London Group Discussion
10:20	Authorship	Pearson/Cohn
10:40	New working groups: 1. Guidelines for imaging tumors 2. Definition of INSS stage 4s 3. Guidelines for scoring threatening symptoms 4. Others?	Pearson/Cohn/Monclair Group Discussion

International Nurses Meeting

May 22; 15:30 - 17:00 : Room 202

Organizer: Junko Ogawa

International Neuroblastoma Pathological Classification (INPC)

May 23; 16:00 -17:30 : Room D

Organizer: Hiro Shimada

International Neuroblastoma Tissue Bank (INTB)

May 23; 17:00 - 18:00 : Room 202

Organizers: Frank Berthold, Akira Nakagawara

2008 International Open Symposium on Childhood Cancer Survivorship

May 24; 13:30 – 16:30 : International Conference Room

Plenary Session: Basic Research 1

9:00-10:15 Hall A & B

Chairpersons: Manfred Schwab, Carol Thiele, Kenji Kadomatsu

Plenary Session: Basic Research 2

10:30-11:45 Hall A & B

Chairpersons: Rogier Versteeg, John M. Maris, Murray D. Norris

Parallel Session: Basic Research 1

13:30-15:00 Hall A

Chairpersons: Angelika Eggert, Michael D Hogarty, Jun-ichiro Fujimoto

Parallel Session: Translational Research 1

13:30-15:00 Hall B

Chairpersons: Barbara Hero, Nili Peylan-Ramu, Oliver Witt

Parallel Session: Basic Research 2

15:15-16:45 Hall A

Chairpersons: Isabelle Janoueix-Lerosey, Jason M. Shohet, Tatsuro Tajiri

Parallel Session: Translational Research 2

15:15-16:45 Hall B

Chairpersons: Victoria Castel, Julie Park, Junko Takita

Selected Poster Session: Basic Research

18:00-19:00 Room C

Chairpersons: Nicole Gross, Dwayne G. Stupack, Tomoro Hishiki

Selected Poster Session: Clinical Study

18:00-19:00 Room D

Chairpersons: Dominique Plantaz, Masaaki Kumagai, Kate Matthay

Plenary Session: Translational Research 1

9:00-10:15 Hall A & B

Chairpersons: Frank Berthold, Robert C. Seeger, Godfrey C. F. Chan

Plenary Session: Translational Research 2

10:30-11:45 Hall A & B

Chairpersons: Per Kogner, Susan L. Cohn, Hitoshi Ikeda

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11:30-11:45 **TR10** **Prediction of Outcome for Children with Metastatic MYCN Non-Amplified Neuroblastoma with a Multi-Gene TaqMan Low Density Array** 100

Shahab Asgharzadeh[1,2], Jill Salo[1], Lingyun Ji[1], Roger Pique-Regi[1], Cathy Wei Yao Liu[1], Hiroyuki Shimada[1,2], Katherine K. Matthay[2,3], Matthias Fischer[4], Andre Oberthuer[4], Frank Berthold[4], Richard Sposto[1,2], Robert C. Seeger[1,2]

[1]Children's Hospital Los Angeles and Saban Research Institute, Keck School of Medicine, University of Southern California, Los Angeles, USA, [2]Children's Oncology Group, Arcadia, USA, [3]UCSF, San Francisco, USA, [4]Children's Hospital, University of Cologne, Cologne, Germany

11:45-12:45 *Lunch (Restaurant NOA)*

Parallel Session: Basic Research 3

13:30-15:00 Hall A

Chairpersons: Gian Paolo Tonini, Yasuhide Hayashi, Thorsten Simon

Parallel Session: Basic Research 4

13:30-14:30 Hall B

Chairpersons: Tommy Martinsson, Hiro Shimada, Michio Kaneko

Parallel Session: Basic Research 5

15:15-16:30 Hall A

Chairpersons: Frank M. W. Westermann, Naohiko Ikegaki, Hajime Okita

Parallel Session: Clinical Study 1

14:30-16:45 Hall B

Chairpersons: Tommy Martinsson, Hiro Shimada, Michio Kaneko

Chairpersons: Ruth Ladenstein, Sylvain Baruchel, Devendra K. Gupta

Selected Poster Session: Translational Research 18:00-19:00 Room C

Chairpersons: Jaume Mora, Darrell Yamashiro, Eiso Hiyama

Plenary Session: Clinical Styudy 1

9:00-10:15 Hall A & B

Chairpersons: Andrew D. J. Peason, Audrey E. Evans, Purna Kurkure

Plenary Session: Clinical Study 2

10:30-11:45 Hall A & B

Chairpersons: Hideo Mugishima, Jin Hua Zhang, Judith Villablanca

Poster Session: Basic Research

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B71 **Presence of 1p Loss and 1q Gain and Absence of 7p Gain Predict Risk of Metastatic Relapse But not Overall Survival Probability in Localized Resectable Neuroblastoma.** 119

Annalisa Pezzolo[1], Elena Rossi[6], Stefania Gimelli[6],Federica Parodi[1], Francesca Negri[2], Gian Paolo Tonini[5], Massimo Conte[3], Angela Pistorio[4], Angela Sementa[2], Vito Pistoia[1], Orsetta Zuffardi[6], Claudio Gambini[2]

[1]Laboratory of Oncology, [2]Laboratory of Pathology, [3]Hematology-Oncology Unit, [4]Epidemiology and Biostatistics Unit, IRCCS G. Gaslini Hospital, Genoa, Italy, [5]Translational Pediatric Oncology, National Institute for Cancer Research (IST), Genoa, Italy, [6]Medical Genetics Department, University of Pavia, Pavia, Italy

B72 **Three New Targets of "Unknown Function" are Associated to Tumoral Progression in Neuroblastoma** 119

Achille Iolascon[1], Veruska Aglio[1], Nicola Orlotti[1], Mario Capasso[1], Gianpaolo Tonini[2], Massimo Zollo[1]

[1]Cattedra di Genetica Medica- DBBM Università Federico II and C.E.I.N.G.E., Naples, [2]IST, Istituto Nazionale Per La Ricerca Sul Cancro, Genova, Italy

B73 **Protein-Protein Interaction Network of AHR and MYCN Involved in Neuroblastoma** 119

Kai-Lin Cheng[1], Hsuan-Cheng Huang[2], Chun-Wei Hsu[2], Wen-Ming Hsu[3], Hsueh-Fen Juan[1,4,5]

[1]Institute of Molecular and Cellular Biology, National Taiwan University, Taipei, Taiwan, [2]Institute of Biomedical Informatics, National Yang-Ming University, Taipei, Taiwan, [3]Department of Surgery, National Taiwan University Hospital and National Taiwan University College of Medicine, Taipei, Taiwan, [4]Departments of Life Science, National Taiwan University, Taipei, Taiwan, [5]Institute of Biomedical Electronics and Bioinformatics, National Taiwan University, Taipei, Taiwan

B74 **Alternative Splicing of *PIK3CD* is Common in Aggressive Neuroblastoma** 119

Susanne Fransson, Katarina Ejeskär

Dept. of Clinical Genetics, Göteborg University, Göteborg, Sweden

B75 **Status and Function of the Tumour Suppressor p14ARF in Neuroblastoma** 120

Daniel Dreidax[1], Kai-Oliver Henrich[1], Marc Zapatka[1], Jessica Berthold[2], Matthias Fischer[2], Frank Westermann[1], Manfred Schwab[1]

[1]German Cancer Research Center, Heidelberg, Germany, [2]University Children's Hospital, Cologne, Germany

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Franck Bourdeaut[1], Carlo Lucchesi[1], Agnès Ribeiro[2], Régine Paris[3], Jérôme Couturier[2], Gaëlle Pierron[2], Loic de Pontual[4], Jeanne Amiel[4], Isabelle Janoueix-Lerosey[1], Michel Peuchmaur[3], Olivier Delattre[1,2]

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Abstracts

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Opening Scientific Lecture 1

OL1 Enjoyment of Science, Serendipitous Discovery, and Regrets of Dilettantism: Still Mysterious Mechanisms of Carcinogenesis

Takashi Sugimura
National Cancer Center and the Japan Academy, Japan

I started my career as a radiotherapist in 1950. An unforgettable experience was as follows. A boy out-patient with testicular tumors scattering in the lung was suffering from dysentery and was sent to the infectious disease hospital. Upon his return to me after release from the hospital, his lung tumors had astonishingly disappeared. Since then, I paid special attention to the spontaneous regression of tumors, including neuroblastoma. In animal experiments, I found a phenotypic reversion in hexokinase isozyme pattern in a subline of slow growing Yoshida sarcoma. The terms of "de-carcinogenesis" and "re-differentiation" were used to describe this phenomenon. I observed the expression of aldolase c, which is specific to brain and nerve tissues, in malignant hepatoma of rat. The term of dis(dys)-differentiation was proposed. During the 2nd World War, 4-nitroquinoline 1-oxide (4NQO) had been synthesized as an anti-malaria agent in Japan, and it was mutagenic to microbes and carcinogenic towards rodents. Metabolic activation pathways of 4NQO are common between microbe and mammalian cells, not involving cytochrome P-450. Thus, 4NQO was useful for mechanistic studies using DNA repair-deficient microbes as well as mammalian cells. The studies on 4NQO led us to the concept that cancer cells arise from DNA damage. Later, by using a metabolic activation system of rat liver, we discovered a series of mutagenic/carcinogenic heterocyclic amine (HCA) compounds formed during the cooking of meat. Despite the low expose dosage of HCAs, some HCAs produced prostate, breast and colon cancers in rats. This study was motivated by smoke which my wife produced by broiling dried fish in our home kitchen. My scientific curiosity flew from one flower to the next flower, just like butterflies. Potent tumor promoters other than TPA were found in fungus and green-blue algae. The science of TPA advanced, but no clear report was available on human cancers resulting from TPA exposure. During studies of disturbed allosteric inhibition (regulation) in hepatoma, a strange new biopolymer was found. That was poly(ADP-ribose) synthesized by a nuclear enzyme from NAD. A splicing abnormality in non-albuminemic rat was analyzed. Some carcinogens yielded cells, being albumin positive with immunostaining. After reaching over 70 years of age, my scientific curiosity remained strong and consistent. I contemplated the fate of many late-phase larvae of cabbage butterflies, and suggested that my colleagues investigate the presence of apoptogenic substances. It turned out that a peptide causing apoptosis of cancer cells was present in late phase of larvae and early phase of pupae. A novel peptide consisting of 850 amino acid residues was purified and named "Pierisin". Serendipitously enough, Pierisin ADP-ribosylated the guanine base in DNA, a new paradigm distinct from ADP-ribosylation on protein, which was discovered 40 years ago. Application of Pierisin to cancer treatment is ongoing. Recalling my entire research life, I enjoyed science very much with my colleagues but regret not devoting deeply enough on each subject, and remained a life long dilettante. Fortunately, my young colleagues are currently expanding the subjects, working with their own interests and enthusiasm. Carcinogenesis involves multiple genetic and epigenetic changes. Still, as indicated by the regression of neuroblastoma, there are many unexplained courses between cancer and normal cells, and many unexplained interactions between cancers and host conditions. Seeing a limited subject illuminated in greater detail, and constructing a new theory are real joys of science. I am hopeful of great progress in the science of neuroblastoma, and the success of this neuroblastoma meeting organized by Prof. Nakagawara.

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Opening Scientific Lecture 2

OL2 Themes of Hereditary Cancer in Children

Alfred G. Knudson
Fox Chase Cancer Center, Philadelphia, USA

Hereditary predisposition to cancer is rare in children, but is well known and, in many cases, the causative mutant gene and its function are known. Collectively the genes are functionally heterogeneous, but several categories are readily recognizable: (1) Direct controllers of birth and death processes, (2) Signal transducers, and (3) DNA repair genes. When new hereditary cancer genes are sought, as for neuroblastoma, it may be useful to consider these categories.

Two of the first genes to be cloned were *RB1* and *TP53*, both well known tumor suppressor genes, the first involved in regulating the birth of cells by the cell cycle, and the second involved in regulating the process of cell death. Both were discovered by the study of familial cancers in children, namely retinoblastoma and rhabdomyosarcoma, respectively, and both impose a predisposition to later cancers, notably sarcomas and lung cancer in the first and breast cancer in the second.

The second category includes the phakomatoses, such as the neurofibromatoses, tuberous sclerosis, and von Hippel-Lindau disease, all of whose genes are tumor suppressors, and a small group of hereditary oncogene disorders, including MEN2 and hereditary gastrointestinal stromal tumor, whose genes are oncogenes.

The third category is large and includes not only dominantly inherited conditions, such as hereditary non-polyposis colon cancer and hereditary breast cancer, but also recessive disorders, such as xeroderma pigmentosum, ataxia telangiectasia and Bloom syndrome. Each of these has taught much about the process of repair of DNA damage, including DNA double-strand breaks (DSBs), and this has stimulated the investigation of endogenous (spontaneous) DSBs. Their existence emphasizes that DSBs in cancer may occur in cells by either exogenous means or by failure to repair endogenous DSBs. Radiation is a known producer of DSBs; furthermore the effect of a given dose depends upon the rate at which it is delivered. The repair of these induced DSBs is optimal when their rate of production is approximately the same as the rate of production of endogenous DSBs.

Persons carrying predisposing genes have greatly increased risks of cancer, with one or more somatic events being necessary. Therefore, attempts should be made to discover whether targeted measures could defer or preclude any necessary further events in such predisposed individuals.

Plenary Lecture 1

PL1 The von Hippel-Lindau Tumor Suppressor Gene: Potential Insights into Paraganglioma and Neuroblastoma

William G. Kaelin
Howard Hughes Medical Institute, Dana-Farber Cancer Institute and Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA

Inactivation of von Hippel-Lindau (*VHL*) tumor suppressor gene plays an important role in clear cell renal carcinoma, hemangioblastoma, and pheochromocytoma (intraadrenal paragangliomas). Individuals with germline *VHL* mutations (VHL disease) are at increased risk for these tumors in an allele-specific manner (genotype-phenotype correlation). The *VHL* gene product (pVHL) has multiple functions including serving as the substrate recognition subunit of an E3 ubiquitin ligase that targets the alpha subunits of the heterodimeric transcription factor HIF (Hypoxia-inducible Factor) for destruction. HIFα must be hydroxylated on one (or both) of two conserved prolyl residues by members of the EglN family (also called PHD or HPH family), which are oxygen-dependent enzymes that also require reduced iron, 2-oxoglutarate, and ascorbic acid, in order to bind to pVHL. Under low oxygen conditions, or in cells lacking wild-type pVHL, HIFα accumulates and activates 100-200 genes involved in adaptation to hypoxia. Deregulation of HIFα (especially HIF2α) appears to play a causal role in clear cell renal carcinoma and almost certainly contributes to the development of hemangioblastomas, which are blood vessel tumors. In contrast, deregulation of HIFα does not appear drive the development of pheochromocytoma. In particular, some VHL families have *VHL* alleles that are essentially wild-type with respect to HIFα regulation and present with familial pheochromocytoma.
Higher metazoans, including people, have three EglN family members (EglN1, EglN2, and EglN3). We made a conditional EglN1 mouse (*EglN1-/-* embryos are not viable) and confirmed cell culture experiments that suggested EglN1 is the primary HIF prolyl hydroxylase. Our recent studies suggest that EglN2 and EglN3 play roles in control of cell proliferation and apoptosis, respectively. We found, for example, that the genes that, when mutated, cause familial paraganglioma define a pathway that is activated in sympathetic neuroblasts during embryological development by growth factor withdrawal. Interestingly, this pathway impinges upon EglN3, which is both necessary and sufficient for apoptosis in this setting. In an unbiased screen for shRNAs that confer protection against EglN3-induced apoptosis, we identified an shRNA directed against *KIF1Bβ*, which maps to 1p36.2. This region of the genome is frequently deleted in a variety of tumors, including neuroblastoma. Notably, this gene is also one of only 6 annotated genes located within a 500 kB homozygous deletion in a neuroblastoma line. Restoration of KIF1Bβ function in this line induces apoptosis and we have identified germline loss of function KIF1Bβ mutations in some neuroblastoma and pheochromocytoma patients, arguing that KIF1Bβ is a potential tumor suppressor gene. Preliminary data suggest that KIF1Bβ haploinsuffiency is sufficient to protect from apoptosis, which might account for the observation that many 1p deleted tumors retain a wild-type KIF1Bβ allele.

Plenary Lecture 2

PL2 Proliferation and Differentiation of Various Stem Cells *in vitro* and *in vivo*

Tatsutoshi Nakahata
Department of Pediatrics, Graduate School of Medicine, Kyoto University, Japan

Abstract
Recent progress of cell biology provides a significant insight into the characterization of various stem cells. There are different kinds of stem cells such as somatic stem cells, embryonic stem (ES) cells and induced pluripotent stem (iPS) cells, which are recently developed by Professor Yamanaka in Kyoto University.
Hematopoietic stem cells (HSC) are one of the well-defined somatic stem cells and possess capabilities of self-renewal and differentiation into various hematopoietic cells, both of which are estimated by a long-term repopulating ability *in vivo* after transplantation. NOD/SCID mouse strain has been found to be an efficient recipient for reconstitution of human HSC, however, the engraftability of human HSC is not satisfactory and complete multi-lineage differentiation has not been achieved in this strain. Engraftment efficiency was slightly improved by using β2 microglobulin deficient NOD/NOD mice as recipients, but no human T cells were detected even if a large number of human HSC are transplanted into the mice.
Recently, we developed a new strain of immunodeficient mice, NOD/SCID /γc-/- (NOG) mice. This mouse carried various immunological abnormalities including complete deficient of NK cell activity in addition to lack of mature lymphocytes, macrophage dysfuncrion and absence of circulating complement. Successful engraftment was achieved even if less than 103 CD34+ cells isolated from human cord blood were transplanted into NOG mice without administration of human cytokines. Multi-lineage reconstitution was confirmed in peripheral blood, bone marrow, spleen, and thymus of NOG mice 3 months after transplantation. We observed human myeloid cells, B cells, T cells, mast cells, NK cells, NKT cells, DC cells, megakaryocytes and erythroid cells in bone marrow of NOG mice. Human IgG, IgA and IgM were detected in the murine sera, indicating functional maturation of the human B lymphocytes.
There has been a great interest in the plasticity of human HSC for a variety of clinical applications such as regenerating medicine of hepatic injury. Several studies have shown that hepatocytes can be generated from HSC. To investigate this phenomenon in human cells, we used a NOG mouse model. Human albumin and alpha-1-antitrypsin-positive cells were invariably detected in the livers of NOG mice after intravenous transplantation of human cord blood CD34+ cells. Human albumin was detected in the murine sera, indicating functional maturation of the human hepatocytes. PCR analysis of recipient livers revealed the expression of a wide variety of human hepatocyte- or cholangiocyte-specific mRNAs. Flow cytometric analysis of recipient liver cells in single-cell suspension demonstrated that human albumin-positive cells were also positive for both murine and human MHC and were negative for human CD45. These results show that human CD34+ cells fuse with hepatocytes of NOG mice, lose their hematopoietic phenotype, and begin hepatocyte-specific gene transcription. These phenomena were not observed when CD34- cells were transplanted. Thus, our model revealed a previously unidentified pathway of human hematopoietic stem/progenitor cell differentiation.
Recent studies have revealed that NOG mice are useful for not only examination of the developmental mechanism of various lineages from human HSC and plasticity of human HSC but also the evaluation of various kind of other human somatic stem cells and the cells differentiated from human ES cells and iPS cells. In this paper, I would like to talk about the differentiation of human ES cells and iPS cells *in vitro and in vivo*, and human cancer stem cells using NOG mice.

Plenary Lecture 3

PL3 The Concept of Dependence Receptors

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Key words: transduction, apoptosis, caspase

Classical signal transduction is initiated by ligand-receptor interactions. An alternative form of signal transduction may be initiated by the withdrawal of trophic ligands (or competition with trophic ligands) from specific receptors referred to as dependence receptors. Fifteen such receptors are now known, including RET, DCC, Unc5H1-4, Ptc, and APP, among others. Since these receptors induce cell death when their trophic ligands are withdrawn, but block cell death when the trophic ligands are present, the expression of these receptors confers a state of dependence on their respective trophic ligands. This process features in developmental cell death, carcinogenesis (including metastasis), neurodegeneration, and subapoptotic events such as neurite retraction and somal atrophy. Mechanistic studies of dependence receptors suggest that these receptors form complexes that include specific caspases. Complex formation appears to be a function of ligand-receptor interaction, and dependence receptors act as molecular switches that appear to exist in at least two conformational states. Complex formation in the absence of ligand leads to caspase activation by a mechanism that is dependent on caspase cleavage of the receptor itself, releasing pro-apoptotic peptides. Thus these receptors create states of dependence on their respective trophic ligands, and may act to integrate extracellular signals in a fashion analogous to the integration provided by dendritic arbors within the nervous system.

Plenary Lecture 4

PL4 The Dependence Receptor Notion: When Apoptosis Regulates Tumor Progression and Metastasis, from a Cell Biology Concept to a Putative Targeted Therapy in Aggressive Neuroblastoma

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Background: Dependence receptors share the ability to induce apoptosis in settings of absence of their respective ligands. As such, these receptors that include p75nr, RET, TrkC, DCC, UNC5H, neogenin, some integrins, and Ptc have been proposed to regulate tumorigenesis. For example, we reported that DCC (Deleted in Colorectal Cancer) and UNC5H (UNC5H1-3), the receptors to the axon guidance cue netrin-1, are such prototype dependence receptors. We thus have proposed that UNC5H and DCC may be considered as tumor suppressors that would induce apoptosis of tumor cells that would grow out of ligand availability. Along this line, both DCC and UNC5H expression appears drastically inhibited in numerous carcinomas including colorectal tumors. Moreover, ectopic expression of netrin-1 or inactivation of UNC5H3 in mice gut leads to an increased tumorigenesis. Thus, the netrin-1 dependence receptors are conditional tumor suppressors.

Methods: We investigated whether netrin-1 dependence receptors, DCC and UNC5H, may also control tumorigenesis of neuroblastoma (NB), the most common extracranial solid tumor of early childhood.

Results: We describe that a large fraction of aggressive NB, rather than to lose DCC or UNC5H as observed in colorectal cancers, have selected a gain of netrin-1 expression, and that high netrin-1 expression is associated with poor outcome especially for patients diagnosed before one year of age. We show that autocrine production of netrin-1 is a tumor growth and dissemination selective advantage in aggressive NB as it blocks the pro-apoptotic activity of the netrin-1 dependence receptors UNC5H. Moreover, we show that disruption of this autocrine loop is associated with inhibition of NB growth and dissemination in different mice and chicken models.

Conclusion: Here we will provide an overview of the implication of the netrin-1 dependence receptors in neuroblastoma and will describe how this may be used to propose a novel therapeutic approach.

Educational Lecture 1

EL1 International Neuroblastoma Risk Groups

Andrew D. J. Pearson, Susan L. Cohn for the INRG Task Force
Section of Paediatrics, Institute of Cancer Research and Royal Marsden Hospital, Surrey, UK, the Department of Pediatrics, The University of Chicago, Chicago, IL, USA

The International Neuroblastoma Risk Group (INRG) classification system is based on survival tree regression analyses of 13 prognostic factors in a cohort of 8,800 children diagnosed with neuroblastoma (NB) between 1990-2002. Stage, age, histological category, tumour grade, *MYCN* status, chromosome 11q status, and DNA ploidy were the most highly statistically significant and clinically relevant. A new pre-treatment INRG staging system (INRGSS) based on tumor imaging has been developed. Sixteen pre-treatment groups (PTG) have been defined based on clinical criteria and statistically significantly different event-free survival (EFS). Patients with 5-year EFS ≥ 85%, >75-<85%, ≥50-≤75%, or <50% were classified as very low-, low-, intermediate-, or high-risk, respectively.

To obtain the benefits of the INRG, co-operative groups must now employ the system in all their future clinical studies. The key for international comparison is the assignment of patients in a given INRG PTG to a single treatment group without splitting that PTG in different treatment subgroups.

Implementation of the INRG requires:

- Utilization of INRGSS.
- Histological classification of localized unresectable tumors, as histology is the major determinant of therapy *in MYCN* non-amplified L2 tumours > 18 months.
- Knowledge of 11q status in subsets. Technologies, such as MPLA, that are not cost prohibitive and will yield rapid and reproducible results are required so that 11q loss can be rapidly assessed in all the relevant population.
- Knowledge of DNA ploidy in patients between 12 and 18 months with *MYCN* non-amplified metastatic disease, as hyperdiploidy differentiates a group that probably do not require myeloablative therapy.

The INRG Task Force is currently defining standard operating procedures for molecular diagnostic testing and criteria for the evaluation of bone marrow metastatic disease and a system for the assessment of metastatic disease by MIBG. We have established an INRG data base that is available for investigator-initiated clinical research studies. This data base will be expanded to include genetic aberrations of tumors identified by genome-wide methods, data that will be critical for further refinement of the INRG risk groups. The INRG Task Force is also developing standardized eligibility criteria and endpoints for Phase II studies. These initiatives will greatly facilitate the development of standardized approaches for risk assessment, comparisons of clinical trials conducted in different regions of the world, and the development of international collaborative studies.

Educational Lecture 2

EL2 The *FRAGILOME* Project and *MYCN*

Manfred Schwab
German Cancer Research Center (DKFZ), Heidelberg, Germany

Genomic instabilityis one of the hallmarks of the cancer cell. A major contribution appears to come from the approximately 100 "common fragile sites" (cFS) in the human genome, which are defined as non-random chromosomal regions prone to undergo breakage and gap formations. Activation of fragile sites, either induced by environmental challlenges or sporadically, appears as a starting point for genomic alterations, particularly microdeletions or gene amplifications through breakage-fusion-bridge (BFB) cycles. The end point of such repeated BFB cycles is an increase of gene dosage of the particular gene(s). A further understanding of the role of cFS in genomic instability and cancer can be expected from a determination of involved DNA sequences, or genes, at fragile site loci. This is the major goal of the *FRAGILOME* project.

The amplification of *MYCN* identifies a group of neuroblastoma patients that have particularly dire prognosis. MYCN has been the first cancer-related gene identified by array-based methods, and its amplification has been prototypic for the clinical use of an oncogene alteration. *MYCN* amplification is of predictive value for identifying neuroblastoma patients that require specific therapeutic regimens and for identifying patients that do not benefit from chemotherapy. Consequently, amplified *MYCN* is employed worldwide as a prognostic marker on which therapy design is based to large extent.

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Educational Lecture 3

EL3 New Approaches to Neuroblastoma Therapy (NANT) Consortium: Phase I Trials Targeting Neuroblastoma

Katherine K. Matthay
UCSF School of Medicine Leader, NANT Consortium

Neuroblastoma, the most common extra-cranial solid tumor of childhood, arises in embryonic cells of the peripheral sympathetic nervous system. Half of the children present with advanced, high-risk disease, with a poor survival, creating an urgent need for more effective therapy. Previous studies have shown improved outcome by the addition of high dose therapy with hematopoietic support, followed by differentiating therapy for minimal residual disease with 13-cis-retinoic acid. However, even with these added modalities, 5-year survival for high-risk patients is less than 40%. Therefore, we formed a consortium of 14 academic institutions in North America with investigators experienced in both bench and clinical research in neuroblastoma to develop therapies targeting this tumor to overcome chemotherapy resistance without adding non-specific toxicity. Current protocols depend on the development of agents targeting norepinephrine transporter, immune mechanisms, genetic and metabolic pathways, and testing these first in vitro in a panel of sensitive and resistant neuroblastoma cell lines, then in vivo in xenograft and transgenic animal models, prior to clinical trials. Selection of therapy is prioritized by agent availability and success and specificity in pre-clinical models. Agents that are shown to be tolerable and have some indication of activity in Phase I testing, proceed to Phase II trials in the COG or to novel combination Phase I therapy. Examples of current studies to be discussed include novel approaches using ^{131}I-MIBG, Trk inhibitor, fenretinide, and immunotherapy. The focus on a single disease with highly experienced institutions promotes careful selection of agents, meticulous central review of response, and concomitant correlative studies with pharmacodynamics, pharmacokinetics, genomics and immunologic studies. Completion of a phase I study in this group also allows assessment of potential activity, and due to the very close interchange with COG, effective mainstreaming of promising agents.

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NANT institutions: Children's Hospital Boston, Dana-Farber Cancer Institute - Boston, MA; Children's Hospital Los Angeles - Los Angeles, CA; Children's Hospital Medical Center - Cincinnati, OH; Children's Hospital of Philadelphia - Philadelphia, PA;Hospital for Sick Children - Toronto, Ontario Canada; Lucile Packard Children's Hospital - Palo Alto, CA; Morgan Stanley Children's Hospital (Columbia) – New York, NY; Texas Children's Cancer Center, Baylor College of Medicine - Houston, TX; University of California, San Francisco - San Francisco, CA; University of Chicago Comer Children's Hospital - Chicago, IL; University of Wisconsin Comprehensive Cancer Center - Madison, WI.

Educational Lecture 4

EL4 Session of the Japanese Heart Inazo Nitobe: The Soul of Japan

Okio Hino
Dept. of Pathology & Oncology, Juntendo University School of Medicine Tokyo, Japan

Cancer is a heritable disorder of somatic cells. The environment and heredity both operate in the origin of human cancer.
The accumulation of mutations, which are likely to occur during continuous cycles of cell division, may eventually transform some normal cells through a multi-stage process. Carcinogenesis looks like an opened **Japanese fan**, because initiated cells grow in several directions and tumors suggest the edge of the fan having many gene abnormalities.

Knudson's 2 hit is the primal force and gene networks might be "federal headship" in carcinogenesis.
This remind us of the formative stimulation of Virchow's & Yamagiwa's carcinogenesis. In 1915, Katsusaburo Yamagiwa (1863-1930) achieved a pioneering work in chemical (coal tar) -induced carcinogenesis by formative stimulation following the hypothesis of Virchow (1821-1902). I want to talk about "**Cancer Philosophy**".

Inazo Nitobe (1862-1933) was the face on the previous 5000 yen and the under secretary general of the League of Nations.
Inazo Nitobe was known internationally as the author of *Bushido: The Soul of Japan*.

I will explain how **Inazo Nitobe** fits in with "*Cancer Philosophy*".

Educational Lecture 5

EL5 The SIOPEN-R-NET Project: Building a European Network for Neuroblastoma Treatment (HR-NBL-1/ESIOP) und Research

Ladenstein Ruth[1], Pötschger Ulrike[1], Schreier Günter[2], B. De Bernardi[3], I. Yaniv[3], D. Valteau-Counet[3], P. Brock[3], V. Castel[3], P. Kogner[3], G. Laureys[3], V. Papadakis[3], J. Malis[3], A. Forjaz De Lacerda[3], P. Ambros[1], A. Pearson[3] for the SIOP Europe Neuroblastoma Group[3]
[1]CCRI - St. Anna Kinderspital, Vienna / International Study Centre and Austrian Research Centres[2]

The SIOPEN-R-NET project (EC grant No. QLRI-CT-2002-01768) aimed to build a European Neuroblastoma Research Network Structure to optimise the use of pre-existing infrastructures and to improve consistency and complementarity through harmonised standard operating procedures. This will improve exchange of reference material and build material resources and repositories for current and future research tasks. A Web based centralised data bank and communication system was developed allowing clinical trial management with remote data entry, electronic data capture, remote randomisation, image transfer, information on trial progress and offers communication tools as well as links between clinical data and research tasks.
The current European high-risk neuroblastoma treatment protocol, the HR-NBL-1/SIOPEN study served as backbone to build these structures. This is a randomised study for children over the age of one with stage 4 disease or stages 2 and 3 with *MycN*-amplified neuroblastoma any age and was activated on 02/02/2002. Currently it is activated in 17 European countries. The protocol consists of a rapid, dose intensive induction chemotherapy (COJEC) adopted from the UK-ENSG 5 protocol with the randomised use of G-CSF [R0] to rapidly reduce bulky disease. It aims to reduce the incidence of local relapse and hence encourages extensive surgical removal of the primary tumour at the end of induction and adds local irradiation to all patients after megatherapy (MGT) / PSCR. It compares the therapeutic benefit and toxicity of two MGT regimens (CEM and BuMel) through randomisation [R1] and attempts to eradicate minimal residual disease with differentiation therapy (13-cis retinoic acid). To date 1012 patients are registered on study via the WEB based study tool, 239 patients participated in R0 and the randomization has been completed. The G-CSF arm had significantly less febrile episodes ($p=0.012$), hospital days ($p=0.01$), days with fever ($p=0.018$) and antibiotic days ($p=0.001$). Reported CTC graded toxicity was also significantly reduced: infection/cycle ($p=0.002$); fever ($p=0.008$); severe leucopenia ($p<0.001$); neutropenia ($p<0.001$); mucositis ($p=0.01$); nausea/vomiting ($p=0.026$) and constipation ($p=0.005$). 403 patients have been randomised for the high dose therapy question in R1 and 19 to R2 so far. Study safety is monitored continuously and surveyed by a data monitoring committee. A number of studies have been performed and are underway within the specialty committees all dealing with standardized diagnostic procedures and response throughout treatments.

Educational Lecture 6

EL6 Epigenetics in Neuroblastomas

Toshikazu Ushijima
Carcinogenesis Division, National Cancer Center Research Institute, Tokyo, Japan

Epigenetic alterations are faithfully inherited in somatic cells, and very common in adult cancers. We found that methylation of multiple CpG islands (CpG island methylator phenotype; CIMP) was strongly associated with poor survival in neuroblastomas (NBLs) (HR=22 in 140 Japanese cases; HR=9.5 in 145 German cases) [Abe, Cancer Res, 65:828, 2005; Abe, Cancer Lett, 247:253, 2007]. Notably, all the cases with *MYCN* amplification (38 Japanese and 23 German cases) with one exception displayed CIMP, and, among cases without *MYCN* amplification, CIMP still retained its effect on survival (HR= 13 and 4.5, respectively). Since CIMP was expected to affect multiple and various genes, effects on survival were analyzed for individual genes and CIMP in a subset of Japanese cases (n=53). HRs conferred by methylation of individul genes, such as *CASP8*, *EMP3*, *HOXA9*, and *NR1/2*, were 0.5-2.0, while that conferred by CIMP was 4.4 (Watanabe, unpublished), indicating that CIMP, rather than methylation of a single gene, was mainly involved in the poor survival of NBL cases. By use of methylated DNA immunoprecipitation and CpG island microarray, it was revealed that CIMP(+) NBLs had 100-1,500 methylated promoter CpG islands while CIMP(-) NBLs had 80-200 (Asada, unpublished). Reversal of methylation of multiple CpG islands by a demethylating agent, 5-aza-2'-deoxycitidine, in combination with 13-*cis*-retinoic acid (RA) in a NBL cell line, NB-39nu, enhanced the differentiation effect of RA synergistically (Abe, Oncology in press). These showed that CIMP is deeply involved in poor survival of NBL cases, and suggested that its reversal could be therapeutically beneficial.

Workshop 1

Translational OMICS in Neuroblastoma

Chairpersons: Javed Khan, *National Cancer Institute, USA*
Miki Ohira, *Chiba Cancer Center Research Institute, Japan*

Summary:
The central dogma in molecular biology states that genetic information is transmitted from the DNA to RNA to proteins which then determine the phenotype of the cell. It has become increasingly clear that the flow of information is not linear but within each cell there exists a complex interaction and feedback loops between DNA, RNA, and a newly discovered species of RNA called micro-RNA (micro-RNA) and protein. The Human Genome Project (HGP) has heralded the way for high throughput analysis of whole genomes and proteomes with the hope that these techniques will identify prognostic biomarkers and novel therapeutic targets to improve the outcome of patients with high-risk neuroblastoma. Many of the technologies have now matured sufficiently to enable us to perform systematic high throughput "omics" analysis of the whole genome and proteome. It is now possible to measure the DNA copy number, perform large scale sequencing, measure the messenger and micro RNA of every gene in the human genome and measure the expression level of 1000s of proteins at once.
The purpose of the educational workshop is to discuss the state of the art applications of omics to decipher the biology of neuroblastoma. We will hear presentations discussing possible tumor suppressor genes located in regions of genomic loss as well as the applications of genome wide association studies to identify neuroblastoma susceptibility genes. There will an update on the role of microRNAs in cancer development. Finally will also discuss ongoing efforts to identify and translate prognostic molecular signatures to the clinic.

Schedule:

Introduction: Javed Khan, National Cancer Institute, USA

Keynote:
1. Garrett Brodeur, The Children's Hospital of Philadelphia and the University of Pennsylvania, USA
"Significance of genomic changes in neuroblastomas, especially 1p deletions"

Invited talk:
2. Alea Mills, Cold Spring Harbor Laboratory, USA
"CHD5 is a tumor suppressor mapping to human 1p36"

Short talks:
3. John Maris, Children's Hospital of Philadelphia, USA
"Approaches to identify the genetic basis of susceptibility to neuroblastoma"

4. Jun Wei, National Cancer Institute, USA
"microRNA profiling and function in Pediatric Cancers"

5. Jo Vandesompele, Center for Medical Genetics, Ghent University Hospital, Belgium
"Real-time PCR based profiling of prognostic gene signatures"

6. Miki Ohira, Chiba Cancer Center Res. Inst., Japan
"Prognosis classification of neuroblastoma by genetic and genomic approaches"

7. Alexander Schramm, University Children's Hospital Essen, Germany
"Proteomics: From high-throughput technology to target validation"

Discussion:

Workshop 2

Neuroblastoma Stem Cell

Chairpersons: David Kaplan, *Hospital for Sick Children, Toronto, Canada*
Frank Speleman, *Ghent University Hospital, Ghent, Belgium*
Takehiko Kamijo, *Chiba Cancer Center Res. Inst., Chiba, Japan*

Summary:
The existence of cancer stem cells in virtually every tumor has transformed our thinking about cancer progression and therapy. They are defined as cells with stem cell properties such as perpetual self-renewal, asymmetric cell division, and expression of stem cell genes and markers, and are capable of faithfully regrowing the tumor from which they were derived from. The cancer stem cell hypothesis rests upon the prospective isolation of distinct populations of tumor-initiating and non-tumor initiating cells, in which a rare fraction of cells in the tumor is responsible for all of the tumor-initiating activity and produces non-tumorigenic progeny. This model is very attractive, as it postulates that successful therapy must eradicate both cancer stem cell and non-stem cell subsets, and therefore the failure of many chemotherapeutic drugs may be due to the lack of efficacy of those drugs on cancer stem cells controlling tumor initiation, progression, angiogenesis, and relapse.
Despite the attractiveness of the cancer stem cell hypothesis, there is still considerable skepticism about its validity. Thus far, isolation of these cells using prospective markers such as CD133 from primary human tumors has only succeeded in isolating subsets that initiate tumors at most at 1 in 100 cells. In addition, cancer stem cells from the same tumor cells have been found in CD133 or side population-positive and negative subsets. Neuroblastoma, which likely originates from a transformed neural crest precursor and that can sometimes be induced to differentiate into neurons and glia, is an ideal candidate for a cancer stem cell-driven tumor. Indeed, as we will hear in this workshop, a high proportion of neuroblastoma cells have stem cell properties and are capable of reinitiating the tumor. However, it has not been demonstrated that neuroblastoma cancer stem cells can generate non-tumor-initiating progeny, nor has a prospective marker for these cells been identified. Talks in this workshop will address whether CD133 is such a prospective, functional or prognostic marker, whether putative neuroblastoma cancer stem cells are prognostic for relapse, the appropriateness of the animal models and culture conditions used to isolate and assess cancer stem cells, and if established neuroblastoma cell lines can reflect a neuroblastoma cancer stem cell population. Ample time will be provided for discussion, focusing upon whether neuroblastoma has a cancer stem cell subset, or if most or all of the tumor and metastatic cells from high risk/relapse patients have a similar probability to induce tumors. The outcome of these discussions will have important implications regarding whether cancer stem cells are important for neuroblastoma prognosis and treatment.

Schedule:

Keynote:
1. David Kaplan, Hospital for Sick Children, Toronto, Canada
"Do cancer stem cells exist in neuroblastoma?"

Invited talk:
2. Hideki Enomoto, MD, PhD, Riken Center for Developmental Biology, Kobe, Japan
"Genetic analysis of RET signaling in neural development and neurocristopathy"

Short talks:
3. Takehiko Kamijo, Chiba Cancer Centre Research Institute, Chiba, Japan
"MYCN regulates stemness-related gene expression in neuroblastoma"

4. Amy McKee, Pediatric Oncology Branch, National Cancer Institute, Bethesda, MD, USA
"A high frequency of tumor-initiating cells with stem cell properties persists in established neuroblastoma cell lines"

5. Nicole Gross, University Hospital, CHUV, Lusanne, Switzerland
"Identification of neuroblastoma specific stem cells markers by micro-array time-course analysis of neurospheres"

6. Hedwig Deubzer, German Cancer Research Center, Heidelberg, Germany "Identification of neuroblastoma stem cells"

7. Frank Speleman, Ghent University Hospital, Ghent, Belgium
"Normal fetal neuroblast and cancer stem cell profiling"

Discussion:

Workshop 3

Translational Targeting Therapies Against High-Risk Neuroblastoma

Chairpersons: Patrick Reynolds, *Children's Hospital, Los Angeles, USA*
Murray Norris, *Children's Cancer Institute, Australia*
Simone Fulda, *Children's Hospital, Ulm University, Germany*

Summary:
Despite aggressive treatment protocols, the prognosis of children with high-risk neuroblastoma remains poor. This highlights the need to develop novel, more effective strategies.
In response to this demand, the goal of this workshop is to discuss concepts and activities in the development of rational, biology-based therapeutics against high-risk neuroblastoma. An overview will be provided on existing consortia on (pre)clinical testing in the U.S. and Europe. Also, examples of experimental therapeutics that are currently under evaluation in preclinical models or early clinical trials in neuroblastoma will be presented. In a roundtable discussion, we will address what are the most promising targets for high-risk neuroblastoma and what are the best preclinical models for their evaluation. In particular, the question what models are required to develop agents that target cancer stem cells will be addressed. In addition, the issue whether it is feasible to develop a drug specifically for neuroblastoma will be raised.

Schedule:

1. Consortia on Preclinical Testing:
- **John Maris**, Children's Hospital of Philadelphia
  Pediatric Preclinical Testing Program (PPTP): what is new for neuroblastoma
- **Andy Pearson**, The Institute of Cancer Research
  SIOPEN Strategy for Neuroblastoma Drug Development leading to the Druggable Cancer Genome

2. Experimental therapeutics:
- **Olaf Witt**, German Cancer Research Center
  HDAC inhibitors
- **Per Kogner**, Karolinska Institute
  Cox, Lox and mTOR inhibition
- **Patrick Reynolds**, Children's Hospital, Los Angeles
  Fenretinide
- **Michael Hogarty**, Children's Hospital of Philadelphia
  ODC as molecular target
- **David Kaplan**, The Hospital for Sick Children MaRs Centre
  Targeting neuroblastoma stem cells

3. Roundtable discussion:
- What are the most promising targets?
- What are the best preclinical models to evaluate experimental therapeutics?
- Cancer stem cells: are we currently hitting the right target and what models do we need to develop therapeutics that target cancer stem cells?
- Is developing a drug specifically for neuroblastoma feasible: how would such a drug be funded in development and beyond and what would be the strategy to obtain regulatory approval? Would a focus on drugs that also have potential for indications beyond be neuroblastoma be preferred?

Plenary Session: Basic Research 1

B1 A Genome-Wide Association Study (GWAS) Identifies Susceptibility Loci to High-Risk Neuroblastoma

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Background: The etiology of neuroblastoma is not known.

Methods: We performed a GWAS by genotyping 1,032 neuroblastoma patients and 2,043 controls of European descent at 550K SNPs. Three independent groups of neuroblastoma cases (N=712) and controls (N=2125) were then genotyped to replicate significant associations.

Results: We observed highly significant association between neuroblastoma and the common minor alleles of three single nucleotide polymorphisms (SNPs) within a 94.2 Kb linkage disequilibrium (LD) block at 6p22 containing the predicted genes *FLJ22536* and *FLJ44180* (P=1.71x10^{-9}-7.01x10^{-10}). Homozygosity for the at-risk G allele of the most significantly associated SNP, rs6939340, resulted in an increased likelihood of developing neuroblastoma of 1.97 (95% CI 1.58-2.44). Subsequent genotyping of these 6p22 SNPs in the three independent case series confirmed our observation of association (combined P at rs6939340=9.33x10^{-15}). Neuroblastoma cases homozygous for the risk alleles at 6p22 were more likely to have Stage 4 disease (P=0.02), *MYCN* amplification (P=0.006), and disease relapse (P=0.01). When only the high-risk patients (N=397) were considered in our discovery case series, in addition to the 6p22 locus we observed several other signals of association including a locus on 2q35, wherein 6 common SNPs in strong LD (r^2 range 0.47-0.96) within the *BARD1* tumor suppressor gene reached genome-wide significance (P=2.25x10^{-8}-2.35x10^{-9}). To date, the *BARD1* association has been replicated in one case series (N=189 high-risk cases and 1178 controls, P=7.89x10^{-7} at rs17487792) and ongoing replication and functional validation efforts at these and other loci will be reported.

Conclusion: Common genetic variations at chromosome bands 2q25 and 6p22 are associated with susceptibility to high-risk neuroblastoma. These results, in conjunction with our data showing that copy number variations are also associated with neuroblastoma susceptibility (*Diskin, ANR 2008*), support a model in which neuroblastomas arise due to the occurrence of complex interaction of common genetic variations.

B2 Prognostic Multigene Expression Classification of Neuroblastoma Patients, a SIOPEN Study

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Background: More accurate assessment of prognosis is of importance to further improve the choice of risk-related therapy in neuroblastoma (NB) patients.

Methods: Re-analysis of seven published microarray gene-expression data sets was performed in parallel with a literature screening of almost 800 abstracts from single-gene studies to identify a list of top candidate prognostic markers for NB. RNA samples from over 400 primary untreated NB-tumours were collected and evaluated for RNA-quality using two PCR-based assays and capillary gel electrophoresis. A validated sample pre-amplification method was applied enabling gene expression profiling of 100's of genes from only 20 ng of total RNA. All prognostic markers were measured using high-throughput RT-qPCR. For data-analysis, samples were divided into a training set and a test set.

Results: Based on our re-analysis and on review of the literature, a list of 59 prognostic markers was established. Approximately 10 % of the RNA samples were discarded due to quality control issues. Of the 366 patients studied, 70 were high-risk patients and 296 intermediate- and low-risk patients according to current SIOPEN risk factors. Median follow-up was 55 months. According to univariate Cox survival analysis, 53 genes (90%) were associated with outcome. Upon training with 15 deceased high risk and 15 low risk patients without events, the PAM classifier distinguished the remaining 334 patients with respect to overall survival and event-free survival (p<0.001). Similar classification performance was seen with a subset of 14 top-ranking genes. Multivariate Cox regression analysis indicated that the PAM classifier is a significant independent predictor after controlling for age, stage and *MYCN* status.

Conclusions: We established a robust and accurate multigene expression predictor, suitable for routine lab tests and ready to be evaluated in prospective studies.

B3 A Genome-Wide Linkage Screen Identifies a Hereditary Neuroblastoma Predisposition Locus at Chromosome 2p24-23

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Background: A subset of neuroblastomas show inheritance as an autosomal dominant trait with incomplete penetrance. There are very few pedigrees of sufficient size for linkage analysis. Low-resolution genetic approaches have not been successful in identifying a narrow genomic region consistent with linkage, a critical first step in identifying the causal gene.

Methods: We performed a genome-wide scan for linkage in eighteen neuroblastoma pedigrees at ~6000 single nucleotide polymorphisms. Parametric and non-parametric analyses were used to identify and refine candidate regions. Candidate genes were resequenced in a panel of 15 probands using Sanger-based or 454 technology.

Results: We discovered a highly significant linkage signal covering a 34 Mb region on chromosome 2p with a maximum non-parametric LOD score of 4.23 (p=0.00001), and 13/18 families screened were consistent with linkage to this locus. No other genomic region was suggestive of linkage in these families, including previously identified 4p16 and 16p12-13 loci. To further refine the 2p region, we performed parametric analyses in which we varied gene frequency and penetrance assumptions across a broad range. All analyses supported the initial inference of linkage, with a maximized LOD score of 6.37 (p=0.0000019) at rs1344063. By mapping informative recombination events, we defined a 16 Mb putative predisposition locus at 2p24-23, a region that includes the *MYCN* oncogene. Resequencing of an 18 Kb region surrounding the *MYCN* gene in probands from each linked family showed no activating mutations or novel sequence variations. Resequencing of other neurodevelopmental regulatory genes including *NAG*, *DDX1*, *GDF7*, and *OSR1* has excluded these as neuroblastoma predisposition genes. Results of ongoing resequencing of a prioritized list of positional candidates will be reported.

Conclusions: A hereditary neuroblastoma predisposition gene is located within a 16 Mb region at 2p24-23. We speculate that inactivation of this gene may also influence the development of non-familial human neuroblastomas.

B4 *KIF1Bβ* is a Haploinsufficient Tumor Suppressor Gene Mapped to Chromosome 1p36.2 in Neuroblastoma

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We have previously found in a neuroblastoma cell line NB1/C201 a 500-kb homozygously deleted region at chromosome 1p36.2 harboring at least six genes. Our array-CGH data using 112 sporadic neuroblastomas also showed that the homozygous deletion was within the smallest region of overlap. Therefore, we transferred each of the six genes into NB1 cells and found that only *KIF1Bβ*, a member of the kinesin superfamily microtuble-based motor protein, induced cell death. This led us to the hypothesis that *KIF1Bβ* could be a tumor suppressor gene mapped to chromosome 1p36 in neuroblastoma.
To prove this, we performed loss of heterozygosity (LOH) analysis, showing that 32% (30/95) tumors examined lost one *KIF1B* allele. The *KIF1B* hemizygous deletion in primary neuroblastoma was significantly correlated with advanced stages (p=0.0013) and *MYCN* amplification (P<0.001). Expression of *KIF1Bβ* was significantly downregulated in advanced stages tumors (p<0.001). In addition, among the tumors with diploid karyotype, those with monoallelic loss of the *KIF1Bβ* locus expressed significantly low mRNA levels of *KIF1Bβ* (p=0.019), suggesting that *KIF1Bβ* is a haploinsufficient tumor suppressor. We found neither significant mutations in both promoter and coding regions nor promoter methylation of the *KIF1Bβ* gene.
The enforced expression of *KIF1Bβ*, but not *KIF1Bβ*, induced massive cell death in neuroblastoma as well as other cancer cell lines, that was p53-independent. In contrast, *KIF1Bβ* knockdown using siRNA resulted in accelerated cell proliferation. The genetic interruption of the *KIF1Bβ* expression in NMuMG mouse mammary gland cells using genetic suppressor element method caused huge tumors in nude mice. The functional analysis using deletion mutants of KIF1Bβ showed that the rod region including the coiled-coil motif was critical to induce cell death. Furthermore, we found that perturbation of G2/M progression might be causally related to apoptotic cell death induced by *KIF1Bβ*.
In conclusion, *KIF1Bβ* acts as a tumor suppressor by gene dosage and that its allelic loss may be involved in the pathogenesis of aggressive neuroblastoma.

Plenary Session: Basic Research 2

B5 *CHD5*, the Best Candidate Tumor Suppressor Gene Deleted from 1p36.31 in Neuroblastomas

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Background: Neuroblastomas are characterized by 1p deletions, suggesting that a tumor suppressor gene (TSG) resides in this region. We mapped the smallest region of consistent deletion (SRD) to a 2-megabase region of 1p36.31 and identified 23 genes in this region. Based on mutation and expression analysis, we identified *CHD5* as the best TSG candidate.

Methods: We measured *CHD5* expression in normal tissues and neuroblastoma cell lines, the methylation status of the *CHD5* promoter in these lines, and the ability of transfected *CHD5* to inhibit clonogenicity and tumorigenicity. We also analyzed the association of candidate TSG expression with prognostic variables and outcome.

Results: *CHD5* encodes a putative chromatin remodeling protein that is expressed preferentially in the nervous system. *CHD5* expression was very low or absent in all neuroblastoma cell lines tested. The promoter was heavily methylated in lines with the lowest expression, but *CHD5* could be upregulated by growing cells in 5-deoxy-2-azacytidine. Transfection with *CHD5* had a limited effect on neuroblastoma cells growing *in vitro*, but clonogenicity and tumorigenicity were markedly abrogated compared to antisense controls. *CHD5* expression was highly associated with clinical and biological features in a panel of 101 primary neuroblastomas. Furthermore, *CHD5* expression was a strong and independent predictor of event-free and overall survival ($p<0.0001$), and it remained predictive of outcome even after correction for *MYCN* amplification and 1p deletion ($p=0.0275$).

Conclusions: We conclude that *CHD5* is the strongest candidate TSG deleted from the 1p36.31 SRD in neuroblastomas, and inactivation of the second allele likely occurs by an epigenetic mechanism.

B6 MYCN Modulates P53 Activity Through Direct Transcriptional Regulation of MicroRNAs

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Introduction: MicroRNAs are endogenous short (22mer) non-coding RNAs expressed from pol-II regulated promoters that have potent tumor suppressor or oncogenic activities in many tumor types. MiRNA expression correlates with MYCN expression and with changes in differentiation in neuroblastoma cell lines and xenograft models. Recently, p53 regulated miRNAs have been shown to act as potent tumor suppressors. We hypothesize that MYCN directly inhibits or activates microRNA transcription through direct DNA binding with oncogenic consequences. MiRNAs down stream of MYCN regulating apoptosis and differentiation are likely important therapeutic targets that can be regulated via SiRNA or other genetic methods.

Methods: We use conditional MYCN expression combined with miRNA array profiling, quantitative PCR, miRNA luciferase sensor constructs and chromatin immunoprecipitation to confirm direct E-box specific binding to and regulation of miRNA promoters.

Results: MYCN positively and negatively regulates multiple miRNAs. mir26a is sharply down-regulated by MYCN expression. MYCN binds selectively to the second E-box in the mir26a-2 promoter region. Target predication algorithms reveal that this miRNA has a number of putative targets involved in ubiquitin/de-ubiquitination reactions. One Target, UBE2D1 is a an E-2 ligase involved in ubiquitination of p53. We demonstrate that p53 stability can be altered by changes in UBE2D1 levels which are controlled by mir26a. MYCN induction reduces mir26 levels, increases UBE2D1 protein levels which reduces p53 activity. mir26a also targets ARF-BP1 (regulates ARF mediated activation of p53) and multiple ubiquitin specific proteases.

Conclusions: MicroRNAs represent a new level of MYCN mediated gene regulation. They act both to degrade mRNA and to suppress translation of proteins controlling differentiation and proliferation. We demonstrate that MYCN can modulate p53 activity through mir26a. In vitro and in vivo testing will determine if exogenously expressed mir26a and other miRNAs can sensitize neurolbastoma to apoptotic stimuli providing support for future therapeutic applications.

B7 The MYCN Oncogene is a Direct Target of miR-34a

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Background: Loss of 1p36 heterozygosity commonly occurs with *MYCN* amplification in neuroblastoma tumors, and both are associated with an aggressive phenotype. Database searches identified 5 microRNAs that map to the commonly deleted region of 1p36 and we hypothesized that the loss of one or more of these microRNAs contributes to the malignant phenotype of *MYCN*-amplified tumors.

Methods: Synthetic microRNAs on 1p36 were introduced into neuroblastoma cell lines with *MYCN* amplification, and the effects of microRNAs on cell growths, apoptosis, and global gene expression were monitored. In addition, a luciferase reporter system was used to validate the targets of miR-34a.

Results: By bioinformatic analysis, we identified that 3 out of the 5 microRNAs target *MYCN*, and of these miR-34a caused the most significant suppression of cell growth through increased apoptosis and decreased DNA synthesis in neuroblastoma cell lines with *MYCN*-amplification. Quantitative RT-PCR showed that primary neuroblastoma tumors with 1p36 loss expressed lower level of miR-34a than those with normal copies of 1p36. Furthermore we demonstrated that miR-34a suppresses MYCN expression using Western blotting. In addition we showed that MYCN is a direct target of miR-34a using a luciferase reporter system. Finally, using a series of mRNA expression profiling experiments, we identified other potential direct targets of miR-34a, and pathway analysis demonstrated that miR-34a suppresses cell cycle genes and induces several neural related genes.

Conclusion: In this study, we demonstrated that miR-34a induces cell growth arrest *via* direct *MYCN* suppression as well as inhibition of other targets. This study reveals an important regulatory role of miR-34a in *MYCN* suppression in neuroblastoma.

B8 A Novel Function of Aurora-A: Kinase-Independent Stabilization of N-Myc Protein in MYCN-Amplified Neuroblastoma

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Background: Amplification of *MYCN* is one of the strongest predictors of aggressive disease, resistance to therapy and poor prognosis in neuroblastoma. *MYCN* encodes a transcription factor of the Myc family, that activates genes acting on cell growth, metabolism, biogenesis, cell cycle progression, apoptosis, and also represses genes. Deregulation of *MYCN* expression by amplification leads to a distinct gene expression profile in neuroblastoma. Although microarray data revealed hundreds of genes deregulated by amplified *MYCN*, only few genes are identified, that explain the aggressive phenotype of these cells.

Results: We have obtained evidence for a novel pathway that regulates the stability of N-Myc protein in human neuroblastoma cells. In an RNA interference screen of 582 shRNA vectors targeting 194 genes, which we had previously identified as overexpressed in *MYCN*-amplified neuroblastoma, we found a small group of genes that were indispensable for the growth of *MYCN*-amplified neuroblastoma cells, but largely dispensable for those without *MYCN*-amplification. One of the genes (*AURKA*) encodes the mitotic kinase Aurora-A, which is amplified and overexpressed in multiple human tumors, including neuroblastoma. Aurora-A overexpression is known to cause genomic instability, aneuploidy and malignant transformation. We found that Aurora-A is required for the stability of N-Myc protein: depletion of Aurora-A by destabilizes N-Myc, whereas overexpression of Aurora-A stabilizes N-Myc. Aurora-A binds to N-Myc in vivo and stabilizes N-Myc. Surprisingly, this occurs independently of its kinase activity. Mechanistically, Aurora-A counteracts the Gsk3/Fbwx7-mediated proteasome degradation of N-Myc thus leading to stabilization of the N-Myc protein.

Conclusions: We have identified a novel mechanism of N-Myc protein stabilization by *AURKA* encoding the mitotic kinase Aurora-A. As the kinase activity of Aurora-A is not necessary for this stabilization, we suggest that kinase independent and non-mitotic properties of AURKA exert its oncogenic function in MYCN-amplified neuroblastoma by stabilizing N-Myc.
This study was supported by an AICR grant.

B9 Neuroblastoma Cells Isolated from Bone Marrow Metastases Contain a Naturally Enriched Tumor-Initiating Cell

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Background: A major impediment to neuroblastoma (NB) research is the lack of cells derived from tumors of different stages of disease and from metastases, and the availability of normal counterparts of NB cells to perform comparative cell biological and signaling studies.

Methods: Putative tumor-initiating cells (TICs) were isolated from tumors and bone marrow (BM) metastases of patients in all NB risk groups, including patients showing no evidence of disease by morphological examination and immunocytochemistry.

Results: Putative NB TICs were isolated and characterized from tumors and BM metastases of patients with high-risk disease and from low-risk tumors. Primary spheres from all tumor risk groups differentiated to form neurons, however differentiation was more robust in cells isolated from low-risk tumors, preserving heterogeneity *in vitro*.
When injected orthotopically, passaged TICs from NB BM samples formed NB tumors that metastasized appropriately with as few as 10 cells, suggesting most cells within the BM metastases are tumorigenic. Interestingly, highly tumorigenic TICs were isolated from the BM of 3 high-risk patients showing no morphological evidence of disease, however the frequency of TICs in 2/3 of these samples was much lower than that in patients with active disease, suggesting that this population of cells may predict clinical behavior and serve as a biomarker for MRD. Cells isolated from low-risk patients were unable to form tumors with as many as 10,000 cells injected orthotopically suggesting that low-risk tumors do not contain a TIC.
TICs can be used for understanding both cellular and molecular events leading to relapse and disease progression, which cannot be studied in established cell lines. Microarray studies comparing NB TICs to non-transformed neural crest precursor cells (SKPs) have thus far identified atypical drug transporter genes, modulators of apoptosis, and a potential marker of the rare TIC in NB tumors.

Conclusions: Our data indicate that high-risk NBs contain a cell with cancer stem cell properties that is enriched in tumor-initiating capacity. These cells may serve as a model system to identify the molecular determinants of NB and to develop new therapeutic strategies for this tumor.

B10 Investigating the Role of *PHOX2B* in Neuroblastoma Development

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Background: *PHOX2B*, a homeodomain transcription factor that regulates sympathetic nervous system development, is mutated in neuroblastoma. In order to understand the link between its developmental role and disease, we examined the effects of abrogating *zphox2b* expression *in vivo* using the zebrafish model.

Methods: Anti-sense morpholinos (MO), that either block translation or proper mRNA splicing of the *zphox2B* gene were injected into one-cell embryos. Sympathetic neurogenesis was analyzed using *in situ* hybridization assays examining dopamine beta hydroxylase (*DBH*) and tyrosine hydroxylase (*TH*) expression in the developing cervical complex (CC).

Results: Normal *zphox2B* expression is first observed in the brain at 12 hours post fertilization (hpf) and can be seen in the CC at 72 hpf and developing enteric neurons by 4dpf. Knockdown of *zphox2B* resulted in decreased numbers of *DBH* and *TH* expressing neurons in the CC that could be rescued by the overexpression of human *PHOX2B*. *DBH* expression was more severely affected, consistent with the fact that *DBH* is a direct target of mammalian *PHOX2B*. Interestingly, both MOs caused increased expression of genes normally expressed earlier in progenitors of noradrenergic neurons, including *zash1a*, in the brain and spinal cord and *zphox2B* itself. Furthermore, forced expression of *hphox2B* in wild type embryos resulted in decreased endogenous *zphox2B* in the brain.

Conclusions: Loss of *zphox2B* expression appears to block the differentiation of sympathetic neurons as indicated by the loss of *DBH* and *TH*, key enzymes involved in noradrenergic cell maturation. *zphox2b* also appears to negatively regulate of its own expression and that of *zash1a*. These data support a model in which *PHOX2B* mutations promote tumorigenesis by blocking noradrenergic neuron differentiation while promoting the expansion of the sympathetic progenitor cells. We are currently phenocopying tumor-derived *PHOX2B* mutations in the zebrafish to further test this model.

Parallel Session: Basic Research 1

B11 *CASZ1*, a Developmental Gene that is Frequently Lost on Chromosome 1p36 Induces Cell Differentiation, Inhibits Growth and Neuroblastoma Tumor Initiating Capability

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Background: The frequent loss of heterozygosity (LOH) on chromosome 1p in neuroblastoma suggests this region contains at least one tumor suppressor or differentiation gene whose inactivation contributes to tumorigenesis. *CASZ1* localizes to 1p36.22, a region of LOH in a subset of poor prognosis NB. Although the function of *CASZ1* in mammals is unknown, its drosophila homolog, *castor*, is a zinc-finger transcription factor known to control neural fate-determination during development. Previously, we have shown that retinoic acid, a key embryonic morphogen, induces *CASZ1* expression.

Methods: A tet-on *CASZ1* expression vector was stably transfected into 3 NB cell lines. The effect of *CASZ1* on cell growth and soft agar colony formation was assessed. Cell migration was measured by scratch assay. *CASZ1* target genes were identified by microarray and confirmed by real-time pcr and western blot analyses.

Results: Genes involved in developmental processes, neuronal development and early markers of neural crest stem cells *p75 (NGFR), TH,* and *GAP43* were induced by *CASZ1*. Over-expression of *CASZ1* induced cell growth arrest and neurite extension. CASZ1 expressing cells had decreased anchorage-independent growth, indicating that *CASZ1* can block the transformed phenotype of NB cells. Interestingly, *CASZ1* also enhances cell adhesion and decreases cell migration. An orthotopic xenograft model showed that *CASZ1* inhibits NB tumor-initiating capability *in vivo*. Treatment with 5-AZA' or an HDAC inhibitor treatment induces *CASZ1* expression, suggesting that promoter methylation and histone deacetylation in addition to 1pLOH contributes to the decrease in *CASZ1* expression in poor prognosis NB.

Conclusions: *CASZ1* regulates cell growth, differentiation, adhesion and migration, which are important events during neural crest development. The loss of *CASZ1* through 1pLOH, promoter methylation, and histone deacetylation may interrupt the commitment of normal neural crest stem cells to a differentiated phenotype and contribute to neuroblast tumorigenesis.

B12 N-Myc Initiates Tumorigenesis in Embryonal Cells by Effects on the Intrinsic Apoptosis Pathway

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Background: Little is known of the mechanisms which cause persistence of embryonal cells beyond birth, which is a necessary pre-requisite for the later formation of most child cancer types. Here we analysed the mechanism of N-Myc-mediated resistance to apoptotic cell death in neuroblasts and granule cell precursors (GCPs), the cell of origin for neuroblastoma and medulloblastoma, respectively.

Methods: Perinatal ganglion cells from N-Myc transgenic mice, which develop neuroblastoma, and GCPs from hemizygous Patched gene knockout mice (Ptc -/+), which develop medulloblastoma, were cultured, subjected to growth factor withdrawal, and analysed for survival and MycN effects on known mediators of apoptosis. Ganglion cells in culture were infected with adenoviral vectors expressing c-Myc, c-src, and N-Myc deletion-mutant constructs.

Results: N-Myc expression increased death resistance of ganglia cells to NGF withdrawal, hypoxia and nutrient deprivation. In contrast, exogenous N-Myc expression in neuroblastoma cell lines derived from N-Myc mice caused spontaneous cell death and sensitivity to death stimuli. Death resistance was also caused by c-Myc, but not c-src, expression. The N-Myc Myc Box II protein domain was sufficient for death resistance. Examination of the intrinsic apoptosis pathway localised the effect of N-Myc to a block in Bax translocation to the mitochondria. N-Myc expression in ganglia increased expression of the SIRT1 and Bmi1 genes, known inhibitors of the p53 pathway. The MDM2 inhibitor, Nutlin-3, reversed death resistance in N-Myc-expressing ganglia. GCPs from Ptc -/+ mice overexpressed N-Myc at a protein, but not mRNA level. A histologic audit of tumor initiation in the model demonstrated an early selection of GCPs which were N-Myc and PCNA positive. Ptc -/+ GCPs exhibited death resistance to sonic hedgehog withdrawal and reduced Bax activation.

Conclusions: Our data indicate a general Myc-driven mechanism for embryonal cancer initiation involving effects on Bax, apoptosis, and key regulators of the p53 pathway.

B13 N-Myc Directly Represses Transcription of the TRKA and p75 Neurotrophin Receptor Genes through the Association with the Sp1 and Miz-1 Transcription Factors

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Background: N-Myc amplification/overexpression has been long proposed as the most critical predictor of outcome for neuroblastoma. Several studies have also shown a strict correlation between high levels of N-Myc and transcription silencing of TRKA and p75 neurotrophin receptor genes suggesting a causal link between N-Myc activity and expression of neurotrophin receptor genes in neuroblastoma. Interestingly, robust expression of TRKA and p75ntr in neuroblastoma appears to be an important predictor of favorable outcome. However, the exact mechanism by which N-Myc causes TRKA and p75ntr silencing is not known. Here, we provide experimental evidence supporting the view that N-Myc is a direct repressor of TRKA and p75ntr gene transcription in neuroblastoma cells.

Methods: We have employed Luciferase reporter, transcription profiling and chromatin immunoprecipitation (ChIP) assays. Specifically we have developed a new ChIP technique, named dual crosslinking ChIP, which allowed the in vivo dissection of a repressor complex at the TRKA and p75ntr promoters. Finally, through a biochemical approach (co-immunoprecitation and GST pull-down assays) we could define the exact nature of the interaction between N-Myc and proteins associated with the TRKA and p75ntr core promoters.

Results: Our results show that: i) N-Myc intracellular protein level is critical for repression of TRKA and p75ntr gene expression. ii) N-Myc targets the TRKA and p75ntr core promoter regions through a direct association with Sp1 and Miz-1, two transcription factors, required to activate TRKA and p75ntr gene transcription. iii) N-Myc contacts Sp1 and Miz-1 by means of two distinct domains forming a ternary complex.

Conclusions: Overall, our findings demonstrate that N-Myc directly represses TRKA and p75ntr gene transcription through association with Sp1 and Miz-1 transcription factors bound to the core promoters, possibly by inhibiting their transactivating function.

B14 The HECT-Domain Ubiquitin Ligase Ureb1 Controls Neural Differentiation and Proliferation by Destabilizing the N-Myc Oncoprotein

Xudong Zhao[1], Julian Ik-Tsen Heng[2], Daniele Guardavaccaro[3], Richeng Jiang[1], Michele Pagano[3], Francois Guillemot[2], Antonio Iavarone[1,4,5], Anna Lasorella[1,4,6]
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Development of the nervous system requires that timely withdrawal from cell cycle be coupled with initiation of differentiation. Ubiquitin-mediated degradation of the N-Myc oncoprotein in neural stem/progenitor cells is proposed to trigger the arrest of proliferation and begin differentiation. It is not well understood how inputs from antimitogenic and differentiation signals lead to elimination of N-Myc. Here we report that the HECT-domain ubiquitin ligase Ureb1 ubiquitinates N-Myc through K48-mediated linkages and targets it for destruction by the proteasome. This process is physiologically implemented by embryonic stem (ES) cells differentiating along the neuronal lineage and in the mouse brain during development. Genetic and RNA interference-mediated inactivation of the *Ureb1* gene impedes N-Myc degradation, prevents exit from cell cycle by opposing the expression of Cdk inhibitors and blocks differentiation through persistent inhibition of early and late markers of neuronal differentiation. Silencing of *N-myc* in cells lacking *Ureb1* restores neural differentiation of ES cells and rescues cell cycle exit and differentiation of the mouse cortex, thus demonstrating that Ureb1 restrains proliferation and enables neuronal differentiation by mediating the degradation of the N-Myc oncoprotein. These findings indicate that Ureb1 links destruction of N-Myc to the quiescent state that complements differentiation in the neural tissue. Genetic and epigenetic events resulting in loss of Ureb1-associated ubiquitin ligase activity may contribute to aberrant accumulation of the N-Myc oncoprotein in neural cancer.

Oral Sessions

B15 MDM2 Haploinsufficiency Delays Tumorigenesis in pth-MYCN Transgenic Mice

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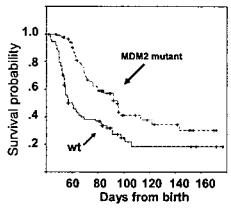
We have generated a novel compound transgenic mouse model of neuroblastoma to test the hypothesis that MDM2 is a critical modulator of MYCN driven tumorigenesis. MDM2 is the major inhibitor of p53 and MDM2 homozygous knockouts are embryonic lethal do un-abated apoptosis. We have previously shown that MDM2 activity is essential to suppress p53-mediated apoptosis in neuroblastoma, and predicted that haploinsuffieciency will result in delayed or deficient tumorigenesis due to elevated p53 tumor suppressor activity.

Method: We backcrossed MDM2$^{+/-}$ mice 8 generations into the SVJ/129 background and crossed these with pTH-MYCN mice to generate MDM2$^{+/-}$ / MYCN$^{+/+}$ compound trangenics. Longitudinal observations were then performed and tumor size, incidence and rates of onset calculated. We also performed genetic and biochemical analysis of the resulting tumors.

Results:



Kaplan-Mier plot of tumor detection and survival for compound transgenic pTH-MYCN mice: Median time to death was 54 days (95% C.I. 49-58 days) for the MDM2wt mice and 94 days (C.I. 84-98 days) for the MDM2$^{-/+}$ mice. Survival distribution as assessed by Log Rank (Chi-Square) analysis gave a p-value of <0.0001. This represents a significant increase in tumor latency and suggests that MDM2 deficiency inhibits tumor formation supporting our hypothesis that MDM2 function is critical for neuroblastoma tumorigenesis.

Conclusions: Haploinsufficiency of MDM2 has dramatic effects on tumorigenesis in pTH-MYCN mice. We propose this is due to altered regulation of p53-mediated tumor suppressor functions in MYCN expressing neuroblasts. Tumor development in this context likely requires additional time to circumvent this activity through genetic or epigenetic alterations in the genes regulating p53. We currently continue to characterize the resulting tumors for mutations in p53, ARF, MDM2 and other associated genes which modulate the p53 response in this tumor model. This animal model represents an important new tool to dissect the role of MDM2 and other oncogenic mechanisms downstream of MYCN.

B16 Novel MYCN Upregulated Genes with Oncogenic Potential in Neuroblastoma

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Background: Amplification of the transcriptional regulator *MYCN* was one of the first genetic parameters used for therapy stratification. Although MYCN overexpression is considered as a crucial event in tumorigenesis, its specific oncogenic contribution remains enigmatic. The extensive influence of MYCN on gene transcription and its fine-tuned temporal and cellular dependency hampers the disentangling of the MYCN transcriptional web. Here, we used a multimodel-multistep strategy to identify and validate consistent and relevant MYCN downstream effectors.

Methods: First, custom cDNA microarray expression profiling of a large series of both amplified and *MYCN* single copy neuroblastsoma (NB) cells, and stably *MYCN* transfected cells versus their parental non-amplified cells provided a list of 77 putative MYCN transcriptional target genes. Second, upon re-analysis of six large microarray studies on primary NB tumors, 59 of these genes were confirmed as MYCN regulated. Third, to discriminate between early (direct) and late (secondary) effectors, we profiled different time points in a cell line with tetracycline controllable MYCN expression. Fourth, the prognostic power of the downstream genes was evaluated using Cox regression analysis of the microarray studies.

Results: A subset of 12 early or late effector genes was found to be prognostically relevant, with 3 genes exerting a role in the folate pathway. All five genes upregulated by MYCN in amplified cells were subsequently silenced using RNA interference. A clear reduction in cell viability was found upon silencing of *MTHFD2* and *SMARCC1*. Further on, *SMARCC1* downregulation induced apoptosis and *MTHFD2* knockdown resulted in stress fiber formation, possibly influencing cell motility and proliferation.

Conclusions: This pipeline of gene expression profiling in different MYCN model systems, combined with meta-analysis of published microarray data and careful data analysis results in the identification of novel MYCN oncogenic mediators with perspectives for molecular therapy.

Parallel Session: Translational Research 1

TR11 Stem Cell Marker CD133 is a Novel MYCN Target and Regulates Aggressiveness in Neuroblastoma

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Background: CD133 is a stem cell marker and expressing on plasma membrane of stem cells in several tissues. Recently, it was reported that hematopoietic, neuronal, colon and other cancer stem cells are selectively expressing CD133; CD133-positive cancer cells had high tumorigenic activity, and resistance to anticancer drugs. Although apparent cancer stem cells have not been identified in neuroblastoma (NB) so far, our preliminary experiments suggest *CD133* expression in I-type NB cell-lines which have stem cell-like and malignant phenotypes. These findings prompted us to study the functional roles of CD133 in NB cells.

Results: To study the functional roles of CD133 in NB cells, we introduced *CD133* cDNA to CD133-low-expressing NB cells by lentivirus. Overexpression of CD133 effectively accelerated the anchorage dependent/independent growth. Furthermore, knockdown of *CD133* by lentivirus-produced shRNA repressed cell growth and reduced the number and size of colonies in soft agar. Intriguingly, in the retinoic acid- and phorbol ester-treated NB cell-lines, CD133 expression was decreased along with differentiation. Next, we found three candidate regions of E-box, which is the direct binding target of MYCN, in upstream of *CD133* 1st exon. These regions were studied by ChIP assay and we could specifically enrich the one of the *CD133* E-box regions with the MYCN antibody relative to an isotype-matched control antibody. Furthermore, *CD133* expression was basically correlated with amplified *MYCN* gene status both in NB cell-lines and unfavorable NB primary tumors in semi-quantitive RT-PCR analysis.

Conclusions: *CD133* seems to be a direct MYCN-transcriptional target gene and related to the aggressiveness of NB. Further analysis of CD133 functional roles will be helpful for development of a new molecular-targeted therapy in NB.

TR12 *In vivo* Anti-Tumor and Anti-Metastatic Activity of Administration of Sunitinib in a Preclinical Neuroblastoma Mouse Model

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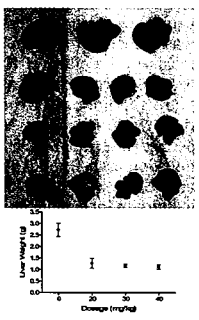
Background: High vascularity is a feature of aggressive, widely disseminated neuroblastoma (NB). Expression of VEGF is nearly ubiquitous in NB primary tumors and all NB cell lines tested to date and high circulating VEGF and bFGF levels correlate with high-risk NB. Sunitinib is a kinase inhibitor targeting platelet-derived growth factor receptors (PDGFRs) and VEGFRs. Preclinical sunitinib studies showed tumor regression, prolonged survival in mammary adenocarcinoma, melanoma and leukemia tumor models.

Aims: To investigate the role of sunitinib in NB tumor growth, angiogenesis and metastasis.

Methods: In localized xenograft model, SK-N-BE(2) cells were injected subcutaneously into the inguinal area of NOD-SCID mice. Treatment was started when tumors reached 0.5cm in diameter. Mice were randomized into control and treatment groups (20mg/kg, 30mg/kg or 40mg/kg/day of sunitinib) and were sacrificed after 14 days of treatment. Tumors were dissected, weighed and analyzed for microvessel density by endothelial cell makers CD31 and CD34. In metastatic model, SK-N-BE(2) cells were injected intravenously through the lateral tail vein. Sunitinib treatment started 7 days after inoculation at the same doses described above. Mice were sacrificed on day 27 and examined for metastases (visually,by weighing and histopathology).

Results: 20mg/kg of sunitinib daily suppressed tumor growth ($p<0.01$ compared to control) but no significant differences between the three dose levels. However, increasing sunitinib doses did decrease the microvessel density in the tissue sections. In the metastatic model, only mice in control group showed signs of distress and developed extensive liver metastasis and ascites. In the sunitinib treatment groups, only few scattered metastatic nodules were observed and a significant difference in liver weight between control and all treatment groups ($p<0.01$) as well as a dose response effect amongst the various dose levels. Histology slides showed at least 90% deduction of number and size of metastatic sites in liver, adrenal gland and bone marrow and 100% in lung, after treatment with sunitinib.

Fig. 4 The effect of sunitinib on NB metastasis



Conclusions: Sunitinib appears significantly inhibit NB tumor growth, angiogenesis and control metastasis. A Minimal residual disease mouse model is currently evalauted.

TR13 Targeted Mcl1 Reduction Inhibits Neuroblastoma In Vitro and In Vivo

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Background: Therapy resistance in neuroblastoma (NB) results partially from overexpression of anti-apoptotic Bcl2 family members (e.g., Mcl1, Bcl2). Apoptosis is restored by depleting such Bcl2-related proteins, or by competitively inhibiting sequestration of activated death proteins. We sought functional validation of this approach in NB.

Methods: Mcl1 and Bcl2 expression was assessed by IHC using a human NB tissue microarray (TMA; N=186 replicate tumor cores). In vitro, Mcl1 and Bcl2 were knocked-down in multiple NB cell lines using siRNA (5-100 nM), singly and in combination with chemotherapy. Cell growth was measured by real-time impedance monitoring (RT-CES, Acea), and apoptosis by caspase activation. In vivo, murine NB xenografts were treated with anti-Mcl1 (Isis) and anti-Bcl2 (Genta) antisense oligonucleotides (ASO).

Results: TMA IHC revealed heterogeneous and redundant expression of Bcl2 proteins, with high expression of Mcl1 (33%), Bcl2 (33%), or both (13%) across NBs. This pattern similarly was demonstrated in NB cell lines (IB). Mcl1 siRNA promoted dose-dependent apoptotic cell death at 5 nM exposure, even in cell lines with high Bcl2 expression (SK-N-AS, KAN, NLF, IMR5), correlating with four-fold Mcl1 mRNA reduction and protein depletion from mitochondria. Conversely, Bcl2 knockdown showed limited growth inhibition (NLF, KCNR). Mcl1 knockdown also potentiated diverse conventional cytotoxics (VP16, mafosfamide, topotecan, cisplatin), at doses one-fourth to one-tenth the monotherapy IC50. Finally, anti-Mcl1 ASO treated xenografts showed tumor growth inhibition, while anti-Bcl2 ASO had limited impact (SK-N-AS, IMR5). These findings parallel in vitro responses to small molecule antagonists against Mcl1 (AT101, Ascenta) and Bcl2 (ABT737, Abbott), see Goldsmith et al., ANR 2008.

Conclusions: Heterogeneity and redundancy of Mcl1/Bcl2 expression in NB may account for therapy resistance. Our data support a prominent functional role for Mcl1 in survival, and suggest that Mcl1 (or dual Mcl1/Bcl2) targeted therapies hold promise for clinical application.

TR14 Immunotherapy of Neuroblastoma (NB) by an Interleukin-21-Secreting Cell Vaccine is Potentiated by CD4+ T Cell Depletion

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Background: IL-21 is the most recently identified member of the IL-2 cytokine family. Here we studied the therapeutic efficacy of IL-21-gene-modified Neuro2a (Neuro2a/IL-21) neuroblastoma (NB) cells as vaccine in a syngeneic metastatic NB model.

Methods: Three doses of viable Neuro2a/IL-21 or empty-vector-Neuro2a (Neuro2a/mock) cells were injected sc in syngeneic A/J mice bearing Neuro2a parental cells (Neuro2a/pc) micrometastases (induced by i.v. challenge) and the percentage of tumor-free survival was evaluated. In vivo depletion studies were performed using anti-asialoGM1, anti-CD8, anti-CD4 and anti-CD25 or irrelevant antibodies. In vitro responses towards Neuro2a/pc and survivin-derived CTL epitopes were assessed by ^{51}Cr-release and Elispot assays.

Results: IT with Neuro2a/IL-21 increased the mean time to tumor onset from 22 to 75 days (P=0.0012 *versus* control mice) leading to a cure rate of 33% at long term, whereas Neuro2a/mock cells had no effect. Splenocytes from cured mice lysed Neuro2a/pc target cells and produced IFN-gamma after in vitro re-stimulation with survivin-synthetic CTL-epitopes. Depletion of CD8+ inhibited the effect of the vaccine, further supporting the role of CTLs. By contrast administration of anti-CD25 (depleting Treg cells) or anti-CD4 mAbs potentiated the effect of IT, leading to a 50% or 80% cure rate. CD4+ cell depletion up-regulated the expression of several cytokine genes such as IL-21, IL-15, and IL-17 both in the bone marrow and spleen.

Conclusions: Our data indicate that IL-21-secreting NB cells are effective as therapeutic vaccine in mice bearing metastatic NB through a specific CTL response involving survivin as antigen. Our data indicate also that the depletion of CD25+ regulatory T cells or of CD4+ cells had a synergistic effect with IT. In case of CD4 T cell depletion this effect may reflect both the upregulation of homeostatic cytokines and removal of T cells and cells that acquire regulatory functions after exposure to tumor cells.
MC is the recipient of the Italian Neuroblastoma Foundation fellowship

TR15 Histone Demethylase LSD1 is Highly Expressed in Poorly Differentiated NB and is a Novel Therapeutic Target

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Background: The importance of epigenetic gene regulation has been established in neuroblastoma for both DNA methylation and histone acetylation. Histone methylation has long been considered to be irreversible and less versatile. Recently, a new class of histone-modifing enzymes, the histone demethylases, has been identified. These enzymes regulate transcription by specifically demethylating histone residues in promoter regions. Here we address the functional significance of the expression of the lysine-specific histone demethylase 1 (LSD1) in neuroblastoma, and identify LSD1 as a novel therapeutic target.

Methods: LSD1 expression was analysed using Affymetrix microarrays (n=102) and on a neuroblastoma tissue microarray (n=99). The effect of LSD1 knock-down or inhibition was analysed in cell-based assays *in vitro*. Nude mice harbouring neuroblastoma xenografts were treated with a small molecular LSD1 inhibitor.

Results: LSD1 was highly expressed in undifferentiated neuroblastomas, while LSD1 levels were reduced in differentiating neuroblastomas and ganglioneuromas. Kaplan-Meier analysis revealed high LSD1 expression to be correlated with adverse outcome. Inducing neuroblastoma cell differentiation *in vitro* with all-trans retinoic acid resulted in significant downregulation of LSD1 expression. Knock-down of LSD1 with siRNA led to decreased cellular viability and the induction of differentiation-associated gene expression patterns. Inhibition of LSD1 with the FDA-approved monoamine oxidase inhibitors, pargyline, clorgyline and tranylcypromine, in neuroblastoma cell lines resulted in an increase of global H3K4 dimethylation and cell death. Treatment of nude mice with tranylcypromine reduced xenograft size *in vivo*.

Conclusions: This suggests that LSD1-mediated histone de-dimethylation might be involved in transcriptional silencing of differention and/or apoptotic programmes inherent in neuroblastoma cells. This makes LSD1 a bona fide target for therapeutic intervention.

TR16 Fenretinide/Lym-X-Sorb Oral Powder Combined with the Oral Microtubule Inhibitor ABT-751 is Highly Active against Multidrug-Resistant Neuroblastoma Xenografts

C. Patrick Reynolds, Nancy Tran, Vanessa Maldonado, Vazgen Khankaldyyan, Hirouki Shimada, Barry J. Maurer
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Background: ABT-751, a colchicine binding-site microtubule inhibitor, and fenretinide [N-(4 hydroxyphenyl) retinamide, 4-HPR] are orally bioavailable drugs that have shown some clinical activity in recurrent neuroblastoma.

Methods: We determined activity of ABT-751 + 4-HPR/Lym-X-Sorb oral powder against progressing subcutaneous xenografts in nu/nu mice from four recurrent (post-chemotherapy) neuroblastoma cell lines (SMS-KCNR, CHLA-90, CHLA-136, and CHLA-140) using an oral 5 day/week-dosing schedule. Apoptosis in tumors was assayed by TUNEL. Cytotoxicity in vitro employed the fluorescence-based DIMSCAN assay while reactive oxygen species (ROS) were measured by flow cytometry.

Results: Combined results (median survival in days) from the four subcutaneous xenograft models (n = 5 per line for each condition) were: control = 28, 4-HPR = 49, ABT-751 = 77, and 4-HPR + ABT-751 = 239. Combining 4-HPR + ABT-751 significantly ($p < 0.01$) enhanced mouse survival (relative to controls or single drugs) in the subcutaneous models (with 5 day/week x 60-90 days and 7-day/3 week dosing schedules) and also in a CHLA-136 disseminated disease model in SCID mice. Tumor cell apoptosis by TUNEL was greater in 4-HPR + ABT-751 treated tumors than with single drugs. When 4-HPR + ABT-751 was stopped at day 66 (all SMS-KCNR mice in response), re-treatment of tumors progressing at day 169 again achieved responses. 4-HPR + ABT-751 was active against xenografts that had progressed on either drug alone. Interestingly, synergy between 4-HPR and ABT-751 in vitro was observed only in 2 of 6 neuroblastoma cell lines. In the CHLA-119 cell line, antioxidants ascorbic acid and α-tocopherol (but not n-acetylcysteine or sodium thiosulfate) suppressed ROS generated by 4-HPR and diminished the synergistic cytotoxicity of 4-HPR + ABT-751 in vitro.

Conclusions: 4-HPR + ABT-751 is a well-tolerated oral drug combination that is highly active in vivo against multiple recurrent neuroblastoma xenograft models and warrants clinical trials.

Parallel Session: Basic Research 2

B17 Neuronal Leucine Rich Repeat Protein 1 (NLRR1) is a Direct Transcriptional Target of N-Myc as Well as C-Myc and Contributes to Aggressiveness of Primary Neuroblastomas

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Background: The NLRR family genes, which encode type I membrane receptors, are developmentally regulated and involved in neurogenesis. However, their ligands are still unknown. We have previously reported that NLRR1 is significantly highly expressed in unfavorable neuroblastomas, whereas NLRR3 expression is downregulated in those tumors. Aggressive neuroblastomas often carry *N-Myc* amplification, nevertheless, why N-Myc contributes to malignant phenotypes of neuroblastomas is still elusive. Here we have identified that *NLRR1* is a novel transcriptional target of both N-Myc and c-Myc, and could be a therapeutic target to develop new drugs against high-risk neuroblastomas.

Results and Discussion: Like our previous observation in primary neuroblasatomas, *NLRR1* mRNA expression was significantly high in neuroblastoma cell lines with *N-Myc* amplification as compared with those with a single copy of *N-Myc* ($p < 0.0001$). We next examined whether or not *NLRR1* mRNA expression was associated with expression of *N-Myc* mRNA in primary neuroblastomas, resulting in that both were significantly positively correlated in 70 sporadic neuroblastomas ($p<0.0001$). We then overexpressed *NLRR1* in neuroblastoma cells, that promoted cell proliferation, whereas knockdown of *NLRR1* mRNA using its siRNA induced apoptotic cell death. The apoptosis of neuroblastoma cells induced by serum starvation was also inhibited by enforced expression of *NLRR1*. Furthermore, in an *N-Myc*-inducible neuroblastoma cell line, SHEP21N, *NLRR1*-siRNA inhibited enhancement of cell growth induced by N-Myc, suggesting that *NLRR1* might be a downstream target of N-Myc. We next examined the promoter region of human *NLRR1* gene and found 5 E-boxes. Luciferase reporter assay and ChIP (Chromatin Immunoprecipitation) analysis revealed that N-Myc is recruited on to the putative E-boxes within the promoter region of *NLRR1* gene. C-Myc was also bound to the promoter of *NLRR1* which is highly expressed in colorectal cancer cell line with *c-Myc* amplification. Thus, NLRR1 may be one of the key players directly targeted by Myc oncoprotein in aggressive neuroblastomas.

B18 Recruitment of Histone Deacetylase 1 by N-Myc as a Mechanism of Transcriptional Repression and Oncogenesis

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Background: Histone deacetylases (HDAC) bind gene promoters and repress transcription. HDAC inhibitors reactivate gene transcription, induce cancer cell differentiation, growth arrest and apoptosis. Myc oncoproteins block cell differentiation, and, promote cell proliferation and malignant transformation, by modulating target gene transcription.

Methods: cDNA microarray was employed to identify target genes of HDAC inhibitors and N-Myc. siRNAs were used to knock down gene expression. Chromatin immuno-precipitation, luciferase assay and protein co-immunoprecipitation were used to verify binding of N-Myc and HDAC1 to target gene promoter and binding of N-Myc to HDAC1, respectively. Immunohistochemistry was carried out to analyse protein expression and co-localisation in neuroblastoma tissues from N-Myc transgenic mice treated with an HDAC inhibitor.

Results: Tissue transglutaminase (TG2) was the gene most significantly repressed by N-Myc in neuroblastoma cells in a cDNA microarray analysis, and was repressed by N-Myc in neuroblastoma cells, and c-Myc in breast cancer cells. Repression of all TG2 protein isoforms by N-Myc in neuroblastoma cells was necessary for the inhibitory effect of N-Myc on neuroblastoma cell differentiation. In contrast, inhibition of the TG2-long isoform alone induced marked neuritic differentiation and growth arrest, suggesting the importance of the two TG2-short isoforms in promoting differentiation. By recruiting HDAC1 protein to an Sp1-binding site in the TG2 gene core promoter, N-Myc acted as a transrepressor, in a manner distinct from its action as a transactivator at E-Box binding sites. HDAC inhibitor treatment blocked the N-Myc-mediated HDAC1 recruitment and TG2 repression *in vitro*. In neuroblastoma-bearing N-Myc transgenic mice, HDAC inhibitor treatment induced TG2 expression and demonstrated marked anti-tumour activity *in vivo*.

Conclusions: Our data indicate the critical roles of HDAC1 and TG2 in Myc-induced oncogenesis, and have significant implications for the use of HDAC inhibitor therapy in Myc-driven oncogenesis.

B19 Transcriptional Repression of *MYCN* is Mediated by TAp63 in Favorable Neuroblastomas

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Background: *p63* is one of the *p53* family genes and is expressed at least in six isoforms. Altenative promoter usage gives rise to wild-type (TAp63) and N-terminally truncated form of p63 (ΔNp63). The C-terminal isoforms including alpha, beta and gamma are generated by alternative splicing. TAp63-gamma is the major isoform expressed in sympathetic neurons during development and plays an essential role in the regulation of apoptotic neuronal cell death. However, the functional significance of TAp63 in the induction of differentiation or apoptosis in neuroblastoma has been elusive.

Methods: Overexpression and siRNA-mediated knockdown of TAp63 were employed to identify its target genes. The expression levels of p53 family members and its target genes were examined in neuroblastoma cells treated with or without 5 μM of *all-trans* retinoic acid (ATRA). The extent of apoptotic cell death and differentiation in response to ATRA was investigated by morphological examination and FACS analysis.

Results: In primary neuroblastomas, TAp63 mRNA was expressed at high levels in favorable subset, whereas expression of ΔNp63 mRNA was rarely detectable in any subsets. The siRNA-mediated knockdown of TAp63 resulted in a strong induction of MYCN in HeLa and HepG2 cells which express MYCN at extremely low levels. In contrast, overexpression of TAp63 suppressed expression of MYCN. The TAp63-mediated suppression of MYCN expression was abrogated by treating the cells with Trichostatin A (TSA), one of the histone deacetylase inhibitors. Among p53 family members (p53, TAp73, ΔNp73, TAp63 and ΔNp63), only TAp63 were specifically induced during ATRA-mediated differentiation and apoptotic cell death in RTBM1 and CHP134 neurobastoma cells, respectively. The change in expression levels of TAp63 mRNA was inversely correlated with that of MYCN mRNA in neuroblastoma cells treated with ATRA.

Conclusions: TAp63 regulates transcriptional repression of MYCN during ATRA-mediated differentiation and/or apoptotic cell death in neuroblastoma cells.

B20 ApoJ/clusterin is a Novel Haploinsufficient Neuroblastoma Suppressor Gene Negatively Regulated by MYCN

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Background: The role of ApoJ/clusterin in cancer is still debated, with evidence that it can be either overexpressed or downregulated in human cancer. Furthermore, ApoJ/clusterin was shown to be a promoter or inhibitor of apoptosis, cell motility and inflammation. We found that expression of the cytoplasmic form of ApoJ/clusterin in neuroblastoma biopsies and surgical resections is significantly associated with differentiating, localised disease, with the majority of undifferentiated, metastatic neuroblastomas showing absence of ApoJ/clusterin protein expression. This finding prompted us to investigate whether ApoJ/clusterin has a direct role in neuroblastoma.

Methods: We crossed MYCN transgenic with ApoJ/clusterin knockout mice to investigate neuroblastoma penetrance *in vivo*. Human neuroblastoma cell lines in which ApoJ/clusterin expression was manipulated were injected in the tail vein of immunodeficient mice. Metastatic neuroblastoma cells were scored in the target organs by FACS analysis. Affymetrix datasets were analysed on-line through the Oncomine website (www.Oncomine.org).

Results: The ApoJ/clusterin mRNA is downregulated in neuroblastomas with unfavourable cytogenetic alterations, such as amplification of the MYCN proto-oncogene and chromosome 1p deletion. ApoJ/clusterin is directly suppressed by MYCN, resulting in the activation of NF-kB. The penetrance of neuroblastomas arising in N-MYC-transgenic mice is significantly increased after deletion of one or two copies of the ApoJ/clusterin gene, providing the first compelling evidence that ApoJ/clusterin is a *bona fide* tumour suppressor gene. In support to this hypothesis, siRNA depletion of ApoJ/clusterin in human neuroblastoma cell lines results in an increase, whereas its overexpression causes a decrease, of experimental metastasis in xenotransplanted mice. Ablation of ApoJ/clusterin is accompanied by activation of NF-kB in developing neuroblastomas and epithelial to mesenchymal transition.

Conclusions: ApoJ/clusterin is a haploinsufficient neuroblastoma suppressor gene. Inhibiting NF-kB and maintaining an epithelial phenotype is likely to be a critical feature of the ApoJ/clusterin tumour suppressive function.

Parallel Session: Translational Research 2

B21 Expression and Sequence Analysis of Candidates for the 1p36.31 Tumor Suppressor Gene Deleted in Neuroblastomas

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Background: Neuroblastomas (NBs) are characterized by 1p deletions, suggesting that a tumor suppressor gene (TSG) resides in this region. We have analyzed over 1,200 tumors using DNA polymorphisms to define the smallest region of deletion (SRD) from 1p36.31 in these tumors. We have identified 23 genes in the 2 Mb SRD, and we have characterized their sequence and expression pattern.

Methods: We examined the expression pattern of 23 genes from the SRD by semi-quantitative RT-PCR in 17 normal tissues and in 20 NB cell lines. We have also analyzed the sequence of all exons of these genes for mutations or inactivating rearrangements.

Results: Most of the 23 genes showed readily detectable expression in 20 NB cell lines, and no correlation with 1p deletion. However, 7 genes (*AJAP1, HES3, GPR153, HES2, ESPN, TNFRSF25, PLEKHG5*) showed uniformly low expression in cell lines, and 2 genes (*CHD5*, *RNF207*) had virtually absent expression, consistent with the expected pattern for a TSG. Five genes (*AJAP1, KCNAB2, CHD5, ACOT7, CAMTA1*) showed preferential neural expression. We sequenced the coding exons of all genes in 30 neuroblastoma cell lines. Although rare mutations were found in 10 of the 23 genes, none showed a pattern of genomic change, and homozygous inactivation by deletion and genomic change was not found for any gene in the region.

Conclusions: The TSG from this region most likely has a low or absent expression pattern in the NB lines and either acts by haploinsufficiency, or the second allele is inactivated by an epigenetic mechanism. Based on a neural pattern of expression as well as consideration of putative function, *AJAP1, TNFRSF25* and especially *CHD5* are the most promising candidates.

B22 Wild-Type CADM1 Expression Suppresses Growth and Invasion of Neuroblastoma Cell Lines

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Background: Loss of chromosome arm 11q has been shown to be an independent predictor for relapse in neuroblastoma, but the critical genes disrupted by this frequent aberration are not known.

Methods: Using a combination of deletion mapping, functional complementation, and mRNA expression data, we identified *CADM1* as a candidate tumor suppressor on 11q and identified a *CADM1* mutation in a neuroblastoma cell line (Attiyeh, ANR 2006). We have cloned 3 different *CADM1* splice variants normally expressed in neuronal tissue into the mammalian expression vector pIRES2-EGFP (Clontech). We have also engineered two point mutants: a nonsense mutant in exon 5 previously described in hepatocellular carcinoma and the C1199A variant in exon 9. We transfected the constructs into the highly tumorigenic neuroblastoma cell lines SK-N-AS (hemizygous for *CADM1* and absent *CADM1* mRNA expression) and NLF (3 copies of *CADM1* with one mutated copy) and assayed for changes in proliferation, contact inhibition, and invasion.

Results: Overexpression of each of the wild-type *CADM1* isoforms resulted in a significant decrease in soft agar colony formation in both cell lines (SK-N-AS: 28.2%-75.4% of empty vector control, p=0.005; NLF: 59.9%-67.0% of control, p=0.001) and a significant decrease in Matrigel invasion (SK-N-AS: 35.6%-51.9% of control, p<0.001; NLF: 39.4%-52.8% of control, p<0.001). There was no effect on cellular proliferation. Unlike wild-type *CADM1*, transfection of the mutant constructs did not have an effect on anchorage-independent growth or invasion.

Conclusions: Wild-type *CADM1* functions to suppress neuroblastoma invasion, suggesting that somatically acquired deletion at 11q results in increased metastatic potential. Additional studies *in vivo* and on primary tumor samples will determine if *CADM1* loss is the major functional consequence of 11q deletion.

TR17 Th CXCR4 Strongly Promotes Neuroblastoma Growth but not Invasion

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Background: Chemokines and their receptors have been involved in tumour growth and metastasis. In particular the CXCR4/CXCL12 axis was reported to mediate organ-specific cancer cells homing and invasion. We investigated the precise role of CXCR4/CXCL12 axis in the malignant behaviour of NB by measuring *in vitro* tumour cell growth, survival, migration, and invasion in response to CXCL12, and *in vivo* using an orthotopic model of metastatic NB.

Methods & Results: CXCR4 overexpression in the non metastatic IGR-NB8 cells (NB8-CXCR4-C3) resulted in enhanced cell proliferation, survival and CXCL12-mediated chemotaxis. *In vivo*, orthotopic implantation of NB8-CXCR4-C3 cells resulted in much faster tumour growth and tumour volumes as compared to controls. Surprisingly, no increase in the frequency of total or organ-selective metastases was observed. CXCR4 overexpression in the metastatic IGR-N91 cells also enhanced *in vivo* tumour growth but did not increase the occurrence of metastases. On the opposite, shRNA-mediated CXCR4 silencing in IGR-N91 cells almost completely abrogated their *in vivo* growth.
Interestingly, high levels of the CXL12 ligand were measured in the nude mice primary tumour environment (adrenal gland) and in the liver, but not in the bone marrow, providing a possible explanation for the lack of CXCR4-mediated organ-selective dissemination of neuroblastoma cells.
Genes and pathways involved in the CXCR4/CXCL12-mediated effects in NB were identified by expression profiles analyses (Affymetrix) of the two transduced cell lines and controls. Ten genes commonly upregulated and 31 downregulated genes in all CXCR4 overexpressing clones were identified that may represent interesting candidate genes involved in CXCR4-mediated growth-promotion.

Conclusions: Our results demonstrate that the CXCR4/CXCL12 axis in NB strongly stimulates tumour growth, survival, and chemotactic migration but is not sufficient to induce or enhance invasion, revealing a tumour type specific growth-promoting effect. Identified genes and pathways activated upon CXCR4 overexpression may be interesting therapeutic targets for tumour growth inhibition.

TR18 Risk Estimation of Neuroblastoma Patients by Gene Expression Based Classification: Ready for Clinical Application?

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Background: To improve risk estimation of neuroblastoma patients, we have recently defined a prognostic 144-gene expression-signature using the PAM-algorithm that distinguished patients with divergent outcome in clinical risk groups. This study aimed at the validation of the PAM-classifier's accuracy in an independent cohort.

Methods: 217 neuroblastoma samples were analyzed using microarrays and classified by the PAM-classifier. Patients were categorized according to the German trial NB2004 criteria (low-risk, n=101; intermediate-risk, n=27; high-risk, n=73; not classified, n=16). Samples were prospectively collected within the NB2004 trial (n=104, median follow-up 1.6 years), and obtained from international collaboration partners (n=113, median follow-up 6.2 years; low-risk, n=27; intermediate-risk, n=19; high-risk, n=53; not classified, n=14). EFS and OS at 3 years were analyzed.

Results: The PAM-classifier distinguished patients with divergent outcome in the total cohort (EFS 0.84±0.04 vs. 0.38±0.05; OS 0.95±0.03 vs. 0.57±0.06; both p<0.001) as well as in the prospective (EFS 0.83±0.05 vs. 0.29±0.11, p=0.002; OS 1.0 vs. 0.56±0.17, p<0.001) and the international (EFS 0.90±0.05 vs. 0.38±0.06; OS 0.94±0.04 vs. 0.54±0.06; both p<0.001) set separately. In the total cohort, patients of the low-risk group were discriminated in subgroups with differing OS (1.0 vs. 0.92±0.05; p=0.016). Patients of both the intermediate-risk and high-risk group were discriminated in subgroups with divergent EFS (1.0 vs. 0.56±0.14, p=0.014; and 0.57±0.16 vs. 0.20±0.06, p=0.011, respectively) and a tendency of differing OS (OS 1.0 vs. 0.86±0.10, p=0.124; and 0.71±0.14 vs. 0.39±0.07, p=0.062, respectively). Separate PAM-classification of risk groups of the international collective revealed no significant survival difference in the low-risk group, whereas patients predicted to be favorable of both the intermediate-risk and high-risk groups had a significantly better EFS (1.0 vs. 0.64±0.15, p=0.03; and 0.57±0.16 vs. 0.21±0.07, p=0.014, respectively).

Conclusions: The PAM-classifier was confirmed to accurately predict patients' outcome in independent cohorts and may contribute to refine current risk stratification systems.

TR19 R2: A Public User-Friendly Web-Tool for Integral Analysis of Clinical and Expression Data 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 is more difficult to extract.

Methods: We designed a web-based program to facilitate functional analysis of mRNA expression data of neuroblastoma. The data were generated by Affymetrix HG U133 plus2.0 analysis of 110 neuroblastomas. We included clinical data, as well as thousands of public microarrays from other tumor types.

Results: The R2 program has a userfriendly 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; ganglioneuroma vs. neuroblastoma). 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 (PAGE analyses, GO, 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. Mapping of expression data to the genome identified amplified regions and revealed novel amplicons in our neuroblastoma series. R2 also calculates Kaplan Meier curves for 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 highly valuable data source for Neuroblastoma researchers. We decided to make the R2 program and databases publicly available. A web-based version is currently implemented and will be available at the ANR2008 meeting. R2 will help researchers in identifying important genes and biological processes in neuroblastoma.

TR20 Segmental Chromosomal Abnormalities are Associated with a Higher Risk of Relapse in Infants with Neuroblastoma – Array-CGH Analysis of Tumours of Infants Included in the INES 99.1, 99.2 and 99.3 Trials. A SIOPEN/ENQUA Collaborative Study

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Background: In the absence of *MYCN* amplification (MNA), infants with neuroblastoma (NB) have an overall good prognosis. However, a subset of these patients may experience relapse, suggesting that this group may be genetically heterogeneous and that recurrent genetic alterations other the MNA could be useful for the identification of a patient subgroup at a higher risk of relapse.

Methods: In order to study the genetic alterations and to determine a pangenomic profile, array-CGH was performed on tumour-DNA from 178 of 245 patients from the participating countries eligible in the INES99.1, 99.2 or 99.3 trials, using an in-house BAC/PAC-array.

Results: Of the interpretable 175 cases, 7 did not show any genomic alteration. Whole chromosome copy number variations only were observed in 132 cases (genomic type 1). In addition to a variable number of whole chromosome copy number variations, segmental alterations recurrently observed in NB occurred in 33 cases (genomic type 2). Three cases showed atypical segmental alterations. A genomic type 2 profile was observed in 8/80 INES99.1, 13/73 INES99.2, and 12/22 INES99.3 cases, respectively (p=0.0002). Chromosome 1p loss (n=9), 11 loss (n=17) and 17q gain (n=31) were associated with a statistically significant poorer progression-free survival (PFS). Moreover, a genomic type 2 profile was associated with a statistically significant poorer 4-year PFS (type 1 tumours: 91%+/-2.1% versus type 2 tumours: 59%+/-8.7%; p<0.0001). In a multivariate model, taking into account the protocol arm, the individual segmental alterations as well as the genomic type, only the genomic type was retained as statistically significant (p<0.0001; Relative Risk 5.2; CI 2.3-11.5).

Conclusion: In infant NB, a genomic type defined by the presence of any segmental chromosome alteration is associated with a higher risk of relapse/ progression and is of higher prognostic impact than the individual genetic markers, making it a powerful tool for the clinical management of these patients.

TR21 Human Exon Array Gene Expression Profiling in Patients with Metastatic Neuroblastoma

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Background: There is great variability in the clinical behavior of metastatic neuroblastomas. Analysis of transcriptomes of these tumors by Human Exon Arrays could provide insight into their biology and help to develop more accurate risk classification.

Methods: 112 neuroblastomas from patients with stage 4 disease were analyzed with Affymetrix Human Exon arrays that include 5.2 million probes (~1.4 million Probe Selection Regions - PSRs). Arrays were normalized and summarized using the Robust Multi-Chip Average (RMA) method at both gene-level and exon-level using core PSRs. A prediction algorithm using Diagonal Linear Discriminant Analysis (DLDA) was used to discover a predictive gene signature for MYCN non-amplified (MYCN-NA) tumors (n=93), and this was compared to the 55-gene signature we previously identified with U133 A+B microarrays (JNCI 98:1193-1203, 2006). DLDA analysis also was performed to identify differentially expressed genes among MYCN-NA and MYCN amplified (MYCN-A) tumors, and top ranking genes were evaluated for alternative splicing usage.

Results: DLDA analysis of MYCN-NA tumors for prediction of progression-free survival (PFS) identified a 13-gene signature using gene-level summarization and a 153-PSR signature using exon-level summarization with cross-validated error rates of 22.8% and 26.8% respectively. The HuEx and U133 A+B signatures showed overlap of >30% for genes identified and included NTRK1, CAMTA1, GNFRa3 being upregulated in low risk tumors. DLDA analysis of MYCN-NA vs. MYCN-A tumors revealed an 8-gene distinguishing signature using gene-level summarization with a cross-validated error rate of 3.9%. In addition to the MYCN gene, CD44 ranked high, having low expression in amplified tumors. However, exon-level analysis revealed that the difference in expression is due to distinct CD44 isoforms in tumors with MYCN amplification.

Conclusions: Human Exon Arrays allow detailed analysis of transcriptomes of neuroblastomas and can identify features for developing more accurate outcome prediction models as well as differences in exon usage.

TR22 A Six-Gene Molecular Signature Detects Neuroblastoma Cells among Hematopoietic Cells with High Sensitivity and Specificity

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Background: Monitoring the response of neuroblastoma cells in blood and bone marrow to therapy with a highly sensitive and specific assay may provide an early surrogate for clinical response and progression-free survival. A standardized multi-gene panel has not been developed for this purpose. This study 1) identified six genes that are highly expressed by neuroblastoma cells but not detectibly by hematopoietic cells; 2) determined optimal conditions for RT and Taqman® Low Density Array (TLDA) PCR; 3) determined the detection limit of the TLDA assay by seeding neuroblastoma cells into PBMC; and 4) compared TLDA and immunocytology assays for evaluating bone marrow and PBSC.

Methods: Two-step RT-PCR gene expression was quantified for CHGA, DCX, DDC, ISL1, PHOX2B, and TH (neuroblastoma signature genes) and for B2M, GAPDH, HPRT1, and SDHA (housekeeping genes) using TLDA.

Results: CHGA, DCX, DDC, ISL1, PHOX2B, and TH were expressed by neuroblastoma primary tumors (threshold cycle, Ct, 23.7, 24.8, 23.9, 22.4, 22.9, 22.0, respectively) but not detectibly by PBMC from normal adults or by BM or PBSC from non-neuroblastoma patients (Ct >40). Optimal detection, based upon seeding neuroblastoma cells into PBMC, was obtained when oligo-dT + gene specific primers were used for cDNA synthesis and when 2500 ng of cDNA was loaded into each port of the TLDA microfluidics card. Seeding SK-N-BE2 or CHLA-255 neuroblastoma cells into PBMC showed reliable detection of one tumor cell per 10^6 PBMC. All bone marrows from patients that were positive by immunocytology were positive by TLDA assay. Furthermore, TLDA evaluation of 19 PBSC samples that were all negative by immunocytology revealed that 90%, 63%, and 32% had detectible expression of 1, 2, or ≥3 neuroblastoma signature genes.

Conclusion: Quantifying six neuroblastoma signature genes with TLDA provides a standard and highly sensitive test for neuroblastoma cells in bone marrow and blood.

Selected Poster Session: Basic Research

B38 LMO3 Acts as an Oncogene via Its Interaction with the Tumour Suppressor, p53, in Neuroblastoma

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Background: LMO3, a member of LIM-only (LMO) protein family, is highly expressed in unfavorable neuroblastomas as compared with favorable ones. Consistent with these results, we previously demonstrated that LMO3 enhances transactivation of Mash1, a cell fate determination gene of sympathoadrenal lineage, and is closely involved in genesis and development of neuroblastoma. However, the molecular mechanisms behind LMO3-mediated oncogenic transformation are not yet clear.

Methods & Results: During cisplatin-induced apoptotic cell death in neuroblastoma-derived SH-SY5Y cells, p53 is known to be phosphorylated and stabilized in the cell nucleus, whereas protein expression of endogenous LMO3 was significantly reduced. LMO3 and p53 co-expression experiments in a p53-null cell line revealed that p53 could target LMO3 for degradation in a proteasome-dependent manner. The possibility of complex formation between LMO3 and p53 was then investigated; co-immunoprecipitation studies showed that these proteins could indeed form a stable complex. To explore the functional significance of this interaction the ability of p53 to activate its target genes in the prescence of LMO3 was tested. Exploiting dual-gene reporter assays and RT-PCR, LMO3 was shown to inhibit p53-mediated activation of $p21^{WAF1}$, bax and p53AIP1.

Conclusions: Our present data suggest that, by physical interaction, LMO3 inhibits transactivation function of p53, whereas p53 decreases protein stability of LMO3. Abnormal LMO3-containing complex formation may be an important mechanism of LMO3-mediated oncogenesis. Therefore, LMO3 might prove to be a useful prognostic indicator for the sensitivity of tumours to DNA-damage inducing therapeutic anti-cancer drugs, such as CDDP. An inhibitor designed to block LMO3 activity may be useful in treatment of aggressive neuroblastoma by activation of the p53 pathway.

B39 Using Whole Genome Based Chromatin Immunoprecipitation (ChIP) to Analyze MYC(N) Functions in Neuroblastoma

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Background: Deregulated MYCN activity is a characteristic feature of malignant progression in a subset of neuroblastomas. The molecular mechanisms by which deregulated MYCN mediates the aggressive phenotype are still poorly understood. Aim of this study is to understand the epigenetic control of MYCN activity in neuroblastomas.

Methods: Chromatin immunoprecipitation (ChIP), a powerful tool to study DNA/protein interactions, was used to analyze MYCN binding to its target genes. Robust criteria for ChIP assay were established by using a set of known MYCN target genes. We adapted the ChIP protocol for large-scale analysis ($ChIP_{chip}$, Agilent 244K microarrays) by using whole genome amplification (WGA) of precipitated DNA. To assess the influence of epigenetic markers on MYCN target gene activation we applied $ChIP_{chip}$ in different *MYCN* amplified and single-copy neuroblastoma cell lines using MYCN/c-MYC antibodies as well as antibodies recognizing histone modifications characteristic for gene activation (H3K4me3), elongation (H3K36me3) and heterochromatin (H3K27me3).

Results: A set of 147 known and putative new MYCN target genes was identified by database and literature search. Microarray and QPCR analysis confirmed differential expression of these genes in different neuroblastoma subtypes and cell lines. $ChIP_{PCR}$ and $ChIP_{chip}$ experiments in WAC2 and IMR5/75 cells verified MYCN binding to 139 of the 147 predicted target genes (95%). For MYCN induced genes, MYCN binding positively correlated with H3K4me3 and H3K36me3, whereas it negatively correlated with H3K27me3 and *vice versa* for MYCN repressed genes.

Conclusions: We successfully used $ChIP_{chip}$ technique in neuroblastoma cell lines to identify and validate new MYCN target genes. The combined ChIP analysis of histone modifications effectively identified DNA binding sites in gene promoters that are activated or repressed by MYCN. We are currently adapting the $ChIP_{chip}$ protocol for the use in primary tumors to identify clinically relevant DNA binding sites of MYCN/c-MYC.

B40 NGFR/$p75^{NTR}$ is Activated through TAp73 during Retinoic Acid-Induced Differentiation of Neuroblastoma Cells

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Background: The *TP73* gene, an homologue of the *TP53* tumor-suppressor gene, maps at 1p36.3, a locus frequently deleted in neuroblastoma (NB). It encodes two major isoforms that differ by the presence (TAp73) or absence (ΔNp73) of the N-terminal transactivating domain. Analyses of knock-out mice have implicated p73 in neurogenesis. However, the downstream signaling pathways related to neuronal differentiation remain elusive. In this regard, we searched for p73 target genes and investigated p73 involvement in retinoic acid (RA)-induced differentiation of NB cells.

Methods: NB cells were infected with adenoviral vectors, or transfected with plasmids or siRNA to ectopically express or inactivate p73. Gene expression profiling was performed on human cancer 1.2 arrays (*Clontech*). The regulation of genes of interest was confirmed by RT-PCR and/or western blot. *NGFR/$p75^{NTR}$* and *$p21^{CDKN1A}$* promoter activity was assessed using Luciferase reporter constructs. *p73* promoter binding was evaluated by chromatin immunoprecipitation-PCR using an anti-p73 antibody (Calbiochem). Neuronal differentiation was monitored by cytomorphology and neurofilaments proteins expression.

Results: Ectopic expression of p73α isoforms led to up-regulation of genes primarily associated with development and neuronal function such as *GDF15/MIC1* (differentiation factor) and *NGFR/$p75^{NTR}$* (neurotrophin receptor). TAp73α and ΔNp73α both activated the *NGFR/$p75^{NTR}$* promoter and endogenous p73 bound this promoter, as evidenced by ChIP, indicating a direct transcriptional regulation. RA treatment of p73-expressing NB cells occasioned an early activation of *NGFR/$p75^{NTR}$* and *$p21^{CDKN1A}$* promoters and transient NGFR/$p75^{NTR}$ protein up-regulation followed by a rapid decrease after 16 hours. A progressive decrease was observed for the p73 protein after 24 hours. Decreased RA-triggered neurofilaments proteins accumulation upon TAp73 siRNA transfection further suggested the implication of the p73-NGFR/$p75^{NTR}$ pathway in the phenotypic response to retinoids.

Conclusions: The role of p73 in RA-induced NGFR/$p75^{NTR}$ and neurofilaments up-regulation suggests that altered sympathetic neuronal differentiation through a dysfunction of this cascade contributes to NB tumor induction.

B41 Gene-Expression Based Classification Predicts the Biology of Metastasized Neuroblastoma under 2 Years of Age Better than Current Clinical Risk Stratification

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Background: The different clinical courses and prognosis of patients with metastasized neuroblastoma (stage 4 and 4S) below 24 months of age are currently estimated by the factors age, stage, and MYCN amplification. The purpose of this study was to investigate whether genome-wide expression profiles would provide a more biological and better basis for risk estimation.

Methods: Gene-expression profiles from fresh frozen tumor samples of 81 patients with metastatic neuroblastoma under 2 years of age (52x stage 4S and 29x stage 4) were performed using a customized 11K (10.263 probes) neuroblastoma microarray. Unsupervised hierarchical cluster analyses and prinicipal component analyses were performed to identify subgroups of the disease. Subsequently, supervised classification was performd utilizing a previously reported 144-gene predictor based on the *prediction analysis of microrarrays* (PAM) algorithm (JCO 24:5070-8;2006).

Results: Although unsupervised analyses of the 81 tumors were able to separate divergent subgoups of the disease, no strict discrimination of stage 4 and stage 4S patients was observed. Gene-expression based classification using the 144-gene classifier predicted a favorable group of patients (n=44) with a 3-year EFS of 0.85±6 and a 3year OS of 0.97±3. In contrast, the predicted unfavorable group (n=37) had a 3-year EFS of 0.42±10 and a 3-year OS of 0.70±9. Eleven out of 12 patients who died of tumor progression demonstrated an unfavorable gene signature. Furthermore, of 18 infants with stage 4S who received an unfavorable PAM prediction, five had MYCN-amplification and four others had progressed to stage 4. In contrast, 10 patients with stage 4 disease who were classified as favorable had an excellent outcome and none had MYCN amplification.

Conclusions: Gene-expression based classification of neuroblastoma patients with metastatic disease under 2 years of age using the 144-gene PAM classifier may represent a new biological basis for an improved risk estimation.

Selected Poster Session: Clinical Study

B42 Gene Expression Profiles Distinguish Two Prognostically Different Subgroups of Neuroblastomas with Loss of Chromosome 11q

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Background: Loss of chromosomal material at 11q is frequently observed in neuroblastoma with poor outcome. It has therefore been suggested that the inactivation of hitherto unknown tumor suppressor gene(s) by 11q deletions characterize a distinct clinico-genetic neuroblastoma subtype. To evaluate this hypothesis, the effect of 11q aberrations on gene expression patterns of subgroups with differing outcome was investigated.

Methods: The impact of our previously described 144-gene expression classifier on EFS and OS was investigated in 41 neuroblastoma patients with 11q alterations. In addition, global gene expression patterns were compared using unsupervised (PCA and hierarchical clustering) and supervised analyses (ANOVA, SAM and analysis of centroid distances) in 110 tumors with normal *MYCN*/1p status separated into four subgroups: (A) favorable 11q normal (no chemotherapy, no event, n=81); (B) unfavorable 11q normal (event despite chemotherapy, n=8); (C) favorable 11q aberrant (n=7); (D) unfavorable 11q aberrant (n=14).

Results: The gene expression classifier separated patients with 11q aberrations into two subgroups with significantly differing EFS ($p<0.01$) and OS ($p=0.02$). Comparison of global gene expression patterns using unsupervised analyses and analysis of centroid distances revealed that unfavorable tumors with 11q aberrations (D) distinctly deviate from both favorable tumors with 11q aberrations (C) and unfavorable tumors without aberrations (B). In contrast, favorable tumours with (C) and without (A) 11q loss were not significantly different. In line with this, 282 and 227 genes were found to be differentially expressed between favorable (C) and unfavorable (D) tumours with 11q aberrations using SAM and ANOVA, respectively, whereas only 2 and 3 genes, respectively, were differentially expressed between favorable tumors with (C) and without (A) 11q aberrations.

Conclusions: Gene expression patterns can distinguish two prognostically different subgroups of neuroblastoma with loss of 11q. Irrespective of the 11q status, neuroblastoma with favorable outcome appear to represent a homogeneous molecular subgroup.

C19 The Treatment of Children over the Age of One Year with Unresectable Localised Neuroblastoma without MYCN Amplification: a SIOPEN Study

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Background: Treatment for children over the age of one year with unresectable, localised neuroblastoma without MYCN amplification varied in Europe in the 1990s, with different intensity and duration of chemotherapy, and sometimes the addition of radiotherapy. This study recommended discontinuing treatment after surgery and planned chemotherapy irrespective of the completeness of surgical excision.

Methods: Following biopsy, six courses of chemotherapy were given, four before and two after surgical resection of the tumour. Courses 1, 3 and 5 comprised carboplatin 200mgs/m^2 and etoposide 150mgs/m^2 each daily for three days. Course 2, 4 and 6 comprised cyclophosphamide 300mgs/m^2 on days 1-5, adriamycin 30mgs/m^2 on days 4 and 5, and vincristine 1.5mgs/m^2 on days 1 and 5.

Results: Between April 2000 and February 2007 one hundred and sixty-five patients were recruited to this study from ten participating European countries. The treatment was well tolerated with only one toxic death (surgical). There are 24 reported relapses at a median time of 15 months (1-40), 17 of them being loco-regional, 5 distant, and 2 combined. Distant metastases occurred in the bone marrow (n=2), lungs (n=2), lymph nodes (n=2), liver, and skeleton (1 each). In one case two different sites were involved. Ten of these patients have subsequently died of disease. Currently the OS for the whole group is 94.2% and EFS 76.1% at 3 years. EFS was significantly better in children less than 24 months than in older children (95% and 60% respectively). Favourable histology (INPC) on central review carries a significantly superior survival to unfavourable histology.

Conclusions: EFS and OS for children aged over 24 months is unsatisfactory, especially for those with poorly or undifferentiated histology. Children identified as low risk could receive a treatment reduction in future studies.

C20 Long Term Outcome and Late Effects after Successful Treatment of Stage 4 Neuroblastoma

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Background: Intensive treatment increased the number of survivors in stage 4 neuroblastoma. Pattern and incidence of long-term toxicities are unkown. We analyzed our data base in order to learn about the frequency of late effects among long term neuroblastoma survivors.

Methods: Data on survival and late effects were prospectively collected during the routine survey of the subsequent neuroblastoma trials every year. Stage 4 neuroblastoma patients of the trials NB79-NB97 diagnosed between 02/1979 and 09/2004 were included, if they survived three years or more from diagnosis.

Results: The three-year-survival increased from 13+/-4% to 60+/-2% between NB79 and NB97. 476 neuroblastoma survivors >3 years were identified. One patient died in complete continuing remission after 3 years. The age at diagnosis was 2.7 years (0.0-25.5). By December 2007, 334/476 patients are still alive with a median observation time of 9.3 years (3.0-24.4). The percentage of patients without reported late effect was 7/12 (58%) in NB79, 22/28 (79%) in NB82, 23/44 (52%) in NB85, 47/175 (27%) in NB90, and 58/217 (27%) in NB97. The total frequency of late effects is shown in the table:

Late effect	n	%
Hearing impairment (with or without hearing aids)	211	44%
Renal impairment	106	22%
Endocrine abnormalities	85	18%
Liver abnormalities incl. focal nodular hepathopathy	30	6%
Visual impairment	17	4%
Developmental delay	14	3%
Scoliosis	14	3%
Horner Bernard syndrome	11	2%
Persistent bone marrow depression	10	2%
Residual transverse myelopathy	10	2%
Cardiomyopathy	8	2%
Motor deficits	7	1%
Peripherial neuropathy	6	1%
Unilateral sympathetic deficit	4	1%
Hypertension	3	1%
Other	62	13%
No late effect reported	157	33%
Total patient number	476	100%

Conclusions: Late effects affect the majority of neuroblastoma survivors. Further treatment protocols need to consider potential toxicities in an effort to cure children from cancer without inducing severe secondary diseases.

C21 A Large-Scale Analysis of *TRK-A* Expression and Neurotrophin Responsiveness in Primary Neuroblastomas

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Background: Neurotrophin receptor genes *TRK-A* and *TRK-B* are well-known prognostic indicators of neuroblastoma expressed preferentially in favorable and unfavorable tumors, respectively. We have been analyzing prognostic factors including *TRK-A* expression in more than 2,000 neuroblastomas found in Japan for 12 years. After excluding mass screening-detected cases, 373 neuroblastomas registered during 1996-2002 were analyzed in this study, to clarify prognostic value of *TRK-A*. Moreover, we performed a large series of primary culture including 195 neuroblastomas with neurotrophins NGF and BDNF, ligands for Trk-A and Trk-B, respectively, and analyzed the effect of responsiveness to them on survival.

Methods: *MYCN* amplification, *TRK-A* expression and DNA ploidy were analyzed by Southern blot, Northern blot and Flow cytometry, respectively. In primary culture study, cells were treated with or without 100 ng/ml of NGF or BDNF.

Results: High expression of *TRK-A* was a good indicator of favorable neuroblastoma with statistical significance. Moreover, in infantile-onset and Shimada-favorable groups which usually have better prognosis, the subgroups with high *TRK-A* expression showed significantly better 5y-EFS than those with low *TRK-A* expression. Oppositely, in DNA diploidy/tetraploidy and INSS stage 3+4 groups that are considered poor prognostic groups, high *TRK-A* subgroups showed statistically better 5y-EFS than low *TRK-A* subgroups. In addition, among 67 *MYCN*-amplified neuroblastomas, only 4 cases highly expressed *TRK-A* and showed 100% of 4y-EFS though the difference was not statistically significant. Primary culture study showed that positive responsivenesses to NGF and to BDNF were closely associated with favorable and unfavorable outcome, respectively ($p<0.0001$ and $p=0.036$), and that BDNF-responsiveness had no significant relationship to the currently known prognostic factors.

Conclusion: The prognostic significance of responsiveness to NGF and BDNF in primary neuroblastoma cells was clearly shown. Activation of NGF/Trk-A as well as inhibition of BDNF/Trk-B pathway might provide a novel therapeutic strategy to treat aggressive neuroblastomas.

C22 Natural Course of Neuroblastoma Detected by Mass Screening: A 10 Years Prospective Study of Observation Program at A Single Institution

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Background: A national-wide mass screening of neuroblastoma (NBL) for 6-month-old infants (MS6M) was started in Japan in 1985. Because favorable biological behaviors of most of the detected tumors were clarified, we started observation program for selected screened cases in 1993. Receiving the report of the study committee of the Ministry of Health, Labor and Welfare in 2003, the MS6M intermitted in most areas of Japan. We will report the clinicopathological findings and the present statue of the cases enrolled in our observation program for evaluation of the program.

Methods: Between 1993 and 2003, 53 of 154 patients with NBL detected by MS6M were enrolled in our program. The criteria for observation included urinary VMA and HVA levels, tumor size, presumed lower stage, and granted informed consent. The patients were divided into four groups according to urinary VMA and HVA levels and tumor size. Surgical removal of tumor was performed in cases which have became inconsistent with the criteria.

Results: Tumor markers level decreased in 39 of 53 cases. In 16 of the 39 cases, the tumor became undetectable by imaging techniques (group A), and in 23 cases, the tumor has been detectable (group B). In eight cases, tumor marker levels varied, and tumor volume gradually increased (group C). In six cases, tumor marker levels and tumor volume increased in short term (group D). All the tumors in the group C and D, and four tumors in the group B were removed. No unfavorable biologic factor was noted in any excised tumor.

Conclusions: This study is one of the largest series of observation program of MS6M, and confirmed more than half of the cases within the criteria could be observed without operation. Hereafter, the observation program may be applicable for incidentally found NBL or NBL in patients with other serious diseases.

C23 Efficacy of Double High-Dose Chemotherapy and Autologous Stem Cell Rescue in Patients over 1 Year of Age with Stage 4 Neuroblastoma: The Korean Society of Pediatric Hematology-Oncology (KSPHO) 6 Year Experience (2000-2005)

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Background: Although the strategy using high-dose chemotherapy and autologous stem cell rescue (HDCT/ASCR) has improved the survival of patients with high-risk neuroblastoma, the survival rate after single HDCT/ASCR has been unsatisfactory. Recently, investigators have examined the efficacy of sequential HDCT/ASCR to further improve the outcome of high-risk neuroblastoma patients. In the present study, the efficacy and toxicity of double HDCT/ASCR were investigated in patients over 1 year of age with stage 4 neuroblastoma.

Methods: Patients over 1 year of age who were newly diagnosed with stage 4 neuroblastoma from January 2000 to December 2005 were enrolled in the KSPHO registry. All patients who were assigned to receive HDCT/ASCR were analyzed to investigate the efficacy and toxicity of double compared to single HDCT/ASCR.

Results: Among 161 patients who were enrolled from 24 hospitals, 141 patients were assigned to receive single or double HDCT/ASCR at diagnosis (single in 70 patients and double in 71). Fifty-seven of 70 patients in the single HDCT group and 58 of 71 patients in the double HDCT group underwent single or double HDCT/ASCR as scheduled. Twenty-five patients in the single HDCT group and 39 in the double HDCT group remained event free with a median follow-up of 56 (24-89) months. Treatment-related death occurred in nine (12.9%) patients in the single HDCT group and seven (9.9%) in the double HDCT group. The probability of 5-year EFS ± 95% confidence interval was higher in the double HDCT group than that in the single HDCT group (51.3% ± 12.5% versus 31.8% ± 11.6%, $P = 0.034$).

Conclusions: The present study demonstrated that double HDCT/ASCR strategy might be better than single HDCT/ASCR strategy in terms of survival in the treatment of patients over 1 year of age with stage 4 neuroblastoma.

Plenary Session: Translational Research 1

TR1 Targeting Neuroblastoma Tumor-Initiating Cells: High-Throughput Screening Strategies to Identify Novel Chemotherapeutics

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Background: Neuroblastoma (NB) is the most common and deadly extra-cranial pediatric solid tumor. The poor prognosis as well as the significant treatment side effects led us to investigate novel NB therapies. Here, we describe the identification of compounds specifically cytotoxic against NB tumor-initiating cells (TICs) from but not normal pediatric stem cells (SKin-derived Precusors, SKPs). We recently isolated a highly enriched population of NB TICs from tumors and bone marrow metastases (Cancer Research 2007) and hypothesize that TICs are related to SKPs. Both originate from the neural crest, express similar neural crest markers, and differentiate *in vitro* into similar cell types. The availability of two neural crest stem cell sources, TICs from the NB tumor and SKPs from human pediatric skin, provides a unique opportunity for therapeutic target discovery.

Results: We established a high-throughput screen in which NB TICs and SKPs were tested against four bioactive compound libraries. Following confirmatory screens, we identified 47 compounds that selectively targeted NB TICs. These included DNA-damaging agents, Na^+/K^+ ATPase inhibitors, and K^+ channel blockers as well as specific antagonists to PKC, NF-κB, and the nicotinic acetylcholine receptor. Identified compounds were cytotoxic against TICs from multiple NB patients while having little effect on normal stem cells in secondary *in vitro* assays. Additionally, identified compounds were more effective against NB TICs than established NB cell lines. In preliminary *in vivo* trials, identified compounds significantly reduced NB xenograft tumor volume when compared to standard therapies. One compound, rapamycin, has been administered to patients on a compassionate basis. Several compounds were also cytotoxic towards breast cancer, human glioblastoma, or human leukemia cancer stem cells, suggesting that this approach may identify drugs with activity against multiple cancer stem cell populations.

Conclusions: These results support the robustness of our assay system and suggest that patient-specific therapeutics as well as novel effectors of neuroblastoma, including drugs that are cytotoxic and that induce differentiation, can be identified using this strategy.

TR2 ODC1 Overexpression is Associated with Poor Outcome in Childhood Neuroblastoma and Represents an Important Therapeutic Target

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Background: The mechanism by which *MYCN* oncogene amplification influences the prognosis of neuroblastoma has not been well defined. Available evidence indicates that ornithine decarboxylase (ODC1) is a critical downstream target for *MYCN*. ODC1 is the rate-limiting enzyme in the synthesis of polyamines and a number of studies have provided evidence for the oncogenic and transforming abilities of ODC1.

Methods: We used Real-Time PCR to analyze *ODC1* expression in a large cohort (n=209) of primary untreated neuroblastomas from patients enrolled on POG biology protocol 9047. To examine ODC1 inhibition as a possible therapy for neuroblastoma, *MYCN* transgenic mice that spontaneously develop neuroblastoma closely mimicking the human disease, were treated with the ODC1 inhibitor, α-difluo romethylornithine (DFMO), either alone or in combination with cytotoxic drugs.

Results: Older age, advanced stage, and *MYCN* amplification were all predictive of poor outcome in this cohort. *ODC1* expression was significantly higher in *MYCN*-amplified tumors by comparison with non-amplified tumors ($p<0.0001$). Kaplan-Meier survival analysis indicated that high levels of *ODC1* expression were strongly predictive of both event-free-survival ($p<0.001$) and overall survival ($p<0.001$) in this cohort as well as in patients whose tumors lacked *MYCN* amplification ($p=0.01$). Furthermore, a functionally important polymorphism within the *ODC1* promoter (G316A), was found to be significantly associated with outcome in patients whose tumors lacked *MYCN* amplification ($p<0.05$). Importantly, DFMO therapy, either concomitant with or following cisplatin treatment, resulted in prolonged tumor-free survival ($p<0.01$) in MYCN transgenic mice, by comparison with cisplatin administered alone. Similar results were obtained with cyclophosphamide treatment. No overt toxicity attributable to DFMO therapy was noted in treated mice.

Conclusions: Collectively, these findings provide strong evidence that ODC1 contributes to the malignant phenotype of neuroblastoma and suggest that targeting this oncogene for suppression of polyamine synthesis is potentially a valuable therapeutic approach for inhibiting neuroblastoma growth.

TR3 Activation of BMP4 Signalling via Inhibition of HDAC11 Represses Neuroblastoma Cell Tumorigenicity both *In Vitro* and *In Vivo*

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Background: The survival rate of children with high-risk neuroblastoma (NB) is dismal despite intensive multimodal therapy. The limited efficacy and the frequent and serious side effects of currently used therapeutic regimens necessitate the development of new, less toxic treatment strategies. Many different histone deacetylase inhibitors (HDACIs) cause differentiation of NB cells in both *in vitro* and *in vivo* preclinical models. However, little is known about the underlying molecular events.

Methods and Results: Using whole genome expression analysis, this study identified bone morphogenetic protein 4 (*BMP4*) as the earliest and strongest regulated gene by both the cyclic tetrapeptide *Helminthosporium carbonum* (HC)-toxin and the carboxylate valproic acid (VPA). Chromatin immunoprecipitation revealed that both HDACIs induce an accumulation of acetylated histone H4 associated with the *BMP4* promotor. siRNA-mediated knock-down of the 11 HDACs belonging to classes I, II and IV showed that inhibition of HDAC11 activity mediates the induction of *BMP4*. The BMP4 antagonist gremlin blocked the HC-toxin induced G0/G1-cell cycle arrest indicating that the secreted protein BMP4 contributes to the cell cycle changes observed. Transient *in vitro* exposure of NB cells to BMP4 activated the SMAD signalling cascade followed by a reduction in both proliferation and colony formation. *In vivo* administration of BMP4 diminished NB formation and growth in mice.

Conclusions: Activation of BMP4 signalling via inhibition of HDAC11 represses neuroblastoma cell tumorigenicity both *in vitro* and *in vivo*. The data highlight both HDAC11 as relevant target for HDACI-mediated therapeutic intervention and the administration of BMP4 as novel strategy against high-risk NB.

TR4 Therapy of Metastases of Drug-Resistant Neuroblastoma Cells in NOD/SCID Mice with Human Natural Killer Cells and Anti-GD2 Antibody Combined with Cyclophosphamide, Zoledronic Acid, and Bevacizumab

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Background: Neuroblastomas that progress are clinically drug-resistant. Activated natural killer (aNK) cells ± anti-tumor antibodies (mAb) can kill drug-resistant cells *in vitro*. However, the efficacy of aNK cells ± mAb against metastases formed by drug-resistant cells *in vivo* is unknown. We evaluated 1) aNK + anti-GD2 mAb ch14.18 therapy against metastases in NOD/SCID mice beginning 7 or 21 days after tumor cell injection (non-detectible and detectible disease with bioluminescent imaging); and 2) aNK + mAb therapy beginning at 21 days combined with agents directed at tumor cells and the microenvironment.

Methods: aNK were generated by culturing NK cells with IL-2 + IL-15. Intravenous injection of luciferase transfected CHLA-255 or CHLA-136 neuroblastoma cells into NOD/SCID mice provided models of drug-resistant metastases. Endpoints were response and tumor growth (defined by bioluminescent imaging) and survival.

Results: When weekly therapy began 7 days after CHLA-255-luc injection, aNK alone had little effect whereas aNK + mAb cured 70% of mice ($P<0.001$). However, weekly aNK + mAb treatment of both CHLA-255-luc and CHLA-136-luc, when begun at day 21, only modestly inhibited growth and prolonged survival. In contrast, aNK + mAb activity was dramatically increased in this setting if mice also received bolus (every 3 weeks) cyclophosphamide and weekly zoledronic acid and bevacizumab. The latter regimen, which was more effective than immunotherapy or chemotherapy alone, caused complete responses at days 28 and 35 ($P<0.001$), slowed growth ($P<0.001$), and prolonged survival ($P<0.001$).

Conclusions: aNK + mAb is active against metastases formed by drug-resistant neuroblastoma cells *in vivo*. Adoptive transfer of aNK cells + mAb can cure mice with minimal disease (day 7) and can modestly prolong survival and decrease growth of established metastases (day 21). aNK + mAb therapy of established metastases is dramatically improved by adding bolus and metronomic cyclophosphamide, zoledronic acid, and bevacizumab.

Plenary Session: Translational Research 2

TR5 Neuropeptide Y and its Y2 Receptors – Potential Targets in Neuroblastoma Therapy

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Neuropeptide Y (NPY) is a sympathetic neurotransmitter, acting via multiple receptors (Y1-Y5R). It has been shown to induce angiogenesis via its Y2Rs. Neuroblastomas release NPY, which results in its elevated plasma levels in patients with advanced disease and a poor clinical outcome. Previously, we have shown that NPY is an endogenous growth factor for neuroblastoma, which stimulates tumor cell proliferation and tumor vascularization. Since both processes are Y2R-mediated, the goal of this study was to assess Y2R as a potential target in neuroblastoma therapy.
In vitro, Y2R antagonist (BIIE0246) reduced growth of neuroblastoma cells, which was associated with decrease in proliferation (BrdU uptake), increase in apoptosis (caspase activity) and blockage of NPY-induced ERK1/2 activation. Consequently, NPY and Y2R siRNAs reduced growth of SK-N-BE(2) cells, confirming that NPY, acting via its Y2Rs, is an autocrine growth factor for neuroblastoma cells. Moreover, the proliferative effect of neuroblastoma conditioned media on endothelial cells was significantly reduced by BIIE0246, indicating that NPY-induced vascularization of neuroblastoma tumors is Y2R-dependent. In vivo, the effect of BIIE0246 on growth of SK-N-BE(2) subcutaneous xenografts was tested in two experimental models – preventive (treatment started two days after cell inoculation) and therapeutic (the treatment initiated two weeks later, in the exponential phase of tumor growth). The antagonist significantly inhibited tumor growth in both experiments, although its effect was more pronounced in the therapeutic model. Analysis of xenograft tissues revealed decrease in proliferation (Ki67) and increase in apoptosis (TUNEL), as well as decrease in vascularization (CD31) and high degree of focal fibrosis/hyalinzation in BIIE0246-treated tumors. The expression of Y2R (IHC) was observed in both tumor and endothelial cells, similarly to the Y2R staining in human tumors. In summary, Y2R is a promising target for neuroblastoma therapy, however effectiveness of Y2R antagonists in treatment of the metastatic disease needs to be determined.

TR6 Genomic Aberration Patterns Predict Relapse in Low-And Intermediate-Risk Neuroblastoma (NB): A Children's Oncology Group (COG) Study

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Background: NB is a childhood malignancy with a heterogeneous clinical course. Sensitive and specific biomarkers of poor outcome for low- and intermediate-risk NB patients do not exist.

Methods: We identified all disease progressions/relapses/disease-deaths (events) from recently closed COG low- (P9641: 903 eligible, 63 events) and intermediate-risk (A3961: 467 eligible, 40 events) NB Phase 3 trials. Tumor DNA from 60 cases was available for whole genome copy number and genotype evaluation at 550K SNPs. Controls were selected from the same studies (93 patients without an event after 3 years). An algorithm was developed to assign genomic copy number and allelic ratio after correcting the SNP array data for sample aneuploidy. Chi-square tests were used to generate nominal p-values for comparisons.

Results: Unsupervised hierarchical clustering of segmental and whole chromosome copy number aberrations (CNAs) identified 3 tumor subsets, 2 highly enriched with cases showing events. Although the proportion of hyperdiploid samples was similar in both groups, specific whole chromosome gains (chromosomes 1, 2, 5, 7, 12, 17, and 22) occurred more frequently in controls (p=0.003-0.043). Segmental gain of 17q was more common in cases (40.0% vs. 18.3%, p=0.003) even though overall 17q gain (including whole chromosome gains) was similarly present in both cohorts (63.3% cases vs. 75.3% controls, p=0.114). Regional aberrations most highly associated with events included loss of 11q (p<0.001), loss of 22q (p=0.002), gain of 17q (p=0.003), and gain of 12q (p=0.020).

Conclusions: Whole genome SNP genotyping detects patterns of CNAs predictive of event occurrence, even in situations where events are rare such as low- and intermediate-risk NB. These data support segmental CNAs as being most highly predictive of events in otherwise favorable NBs, and that specific whole chromosome gains are associated with fewer events. This can be used to identify patients eligible for chemotherapy reduction and patients requiring intensification.

TR7 The Overall Genomic Pattern is a Predictor of Outcome in Neuroblastoma

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Background: Various genetic alterations bring prognostic information in neuroblastoma (NB), a frequent and severe paediatric cancer. For a comprehensive overview of these alterations, their association and clinical significance, we have conducted a whole-genome DNA copy number analysis.

Methods: A series of 493 NB samples was investigated by array-based comparative genomic hybridization in two consecutive steps. Ploidy data were also integrated.

Results: Genomic analysis identified several types of profiles. Near-triploid tumours presenting exclusively whole chromosome copy number variations were associated with an excellent survival. No disease related death was observed in this group. In contrast, tumours with segmental chromosome abnormalities, whatever the individual alterations and the ploidy context, characterized patients with a high risk of relapse. For tumours with a combination of numerical and segmental abnormalities, the significance of the latter was clearly dominant with respect to prognosis. In a multivariate analysis, taking into account the genomic profile, but also previously described individual genetic and clinical markers with prognostic significance, the presence of segmental alterations was the strongest predictor of relapse (p<0.001), the other significant variables being age (p=0.004), stage 4 (p=0.005) and *MYCN* amplification (p=0.02). Finally, within tumours showing segmental abnormalities, stage 4, age, *MYCN* amplification, 1p and 11q deletions and 1q gain, were independent predictors of a decreased overall survival.

Conclusions: The overall genomic pattern, which probably unravels particular genomic instability mechanisms, rather than the analysis of individual markers, is essential to predict relapse in NB patients and adds important prognostic information to conventional indicators.

TR8 Segmental Chromosomal Alterations Have Prognostic Impact In Neuroblastoma: A Report From The INRG Project

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Background: MYCN amplification (MNA) is the strongest genetic prognostic marker in neuroblastoma. The prognostic impact of other genetic alterations in tumors without MNA is less well established. The hypothesis that any segmental alteration might be of prognostic impact in tumors without MNA was tested in the INRG dataset.

Methods: Genetic and clinical data were retrieved from the INRG dataset. The presence of any of the three segmental chromosomal alterations in a tumor (chromosome 1p deletion and/or 11q deletion and/or 17q gain) was taken into account to define a "segmental" genomic type. Tumors with unaltered status of all three chromosome arms were classified as having no segmental changes.

Results: Within the 8800 patient cohort, 5947 lacked MNA, and of these, 505 could be attributed to a genomic type: 397 cases had a "segmental" genomic type, whereas an absence of the three segmental alterations was demonstrated in 108 cases. In this patient cohort (n=505), a "segmental" genomic type was more frequent in patients >18 months at diagnosis and in stage 4 disease ($p<0.0001$). In univariate analysis, chromosome 1p deletion, 11q deletion and 17q gain were all associated with a poorer event-free survival (EFS) ($p=0.03$, $p<0.0001$ and $p=0.0001$, respectively), as was diploidy ($p=0.02$). In multivariate analysis "age", "stage", and a "segmental" genomic type retained prognostic significance, whereas the individual genetic markers did not ($p<0.0001$ and RR=2.56; $p=0.0002$ and RR=1.8; $p=0.01$ and RR=1.7, respectively). In particular, a "segmental" genomic type was associated with a significantly poorer EFS in those 86 patients >18 months with non-stage 4 disease ($p<0.02$).

Conclusion: In neuroblastoma patients without MNA, a genomic profile characterized by any segmental alteration adds prognostic information to the clinical markers "age" and "stage", suggesting that a panel of genetic markers will be useful for risk assignment and treatment stratification of neuroblastoma patients without MNA.

TR9 Comparison of Early Passage Neuroblastoma Cell Lines to Matched Primary Tumors Using SNP Arrays

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Background: Stromal contamination is a major confounding factor in the analysis of primary solid tumor samples by single nucleotide polymorphism (SNP) arrays. As we propose to employ genome-wide SNP array analysis as a diagnostic platform for neuroblastoma, the sensitivity, specificity, and accuracy of these studies must be optimized.

Methods: In order to minimize the effect of stroma, we derived early passage cell lines from ten primary tumors, and compared their genomic signatures with those of the matched primary tumors and normal blood samples by 100K SNP array analysis.

Results: For raw LOH (loss of heterozygosity) calls, the average concordance between tumor and cell line was 96% (range 91%-99%). The average agreement for raw copy number alterations (CNA) was 71% (range 43%-87%). In general, there were more LOH events in derived cell lines compared to tumor samples (mean increase 2.5%±1.8%). We have developed an algorithm to show that presence of stroma contributes to under-reporting of LOH and CNAs. Notable findings in this small sample set were uniparental disomy of chromosome 11p as well as on three novel regions on chromosomes 1q, 14q, and 15q. Most samples had chromosome 17q gain; in one, gain was accompanied by LOH, which was verified in the derived cell line. Chromosome 10q LOH was seen in 3 samples, all with LOH of chromosome 1p, *MYCN* amplification, and 17q gain. A novel area of amplification on chromosome 11p15 was also noted in both the tumor and cell line.

Conclusions: Our analysis demonstrates that there were significantly more LOH and CNA events in derived cell lines compared to the original tumor samples. While these may in part be due to clonal selection or acquired mutations, we believe that contamination by normal stromal elements may be a major contributing factor in underestimation of genetic events.

TR10 Prediction of Outcome for Children with Metastatic MYCN Non-Amplified Neuroblastoma with a Multi-Gene TaqMan Low Density Array

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Background: A clinically applicable assay that predicts progression-free survival (PFS) for patients with stage 4 MYCN non-amplified (MYCN-NA) neuroblastoma diagnosed >18 months of age is not available. We developed a prediction model for these patients based upon microarray gene expression profiles of their tumors (JNCI 98:1193-1203, 2006). Quantifying gene expression with a multi-gene TaqMan Low Density Array (TLDA) may provide a clinically applicable assay for predicting PFS.

Methods: Stage 4 MYCN-NA neuroblastomas from patients in Children's Cancer Group (training set, n=129) and German Cooperative Group (test set, n=51) trials were tested. Expression of 44 genes (32 from the microarray signature and 12 related to inflammation) was determined with a TLDA assay. After normalizing expression of the 44 genes to housekeeping genes, a diagonal linear discriminant analysis (DLDA) model was developed on the training set and optimized to predict failure for patients >18 months. Leave-one-out cross validation (LOOCV) was applied to the training set, and the classifier was applied to the test set to estimate classification error.

Results: Area under the curve (AUC) values of the ROC from LOOCV for training and test sets were 78% and 80% for all ages and 59% and 75% for >18 months. Based on ROC analysis of training data of the >18 months group, the final DLDA model yielded two risk groups. In the training data, PFS was 58%±5.3% and 19% ±6.1% for entire group and 34%±6.5% and 15%±5.8% for the >18 months group. In the test data, PFS was 59%±9.2% and 12%±11% for the entire group and 42%±11% and 14%±13% for the >18 months group.

Conclusion: These data support using the TLDA platform for a clinically applicable assay to identify patients with ultra-high risk tumors lacking MYCN amplification.

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B23 Expression Profiling of a New Class of Non-Coding RNAs in Human Neuroblastoma

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Background: Small non-coding RNAs are emerging as key players in a number of different pathways involved in development and cancer. Among those small RNAs, microRNAs (miRNA) are emerging as important players in oncogenesis. miRNA expression profiling discriminates tumour subentities and they have been shown to act either as oncogenes or tumour-suppressor genes. Recently, the expression of a new class of non-coding RNAs (ncRNAs) has been linked to cancer. These RNAs, termed transcribed ultraconserved regions (T-UCR), are encoded by DNA sequences of at least 200 bp long showing 100% sequence homology between human, mouse and rat. Like miRNAs, these T-UCRs are frequently located at cancer associated genomic regions, can act as oncogenes and have distinct signatures in human leukaemia's and carcinomas.

Methods: To evaluate T-UCR gene expression in neuroblastoma (NB), we designed, validated and applied real-time quantitative PCR assays enabling expression profiling of all 482 T-UCRs in two 384-well plates for 51 NB tumour samples.

Results: T-UCR gene expression signatures of normal neuroblast precursor cells, a carefully selected cohort of representative NB tumour samples and NB cell lines revealed differentially expressed T-UCR genes (1) between tumour and precursor cells, (2) between different prognostic and genetic NB subgroups and (3) between MYCN amplified and MYCN single copy cells. Possible tumour-suppressor or oncogenic properties of the differentially expressed T-UCRs are currently being evaluated.

Conclusions: T-UCR genes have distinct expression signatures in NB tumour subgroups. Therefore, the role of ncRNAs in NB tumorigenesis might be greater then previously expected. To further explore the T-UCR profiles, integrated analysis with available mRNA and miRNA signatures and gene copy number profiles of our tumour cohort will be performed and presented.

B24 ncRAN, a Novel Non-Coding RNA Mapped to Chromosome 17q25.1, Promotes Cell Proliferation and Transformation and Its Expression Predicts Poor Ontcome in Neuroblatoma

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Background: An abundant class of non-coding RNA including microRNAs, have been shown to repress the expression of important cancer-related genes and might provide useful clues in the diagnosis and treatment of cancer. The object of this study was to investigate the functional role of a novel large non-coding RNA termed as *ncRAN* (non-coding RNA expressed in aggressive neuroblastoma) mapped in the region of chromosome 17q gain that was identified from our Neuroblastoma (NB) cDNA Project and comprehensive microarray analyses.

Methods: For genes expression analysis, we used *in-house* cDNA microarray. The relationship of *ncRAN* expression levels with clinical outcome was analyzed by log-rank test. Soft agar assay was performed to examine the possible transformation potential of *ncRAN*. Cell proliferation was evaluated by siRNA-mediated knockdown and MTT assay. *In vitro* transcription/translation experiments were carried out.

Results: The novel *ncRAN* gene (*Nbla10727*, *Nbla12061*) was mapped to chromosome 17q25.1. High expression levels of two *ncRAN* splicing variants (2186 bp and 2087 bp) were significantly correlated with poor prognosis in 136 primary NBs (Kaplain-Meier survival curves, $p<0.001$). The approximate transcript size of *ncRAN* identified by Northern blot was 2.3 kb in neuroblastoma cell lines. Enforced expression of *ncRAN* resulted in an acceralated cell growth and induction of malignant transformation. Knockdown of *ncRAN* with *siRNA* led to a significant inhibition of the cell growth in SH-SY5Y neuroblastoma cells. Bioinformatic analysis indicated that there did not exist any putative translation start site with Kozak sequence. Consistent with these observations, we could not detect ncRAN expression at protein level as examined by *in vitro* as well as *in vivo* transcription/translation experiments.

Conclusions: The *ncRAN* may be a novel non-coding RNA highly expressed in malignant subset of NB with 17q gain. The development of the tools targeting *ncRAN* should be novel therapeutic strategies to treat high-risk NBs.

B25 Unmasking of Epigenetically Silenced MicroRNAs in Neuroblastoma

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Background: MicroRNAs (miRNAs) are small non-coding RNAs that negatively regulate gene expression at the post-transcriptional level. Down regulation of miRNA expression has been shown to be directly implicated in cancer development. Based on observations in coding tumour suppressor genes, it can be expected that abnormal DNA methylation at miRNA CpG islands can also contribute to tumour formation.

Methods: To identify aberrantly methylated miRNAs in neuroblastoma (NB), we measured the expression level of 384 miRNAs in a panel of 7 NB cell lines before and after treatment with a demethylating agent (5-aza-2'-deoxycytidine, DAC) or a histone deacetylase inhibitor (trichostatin A, TSA), or a combination of both. We used a real-time PCR based stem-loop reverse transcription primer method (Applied Biosystems) using only 10 ng of total RNA to synthesize cDNA of all miRNAs in a megaplex reaction, followed by pre-amplification using limited cycle PCR. miRNA profiles were also determined for 51 well-documented NB tumours and normal NB progenitor cells that have been isolated by laser capture microdissection from fetal adrenal glands.

Results: Based upon the association with a CpG island and the absence of expression in a subset of NB tumours, six reactivated miRNAs were identified as strong candidate suppressor miRNAs. Two of those miRNAs, i.e. miR-34b and miR-34c, belong to the same cluster located on 11q23.1, a region critically deleted in a subset of aggressive NB. Functional assessment using miR-34b/c mimics demonstrated significant cell viability reduction.

Conclusions: miRNA profiling successfully identified specific miRNAs silenced by CpG island hypermethylation in NB. Hence, this analysis may enable the identification of epigenetically regulated miRNAs that contribute to NB pathogenesis and which represent new targets for therapy.

B26 MYCN Regulates Oncogenic and Tumour Suppressor miRNA Networks in Neuroblastoma

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Background: The specific contribution of MYCN to neuroblastoma (NB) tumour development remains poorly understood. Recent studies have pointed at a critical role of non-coding RNAs in signaling pathways disturbed in cancer and therefore miRNAs should be regarded as important candidate MYCN transcriptional targets.

Methods: To identify MYCN regulated miRNAs, we profiled the expression of 384 miRNAs in 12 NB cell lines and 51 primary untreated tumours using real-time quantitative PCR (RT-qPCR), preceded by stem-loop megaplex reverse transcription (RT). miRNA detection sensitivity was increased by preamplification of the RT product. The high specificity of RT-qPCR together with a superior sensitivity makes this approach the method of choice for miRNA expression profiling.

Results: Comparison of *MYCN* single copy with *MYCN* amplified NB cells revealed both known and novel differentially expressed miRNAs, miRNA clusters and miRNA families. Transfection of NB cell lines with anti-mirs and miRNA precursors directed against the up- and downregulated miRNAs respectively resulted in a profound decrease of cell viability indicative for oncogenic and tumour suppressor activity. Further, computationally predicted targets of the differentially expressed miRNAs were significantly enriched for known MYCN downstream genes. A substantial number of these predicted target genes possess 3'UTR binding sites for multiple candidate MYCN regulated miRNAs possibly suggesting cooperative regulation of gene expression. CAMTA1, a putative NB tumour suppressor located on 1p36, ZBTB4, a transcriptional repressor and KIAA1128 are downregulated by MYCN and predicted targets of 5 different MYCN regulated miRNAs.
A combination of *in silico* promoter analyses and experimental procedures such as chromatin immunoprecipitation are currently being applied to confirm MYCN binding to miRNA promoter motifs.

Conclusions: These findings indicate that miRNAs are indeed fundamental components of the MYCN transcriptional network that mediate the MYCN tumorigenic program.

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B27 MYCN Regulates Oncogenic MicroRNAs in Neuroblastoma

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Background: *MYCN* amplification is a common feature of aggressive tumour biology in neuroblastoma. The MYCN transcription factor has been demonstrated to induce or repress expression of numerous genes. MicroRNAs (miRNA) are a recently discovered class of short RNAs that repress translation and promote mRNA degradation by sequence-specific interaction with mRNA. Here, we sought to analyse the role of MYCN in regulation of miRNA expression.

Methods: Using a miRNA microarray containing 384 different miRNAs and a set of 160 miRNA real-time PCR assays, miRNA expression was analysed in 24 primary neuroblastomas and in a neuroblastoma cell line conditionally expressing MYCN. A model system to conditionally overexpress or knock down the identified miRNAs was established.

Results: Seven miRNAs were identified that are induced by MYCN *in vitro* and are upregulated in primary neuroblastomas with *MYCN* amplification. Three of the seven miRNAs belong to the miR-17 cluster, that has previously been shown to be regulated by c-Myc. The miR-17-92 polycistron acts as an oncogene in haematopoietic progenitor cells. We show here that miR-221 is also induced by MYCN in neuroblastoma. Previous studies have reported miR-221 to be overexpressed in several other cancer entities, but its regulation has never before been associated with Myc. For further functional analysis, we conditionally overexpressed or knocked–down miR-17-92 and miR-221 in neuroblastoma cell lines, and subsequently use the Amersham DIGE System to identify miRNA target genes using high-throughput proteomic analysis.

Conclusions: Here we present evidence of miRNA dysregulation in neuroblastoma and report miRNA induction to be a new mechanism of gene expression downregulation by MYCN.

B28 A Novel MicroRNA Targeting Inhibitor-of-Apoptosis Proteins Acts as a Tumour Suppressor in Neuroblastoma

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Background: Neuroblastoma (NB) is a childhood tumour of the peripheral nervous system that occurs in neural creast cells colonizing the adrenal medulla in early developmental phases of embryogenesis. In an attempt to find new miRNAs involved in NB, we here report the role of 6A7 miRNA

Methods: We have identified a novel microRNA (6.A7 miRNA) with expression confined to the central and peripheral nervous system of the adrenal gland during development. Luciferase experiments, Caspase assays, FACs analyses, RT-cDNA PCR quantitative analyses, rescue experiments, immunohistochemistry analyses, "insitu miRNA-LNA" detection assays, BLI imaging technology on orthotopic/ xenograft neuroblastoma animal models were here applied to study 6A7 function.

Results: Quantitative expression analyses of 6.A7 miRNA in 93 neuroblastoma cohorts showed that it has prognostic value as an independent factor of *N-MYC* gene copy number amplification. Overexpression of 6.A7 miRNA induced down-regulation of inhibitors-of-apoptosis proteins (*XIAP*, *NAIP* and *Survivin*) both *in vitro* and *in vivo*

Conclusions: We thus provide here evidence of a new oncosuppressor function for 6.A7 miRNA, highlighting the use of this oncosuppressor miRNA as a therapeutic concept of 'one hit, multiple targets', a valuable approach for future clinical applications in neuroblastoma.

B34 Netrin-1 Acts as a Survival Factor for Aggressive Neuroblastoma

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Background: Dependence receptors are receptors that induce apoptosis in settings of absence of their ligand. As pro-apoptotic entities, they have been shown to regulate tumorigenesis as described in different solid tumors like colorectal or breast cancer. We investigated whether netrin-1 dependence receptors, DCC (Deleted in Colorectal Cancer) and UNC5H, may also control tumorigenesis of neuroblastoma (NB), the most common extracranial solid tumour of early childhood that is often diagnosed as a disseminated disease (stage 4).

Methods: We analysed expression of netrin-1 and its dependence receptors DCC and UNC5H by Q-RT-PCR in 102 stage 4 NB and correlated it with patient overall survival. We then used NB cell lines expressing high or low level of netrin-1 to monitor netrin-1 effect on NB cells survival, by using two strategies inhibiting netrin-1 expression (siRNA), and netrin-1 interaction with its receptors (inhibitory peptide). We next assessed, both in chicken and mouse models, whether interfering with netrin-1 is a relevant tool to limit/inhibit NB progression and dissemination in vivo.

Results: We describe that a large fraction of aggressive NB have selected a gain of netrin-1 expression, and that high netrin-1 expression is associated with poor outcome especially for patients diagnosed before one year of age. We show that autocrine production of netrin-1 is a tumour growth and dissemination selective advantage in aggressive NB as it blocks the pro-apoptotic activity of the netrin-1 dependence receptors UNC5H.

Conclusion: We show that netrin-1 up-regulation is a potent marker for poor prognosis in NB stage 4 diagnosed infants. Moreover, we propose that interference with the netrin-1 autocrine loop in NB cells could represent an alternative therapeutic strategy as disruption of this loop triggers NB cell death in vitro and leads to the prevention or regression of metastatic NB lesions in chicken and mouse models.

B35 Unc5H4, a Novel Dependence Receptor of Netrin-1 for Survival, is a Direct Target of p53 and Its Expression Levels are Downregulated in High-Risk Neuroblastomas

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Background: We have previously reported that expression of DCC tumor suppressor is downregulated in advanced stages of neuroblastoma (Clin. Cancer Res., 1996). The lignad of DCC is a Netrin-1 which inhibits DCC intracellular signals inducing cell death. Recently, it revealed that Netrin-1 also binds to the Unc5H family receptors with a death domain and inhibits the receptor-mediated apoptotic signals. Therefore, both DCC and Unc5H receptors have been defined as dependence receptors of Netrin-1 for survival.

Results and Discussion: Differential screening of the novel genes obtained from the CCC Neuroblastoma cDNA Project has identified a novel human gene *Unc5H4 (Nbla00613)*. Among the Unc5H family, only *Unc5H4* showed significantly higher expression in favorable neuroblastomas than unfavorable ones, and its high expression was associated with favorable prognosis (n=108, p=0.003). Cox hazard model showed that Unc5H4 expression was independent prognostic indicator from age, stage, *TrkA* expression and DNA ploidy, but not *MYCN* amplification. Our immunohistochemical analysis of 11 primary neuroblasotmas using specific antibody we generated also supported the above results. These suggested that Unc5H4 is involved in regulating growth, differentiation and apoptosis in primary neuroblastoma. Enforced expression of Unc5H4 in cells inhibited growth, whereas its knockdown by using siRNA increased sensitivity to adriamycin and enhanced apoptosis. Furthermore, the Unc5H4 intracellular domain including the death domain was cleaved by caspases. Both p53 and p73 directly bound to the *Unc5H4* promoter to transactivate its expression and enhanced cell death. Furthermore, colony formation assays revealed that Unc5H4-mediated inhibition of anchorage-independent cell growth was strongly suppressed in both SK-N-AS neuroblastoma cell line and H1299 cells without the p53 gene, suggesting that one of the downstream targets of Unc5H4 is also p53. Thus, the dependence receptor Unc5H4 forms a positive feedback loop with p53, and its activation by activated caspases triggered by other proapoptotic signals like NGF depletion amplifies the apoptotic circuit. This might occur during regression of neuroblastoma and be blocked in high-risk tumors.

Parallel Session: Basic Research 5

B36 Delineation of Notch Signaling Reveals an Integrated Network of CDK2, MYCN and Notch Genes Involved in Neuroblastoma Development

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Background: Notch signaling is an evolutionary conserved mechanism of intercellular communication involved in development and cancer. Notch receptors and their ligands can play tumor-suppressive as well as oncogenic functions, depending on cellular context. Aberrant expression of Notch signaling is well established in various tumour types, however the role of Notch signaling in neuroblastoma pathogenesis remains largely unknown.

Methods: We used gene expression profiling to analyze Delta-Notch pathway components in 110 primary neuroblastomas. Furthermore, Notch signaling activity was analyzed on protein level in 24 neuroblastoma cel lines. To elucidate the relevance of Notch signalling in neuroblastoma, we applied a systematic approach of inducible expression or lentiviral mediated shRNA of essential Notch pathway components (e.g. Notch intracellular domains, notch ligands and target genes). Gene expression profiling of time-course experiments was used to identify target genes.

Results: Kaplan-Meier survival analysis revealed strong correlations of Notch pathway gene expression with poor patient prognosis. Indeed, Notch over-expression revealed an essential role in G1 cell cycle progression. In addition, Notch signaling rescued cells from low-serum induced apoptosis indicating a possible role in neuroblastoma cell survival.
Notch target genes were analyzed with respect to target genes from other major pathways in neuroblastoma, e.g. MYCN and CDK2. Surprisingly we observed substantial overlap in target genes from CDK2 knock down and MYCN over-expression experiments, with significant enrichment of genes involved in cell cycle progression, apoptosis and extra-cellular matrix interaction. Comparison of Notch and CDK2 target genes revealed a significant number of regulated genes with a presumed role in cell cycle progression and apoptosis.

Conclusions: Notch signaling emerges as a major pathway in neuroblastoma that interacts with core genes like CDK2 and MYCN to ensure cell cycle progression and cell survival. This is reflected in the poor patient prognosis associated with Notch expression in tumours.

B37 The Wnt/β-catenin Signalling Pathway Cooperates with *MDR1* Gene-Encoded P-Glycoprotein in Multi-Drug Resistant Neuroblastoma Cells

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Background: The development of multi-drug resistance (MDR) represents a major obstacle for a successful therapy of high risk Neuroblastoma (NB). Elucidation of the complex mechanisms responsible for the development of drug resistance of these tumours is therefore strongly needed.

Methods: To address the mechanisms involved in multi-drug resistance, two doxorubicin-resistant (DoxR) NB cell lines (IGRN-91R and LAN-1R) were generated and analysed for their gene expression profile.

Results: These cells overexpress the *MDR1* gene-encoded P-glycoprotein and are cross-resistant to other MDR1 and non-MDR1 substrate drugs. As the P-gp inhibitor verapamil was not able to restore 100% of drug sensitivity, the identification of other potentially MDR-related deregulated molecules was investigated. Microarray expression profiles of resistant versus sensitive parental cell lines only showed a limited number of differentially expressed genes in IGRN-91-DoxR cells. As expected, the *MDR1* gene was included in the 16 upregulated transcripts, while the highest overexpressed transcript was the frizzled-1 (*FZD1*) receptor gene, belonging to the wnt/β-catenin pathway. FZD1 overexpression in DoxR cells was confirmed at the mRNA and protein levels. Functional analyses further indicated that FZD1 overexpression is associated with a sustained activation of the wnt/β-catenin pathway. Moreover, microRNA-adapted small hairpin RNA method used to specifically silence the *FZD1* gene in the two DoxR cell lines, reveals that FZD1 knock-down in the LAN-1R cells is associated with a sensitisation of these cells to chemotherapeutic drugs such as doxorubicin, taxol and etoposide.

Conclusion: This report represents the first implication of pathological activation of the wnt/β-catenin pathway in the aggressive and drug-resistant behaviour of NB. In addition to providing new insights into the mechanisms of drug resistance and tumorigenesis, these findings could lead to identification of specific targets to treat aggressive and resistant NB.

B29 Highly Tumorigenic and Tumor Stem Cell Like Neuroblastoma Side-Population Cells are Localized in Their Hypoxic Niche

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Background: Localization of neuroblastoma tumor stem cells (TSC) in their *in vivo* niche is not known. We recently isolated a highly tumorigenic, TSC like migratory SP (SPm) cells from neuroblastoma using a Boyden Chamber against a SDF-1α enriched bone marrow stromal cell conditioned media [1]. Here we investigated the localization of SPm fraction in the hypoxic zone *in vivo*.

Methods: Pimonidazole was used to label hypoxic cells *in vivo*. Quantum nano crystal was used for cell tracking. SPm cells were obtained from SK-N-BE(2) cell line.

Results: The majority of the pimonidazole positive SP cells (84%; p= 0.0077) were in the SPm fraction. Exposure to *in vitro* hypoxia increased the number of SPm fraction by 29% (p=0.0067). The post-hypoxia SPm (the $SPm_{(hox)}$) fraction showed a hundred fold enrichment of tumor initiating cells (Maximum likelihood analysis) compared to SPm fraction. When quantum-dot labeled $SPm_{(hox)}$ cells were injected *in vivo*, the labeled cells were seen mostly in the hypoxic zone of the tumor. More than 50% of $SPm_{(hox)}$ showed strong Oct-4 expression. Immunohistochemistry and flow cytometry analysis showed that majority of Oct-4 positive cells are localized in the hypoxic zones *in vivo (65%; p = 0.042)*. Since HIF-1α/VEGFR1 autocrine loop is active in SK-N-BE (2) [2], and downregulation of VEGFR1 reduces Oct-4 expression in SK-N-BE (2) SP cells [3], post siRNA VEGR1 treated SP cells were investigated for Oct-4 expression and tumorigenic potential. The siRNA knockdown reduced the Oct-4 positive $SPm_{(hox)}$ fraction and subsequent tumorigenic potential.

Conclusions: Conclusion: Hypoxia may act as a niche for neuroblastoma TSCs, where VEGF autocrine signaling may maintain TSC survival and or stemness.
1. Das B et al. Stem Cells. 2007; under revision.
2. Das B et al. Cancer Res. 2005;65(16)
3. Tsuchida R*, Das B*et al. Oncogene (In Press) * equal contribution.

B30 Combined Genomic and Molecular Signatures of Neuroblastoma: Implication of Their Clinical Application

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Background: Neuroblastoma (NBL) shows various clinical behaviors from spontaneous regression to aggressive growth. To understand the pathogenesis and to provide high quality prognostic tools for NBL, we here analyzed the combined genomic and expression signatures of NBLs in detail.

Methods & Results: aCGH of 234 tumors by using 2,464 cones-BAC arrays demonstrated there exist three genomic groups (GG) of chromosomal aberrations in NBL: silent (GGS), partial gains and/or losses (GGP) and whole gains and/or losses (GGW). Each GG was further segregated into subgroups with different clinical outcomes clearly defined by *MYCN* amplification (*MYCN*-amp), 1p loss, 11q loss and 17q gain. In all of them, *MYCN*-amp caused serious outcomes to patients. Interestingly, long survivors (>5yrs) with *MYCN*-amp were mostly found in GGP with 1p loss but without 11q loss (5-yr survival rate: 42%), whereas those were very rare in GGPa with both 1p and 11q losses (0%) as well as GGSa (0%). We previously finished gene expression profiling of 136 NBLs by an in-house, 5,340 NBL-derived genes chip and identified the molecular signatures strongly related to the prognosis of NBLs. The multivariate analysis indicated that the genomic and gene expression signatures were mutually independent prognostic indicators for all (n=40, p=0.045 and p=0.002, respectively) as well as sporadic NBL without *MYCN*-amp (n=30, p=0.031 and p=0.034, respectively). The genomic signature in addition to the molecular signature may help construct a novel risk classification system to predict prognosis of NBLs.

Conclusions: The existence of GGS, especially GGSa, is very important. The pathogengesis of *MYCN*-TG mice, which is very unfavorable, might be similar to the human GGSa. On the other hand, the prognosis of the GGSs is favorable (91%). Since genomic and molecular signatures were mutually independent, combination of both may contributes to establishing personalized medicine of neuroblastoma.

B31 A Genome-Wide Association Study (GWAS) of Copy Number Variants (CNVs) Identifies Neuroblastoma Predisposition Loci

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Background: CNVs may predispose to human disease.

Methods: We performed a GWAS of CNVs by genotyping 846 neuroblastoma patients and 803 controls (discovery) and 393 cases and 1148 controls (replication) at 550K SNPs.

Results: We observed highly significant associations between neuroblastoma and three distinct regions of copy number loss. The first encompassed 7 consecutive SNPs at 1q21 (P=2.30x10^{-10}-3.51x10^{-14}) within a known CNV spanning up to 1.6 Mb. The region contains many segmental duplications and includes a cluster of "neuroblastoma breakpoint family" (*NBPF*) genes initially identified as paralogs to *NBPF1*, a gene disrupted at 1p36 in a constitutional translocation from a neuroblastoma patient (Vandepole et al 2005). We validated heritability of the CNV in available trios, and review of paired tumor data confirmed all deletions, consistent with these being germline alterations. We also observed significant association of loss on chromosome 7 at two regions spanning *TCRG* (P=6.93x10-9-4.85x10^{-18}) and a single SNP within *TCRVB* (P=5.98x10^{-8}). T-cell receptor deletions were not present in paired tumor DNA, suggesting an oligoclonal expansion of lymphocytes in the blood of these patients. The most significant association was a 76 Kb gain at a 6q CNV present in 11.3% of cases and 0.5% of controls (P=5.93x10^{-18}-4.19x10^{-24}). Other regions of gain at 4q and 17q also reached genome-wide significance. The *TCRG* and *TCRVB* deletions showed robust replication (P=5.8x10^{-16} and P=1.2x10^{-8} respectively), as did the deletion at 1q21 (P=1.5x10^{-4}), while the regions of gain showed modest (6q P=0.005; 4q P=0.03), or no (17q P=0.22) evidence for replication.

Conclusions: Common CNVs are associated with susceptibility to neuroblastoma. Together with our discovery of common SNPs associated with neuroblastoma (*Maris, ANR 2008*), these data support a model of multiple common genetic variants cooperating in the etiology of this disease and are the first report of CNVs being associated with human cancer.

B32 Sub-Classification and Individual Survival Time Prediction from Gene-Expression Data of Neuroblastoma Patients Using CASPAR

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Background: Current gene-expression classification of neuroblastoma patients predict subgroups of the disease (favorable vs. unfavorable) with divergent outcome. Using a novel algorithm termed CASPAR, we sought to predict individual survival time (as a continuous variable) for neuroblastoma patients from gene-expression data of their tumors.

Methods: Gene-expression profiles were generated from neuroblastoma samples of 256 patients using a customized oligonucleotide-microarray. Then, the CASPAR algorithm was combined with a leave-one-out cross-validation to predict individual times for event-free (EFS) and overall survival (OS) for the total cohort and for subcohorts of patients, including: 1. stage 4 disease (n=67), 2. *MYCN*-amplified disease (n=32), 3. 1p-deletion (n=53), 4. 11q-deletion (n=56), 5. 17q-gain (n=78), 6. patients stratified as high-risk (n=79) or 7. stratified as intermediate- or high-risk (n=94) by the German neuroblastoma trial NB2004 and 8. patients predicted as unfavorable by a recently described, superior gene-expression classifier (n=83; (JCO 24:5070-8;2006)). Prediction accuracy of individual survival times was assessed by Kaplan-Meier analyses, receiver operator characteristics (ROC) curve analyses and time-dependent ROC analyses.

Results: In the whole cohort, CASPAR separated patients with divergent outcome (survival probability 5y-EFS 0.82±0.06 (predicted long EFS) vs. 0.33±0.14 (predicted short EFS) and 5y-OS 0.94±0.04 (long) vs. 0.38±0.17 (short); both p<0.0001). Furthermore, CASPAR demonstrated a high accuracy in predicting patients' individual survival time as indicated by time-dependent ROC analyses (mean area-under-curve for the first ten years EFS 0.82±0.06, OS 0.92±0.04). Moreover, with the exception of MYCN-amplified patients, CASPAR significantly distinguished short (<5y) from long (>5y) survivors in all sub-cohorts with unfavorable markers (stage 4 disease; p<0.001); 11q-deletion (p<0.001), 1p-deletion (p=0.03), 17q-gain (p<0.001), NB2004 high-risk (p<0.001), NB2004 high- and intermediate-risk (p<0.001); unfavorable gene-expression prediction (p<0.001)).

Conclusions: The CASPAR algorithm allows to accurately predict *individual* survival times for neuroblastoma patients from gene-expression data.

B33 Meta-Analysis of Array CGH Datasets Reveals New Genomic Markers for Improved Prediction of Neuroblastoma Treatment Failure

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Background: Array CGH profiles are prognostically relevant in neuroblastoma (NB) patients. This prognostic relevance is further explored using a cross-platform meta-analysis of 422 tumors in order to detect DNA imbalances with additional power for predicting treatment failure for high risk NB.

Methods and Results: Array CGH data were imported in arrayCGHbase. Using circular binary segmentation on standard deviation profiles based on the copy number ratios across all samples, various segments displaying DNA imbalances were identified. For each segment a genomic position was selected for unbiased hierarchical clustering which yielded four clusters corresponding to three known clinico-genomic subgroups (1: numerical aberrations only, 2A: 11q deletion, 2B: *MYCN* amplification (MNA)) and a fourth silent subgroup with no or few genomic aberrations. In order to assess the prognostic significance of the genomic aberrations, we performed multiple testing corrected log-rank analysis for all positions in the genome. This confirmed the power of established prognostic markers (e.g. 1p-, 11q-, 17q+, MNA). Of particular interest, we identified genomic markers that further improve the power for predicting treatment failure in high-risk tumors. Gain of 1q is present in 1 low-risk and 29 high-risk patients of which 20 did not survive (present in 15% MNA tumors of which 75% died and in 9% of type 2A tumors of which 55% died). Other rare but recurrent CNAs in patients that almost invariably failed multi-modal treatment are high level *ALK* (2p23) gain (in type 2A tumors) or amplification (in MNA tumors, as distinct amplicon) and 6q loss (observed in 8 non-survivors).

Conclusions: This first successful cross-platform array CGH meta-analysis in NB demonstrates (1) unbiased recognition of four major genomic subgroups with prognostic significance and (2) particular imbalances identifying subsets of ultra high-risk patients.

Parallel Session: Clinical Study 1

C11 Effective Intrathecal Radioimmunotherapy-Based Salvage Regimen for Metastatic Central Nervous System (CNS) Neuroblastoma (NB)

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Background: Recurrent metastatic NB is difficult to cure, particularly in patients with CNS disease. This complication was uniformly fatal in our previous published experience, median time to death of 6.7 months. We evaluated the addition of antibody-based intrathecal radioimmunotherapy (RIT) targeting minimal residual disease following surgery, craniospinal radiation, and chemotherapy.

Methods: 44 patients with recurrent CNS NB treated at Memorial Sloan-Kettering Cancer Center over a 20 year period are the subject of this report. 30 patients (group 1) were treated with various combinations of surgery, chemotherapy, and radiation therapy. Since 2003, 14 patients (group 2) were treated with surgical resection of parenchymal lesions, 1080-2160 cGy craniospinal radiation, intravenous irinotecan plus oral temozolomide, intrathecal RIT with ^{131}I-3F8 or ^{131}I-8H9, intravenous anti-GD2 monoclonal antibody plus Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF), and oral temozolomide.

Results: All 30 patients in group 1 died of progressive disease, median time to death was 5.5 months from the CNS event. In contrast, 13 of 14 patients in group 2 are alive and free of NB 6-53 months since CNS event; 1 patient with leptomeningeal NB died of infection 22 months after the detection of CNS disease, no NB was found at autopsy. 1 patient remains on treatment for refractory systemic NB. Myelosuppression following craniospinal radiation therapy and chemotherapy was common; 5 patients received stem cell support to minimize hematologic toxicity. Three patients remain on oral supplements for treatment-related hypothyroidism. One patient with a 7-year history of NB is being treated for MLL-associated secondary leukemia that developed 3.5 years after detection of CNS disease.

Conclusion: Effective control of CNS NB is possible with intrathecal RIT-based treatment. This multimodality treatment regimen is well tolerated by young patients despite prior intensive cytotoxic therapies, and has the potential to increase survival.

C12 Potential Prognostic Value of ^{123}I mIBG Scintigraphy in High Risk Neuroblastoma: Development of a Semi-Quantitative Reporting Method

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Background: A reproducible, semi-quantitative reporting method is required to assess the potential of ^{123}I meta-IodoBenzylGuanidine [mIBG]imaging as an independent prognostic indicator in high risk neuroblastoma. The development and testing of a new technique for ^{123}I mIBG image evaluation by an international panel of 7 nuclear medicine specialists is described.

Methods: Patterns of skeletal and soft tissue disease on planar ^{123}I mIBG images were defined and assigned numerical scores within specified anatomical body areas. 246 anonymised, electronic ^{123}I mIBG scans stored on the SIOPEN R NET database were scored as 132 unblinded pairs [pre- and post- induction chemotherapy] and then re-scored individually, in random order, as a blinded study. Interclass correlation coefficients [ICC] were calculated to determine inter- and intra-observer error for unblinded and blinded scores respectively.

Results:

	Pre treatment ICC	Post induction ICC
Unblinded study [132 data pairs]	0.95	0.98
Blinded study [246 individual scans]	0.95	0.99

Tumour burden varied significantly between patients both at diagnosis and post treatment ($p<0.0001$).

Conclusions: The semi-quantitative scoring method developed: 1) provides a reliable, reproducible objective measure of disease extent and treatment response in high risk neuroblastoma 2) is valid across the expected disease spectrum in this population 3) provides the basis for determining the prognostic value of ^{123}I mIBG scintigraphy in high risk neuroblastoma.
The project was sponsored by the Adam's Hats Charity

C13 Late Sequelae of Localized Neuroblastoma Presenting with Epidural Compression. A Study of the Italian and French Neuroblastoma Groups

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ABSTRACT. To report on late sequelae of a cohort of children with localized neuroblastoma presenting with symptomatic epidural compression (EC) and correlate them with clinical features and tumour characteristics at diagnosis, and treatment.

Patients and Methods. 101 children with localized neuroblastoma presenting with EC and surviving more than 2 years from time of diagnosis were identified in the Italian and French Neuroblastoma Registries. Adequate information were obtained for 98.

Results. Symptoms of EC at diagnosis included motor deficit in 94/98 cases and sphyncteric deficits in 33. Initial treatment consisted of chemotherapy in 66 cases, neurosurgical decompression in 29 and radiotherapy in 3. Chemotherapy was chosen more frequently for children of younger age and stage 3 disease. Overall treatment consisted of chemotherapy alone in 44 cases, neurosurgery ± chemotherapy in 38, radiotherapy and chemotherapy ± neurosurgery in 16. After a median follow-up of 7.3 years, 57 children (58.2%) had one or more sequelae. Children treated by chemotherapy alone had overall fewer sequelae. Motor deficit, bladder and bowel dysfunctions at time of visit correlated with severity of motor deficit and bladder and bowel dysfunctions at time of diagnosis. Orthopedic sequelae at last visit correlated with motor deficit at diagnosis, age above 10 years at time of last visit and overall treatment including neurosurgery and/or radiotherapy.

Conclusion. More than one half of children with localized neuroblastoma and symptomatic EC enrolled into this study had at least one or more sequelae. The severity of motor deficit at diagnosis was the major factor accounting for the development and severity of sequelae.

C14 Crossing the Midline does not Influence Outcome in Infants with Stage 4S Neuroblastoma

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Background: The International Neuroblastoma Staging System (INSS) confines stage 4S disease to small primary tumors stage 1 or 2 with typical metastases in liver, bone marrow and skin. In the German Neuroblastoma trials, this limitation was not observed and primary tumors stage 3 were accepted in infants with stage 4S metastatic pattern. We now analyzed the outcome of stage 4S patients with stage 3 compared to stage 1 or 2 primary tumors.

Methods: Clinical data, molecular data and outcome in stage 4S patients diagnosed between 1990 and 2006 were analyzed and correlated to primary size at diagnosis.

Results: Data on primary tumor extension were available in 134/264 infants with stage 4S. Primary tumors were classified as stage 1 (completely resected) in 45, as stage 2 in 45 and as stage 3 (crossing the midline or contralateral lymph node involvement) in 44 patients. Stage of the primary (stage 3 compared to stage 1 or 2) was not associated with age at diagnosis (median 3.7 vs. 2.9 months, p=0.69), MYCN amplification (7% vs. 13%, p=0.38), and 1p aberrations (16% vs. 20%, p=0.78), but with higher tumor volume (median 69 vs. 31 ml, p=0.001), higher frequency of bone marrow metastases (80% vs. 54%, p=0.007) and lower frequency of liver metastases (57% vs. 79%, p=0.01).
Twelve out of 44 patients with stage 3 primary tumors showed regression without cytotoxic treatment. Neither primary stage (stage 3 vs. stage 1,2: 3-y-OS: 0.88+/-0.05 vs. 0.91+/-0.03; p=0.65) nor tumor volume (>50 ml: 3-y-OS: 0.90+/-0.05 vs. <50ml: 0.90+/-0.04; p=0.80) were associated with poor outcome, while patients with MYCN amplification fared worse (3-y-OS: 0.59+/-0.14 vs. 94+/-0.02; p<0.001).

Conclusions: Primary stage 1, 2 or 3 tumors appear to have similar biology in stage 4S neuroblastoma. The inclusion of stage 3 primaries in the definition of 4S disease is suggested.

C15 Peripheral Neuroblastic Tumours of the Stroma-Rich and Stroma-Dominant Histotypes are Distinguished by Different DNA Content and Patterns. Analysis of 50 Italian Cases

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Background: Peripheral neuroblastic tumours of the ganglioneuroblastoma (GNB) "intermixed" and ganglioneuroma (GN) maturing histotypes are clearly distinguished on histopathological grounds on the basis of precise, although subtle, morphological differences (International Neuroblastoma Pathology Committee classification, 1999). In spite of this, these two kinds of tumour present a strikingly uniform benign clinical behaviour. We therefore investigated the DNA content of a series of 50 cases in order to pick out possible biological differences.

Methods: DNA content was analysed by means of a cytofluorimetric assay on formalin-fixed, paraffin-embedded samples from 25 GN and 25 GNB intermixed belonging to the Italian Neuroblastoma Registry (1979-2007). Cytofluorimetric examination was carried out under a cytofluorimeter (FACScan Becton-Dickinson) on at least 20,000 events. The results were analysed by means of Modfit software.

Results: The histograms obtained from the cytofluorimetric analyses displayed two clearly different patterns. In the 25GN, only one diploid cell population was identified, represented by a large-base peak with a high variation coefficient (essentially representing Schwann cells). Of the 25 GNB intermixed, 20 showed a large-base diploid peak (representing Schwann cells) together with a small aneuploid peak, whose DNA index was near triploid (this aneuploid population corresponding to the neuroblastic cells). In the remaining 5 cases of GNB intermixed, the DNA content turned out to be diploid. These 5 cases underwent further analysis on areas which evidenced distinct nests of neuroblastic cells on light microscopy. On these new samples, analysis yielded a DNA index different from 1.

Conclusions: Our results indicate that the distinction between GN and GNB intermixed is warranted not only on pure morphological grounds but also on the grounds of significant biological differences.

C16 Management and Outcome of INSS Stage 3 Neuroblastoma: the Memorial Sloan-Kettering Cancer Center Experience

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Background: The management of patients with INSS Stage 3 neuroblastoma (NB) worldwide is not consistent. Most patients receive chemotherapy, many with stage-4-like dose-intensity. We describe a single center approach at Memorial Sloan-Kettering Cancer Center (MSKCC) from 1987-2007, minimizing therapy except for those with *MYCN*-amplified disease.

Methods: In this retrospective analysis, 64 patients were divided into 3 groups: (A) 39 without *MYCN*-amplified tumors, (B) 15 with *MYCN*-amplified tumors first evaluated at MSKCC prior to relapse and (C) 10 with *MYCN*-amplified tumors evaluated at MSKCC after relapse or progression. Progression-free survival (PFS) and overall survival (OS) were analyzed by Kaplan Meier analysis.

Results: Group A: 14 patients were treated with surgery alone and 25 with surgery at MSKCC after a short course of prior chemotherapy (at other institutions) followed by discontinuation of all further treatment. PFS and OS probabilities for Group A were both >90% 19 years post-diagnosis. Group B: all 15 underwent dose-intensive induction, gross total resection of primary tumor followed by local radiation. 13 achieved CR/VGPR, and 10 received megatherapy/ stem cell transplant. 11/15 also received 3F8-based immunotherapy, 10 remain free of disease. PFS and OS probabilities of the immunotherapy group were both >90% 10 years post-diagnosis. Group C consisted of 10 patients for whom treatment at relapse included surgery and/or chemotherapy. 6 also received 3F8-based immunotherapy: 2/6 survive progression-free 2.2 and 6.2 years after relapse. All other patients in group C died of progressive disease.

Conclusions: Patients with non-*MYCN*-amplified stage 3 NB can be successfully treated with early surgery without the need for continuation of chemotherapy. Radiotherapy is not necessary for disease control. Induction of remission in patients with *MYCN*-amplified disease requires dose-intensive multimodality therapy. 3F8-based immunotherapy may prolong survival for both first or second remission. The role of myeloablative therapy remains to be carefully assessed.

C17 T Cell Augmentation Given Immediately after SCT Produces Rapid T Cell Recovery and Robust Vaccine Responses

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Background: Chemotherapy-based approaches against NB have reached limits of efficacy and tolerability. A tumor vaccine could improve outcome, but must be delivered post SCT when there is no recovery of T cell function. Vaccine responses are absent for 6-12 months post autologous SCT. In this study we tested the impact of post-SCT T cell augmentation (TCA), where autologous activated T cells are delivered immediately post-SCT period, on T cell recovery and vaccine responses.

Methods: We tested TCA in NB in 2 phases, in the context of a trial of HRNB therapy consisting of induction chemotherapy followed by tandem SCT including TBI in SCT#2, which has yielded a 3yr EFS of 55% in a large Phase II study. Initially, TCA was given at day 12 post-SCT (D+12) in 10 pts. We then did a randomized pilot study (N=30) of TCA at D+2 or at D+90, assessing CD4 and CD8 T cell recovery, and giving a conjugate pneumococcal vaccine (Prevnar) on D+12 and D+60 to assess functional immune recovery.

Results: TCA given at D+12 or D+2 after a highly immunosuppressive tandem transplant results in rapid improvement in CD4 (Table) and CD8 T cell counts. The D+2 TCA group displayed Prevnar response well above protective levels in almost all pts immunized (some as early as D+30), an effect not seen in the late TCA pts. 4 pts have also shown an engraftment syndrome after early TCA, consisting of fever and a rash pathologically and clinically indistinguishable from GVHD, with 1/4 pts briefly receiving steroid therapy.

CD4 Count Recovery after SCT and TCA

postSCT	No TCA	D12 TCA	D2 TCA	Eng Syn
D0	0	10	5	0
D30	22	378	1500	1926
D60	87	560	931	1357

Conclusions: This trial, together with data showing survivin to be a tumor antigen relevant to T effector responses in NB, suggest a study design where of TCA to restore cellular immunity post SCT, followed by an anti-tumor vaccine. A survivin peptide vaccine is in Phase I and could be tested in this setting.

C18 Analysis of Copy Number Gain of *MYCN* in Neuroblastoma – a Children's Oncology Group Study

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Background: *MYCN* amplification predicts a poor outcome in neuroblastoma and is used for risk stratification. The COG defines *MYCN* amplification as >10 copies/ cell; however, subthreshold copy number gain in the absence of hyperdiploidy occurs. Our aim was to determine whether this gain has prognostic significance.

Methods: Tumors received at the COG Neuroblastoma Reference Laboratory from 2004-2006 were analyzed with a *MYCN* locus-specific probe (Vysis Inc). Tumors scored as having ≥5 signals were reanalyzed with dual colored *MYCN* and ch2 centromere probes. The centromere probe provided an internal control to distinguish between aneusomy versus *MYCN* amplification. Tumors were grouped into multiple categories with copy numbers ranging from 2-7 and two borderline categories: A) ≥2-8 copies and B) ≥2-10 copies but not clearly amplified. Kaplan-Meier survival curves were plotted and categories were compared with a logrank test. To determine if 8 copies would be a more optimal cut-off the cohort was dichotomized into high versus low copy number and compared using a Cox regression model.

Results: Of 1066 tumors, 191 (18%) had *MYCN* amplification. Copy number gain was seen in 26 additional tumors (2.4%). Of the 1,066, 929 had sufficient outcome data. The outcomes of the copy number gain categories were not significantly different from the non-amplified group. Moreover, the outcomes of both borderline categories were different from the truly *MYCN* amplified tumors [(A: EFS: p=0.0008; OS: p<0.0001); (B: EFS: p=0.0503; OS: p=0.0073)]. The comparison of low versus high *MYCN* copy number was highly significant using either the 8 or the 10 copy cut-off; however, hazard ratios for EFS/OS were higher with the 10 copy cut-off.

Conclusion: *MYCN* gain is not predictive of a poor prognosis in neuroblastoma. There is no evidence to support a change in the consensus, albeit arbitrary, definition of >10 copies/cell to define *MYCN* amplification for risk stratification.

Selected Poster Session: Translational Research

TR23 Identification of Neuroblastoma Therapeutic Targets Using an Integrative Genomics Strategy in the Murine and Human Systems

Naomi Balamuth[1], Qun Wang[1], Andrew Wood[1], Zhe Zhang[1], Jay Jaganathan[1], Eric Rappaport[1], Rosalind Manning[1], Bruce Pawel[1], Eric Sekyere[2], Joanna Keating[2], Glenn Marshall[2], John M. Maris[1]
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Background: New therapeutic targets are needed for high-risk neuroblastoma.

Methods: Genome-wide oligonucletide-based gene expression was measured in a series of sympathetic ganglia (precursor lesions; N=5 at birth, N=2 at day of life 7, N=2 at day of life 14) and tumor samples (N=6 at 4 different sizes from 0.08±0.03 to 2.01±0.59 grams) harvested from *MYCN*$^{+/+}$ transgenic mice. Genes showing a linear increasing or decreasing expression across this sample set were selected (FDR<0.05). We then determined which potential oncogenes were differentially overexpressed in *MYCN* amplified human neuroblastomas (N=20) compared to biologically favorable localized neuroblastomas (N=27). Finally, genes encoding potentially druggable targets were prioritized for immunohistochemical and functional validation.

Results: We identified 93 murine genes showing expression highly associated with tumor progression ($p < 0.01$), and some of these genes were also differentially overexpressed in human *MYCN* amplified neuroblastomas. *CENPE, EP-CAM, GPR49* and *IMPDH2* were prioritized for further study as each is reported as a human oncogene, and targeted inhibition strategies are in development. siRNA knockdown of *Cenpe* in a murine *MYCN*$^{+/+}$ cell line showed clear growth inhibition. In addition, a small molecule *CENPE* inhibitor currently in early phase clinical trials showed broad activity against a panel of 21 neuroblastoma cell lines (median IC50=47 nM; range 27-267 nM) as well as significant single agent activity against the NB-1643 xenograft ($p<0.0001$). Immunohistochemical and functional validations studies for this and other prioritized targets are ongoing and will be reported.

Conclusion: The *MYCN* transgenic mouse can be used to identify novel therapeutic targets relevant to human neuroblastoma. *CENPE, EP-CAM, GPR49* and *IMPDH2* are lead candidates for development of new treatment approaches. *CENPE* inhibition shows significant single agent activity in preclinical models, both validating this approach and suggested that *CENPE* inhibitors should be prioritized for clinical development.

TR24 Perifosine, an Akt Inhibitor, Sensitizes Neuroblastoma to Etoposide Treatment

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Cell & Molecular Biology Section, Pediatric Oncology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, USA

Background: BDNF , TrkB and activated Akt are expressed in unfavorable, chemo-resistant NB tumors. The aim of this study is to develop a mouse model to evaluate the efficacy of combining small molecule inhibitors of the PI-3 kinase/Akt pathway with cytotoxic drugs to develop more effective, less toxic therapy for poor prognosis NB patients.

Methods: A tetracycline (Tet)-regulated TrkB-expressing NB cell line is used. In vitro, cells were pretreated with perifosine, an Akt inhibitor, followed by etoposide prior to BDNF administration. To develop a mouse model, NB cells were injected at subcutaneous or orthotopic (periadrenal) sites in mice. Mice received water with or without Tet. Mice with tumors were randomized to receive etoposide alone or in combination with perifosine.

Results: *In vitro*, activation of Akt via TrkB causes a 50% reduction in the cytotoxic effects of chemotherapeutic drugs. Pretreatment of NB with perifosine blocked the BDNF-induced phosphorylation of Akt. Perifosine sensitizes NB cells to etoposide, and blocks the BDNF protection of NB cells from etoposide-induced cell death. In vivo, TrkB expression in tumors from either subcutaneous or orthotopic sites in mice without Tet in water was about 3-fold higher than the TrkB levels in tumors from mice with Tet in water. Tumor bearing mice were treated with etoposide at 10 mg/kg and 20 mg/kg doses. There was a 40% reduction in tumor size in low TrkB-expressing tumors with 10 mg/kg etoposide, while the tumors expressing high levels of TrkB required 20 mg/kg etoposide to cause a comparable reduction. In mice with high TrkB-expressing tumors, perifosine administration sensitized these tumors to etoposide (10mg/kg) treatment that resulted in a 50% reduction in tumor size.

Conclusions: Combining perifosine, an Akt inhibitor, with the chemotherapeutic etoposide increases the treatment efficacy in high TrkB-expressing cells and tumors.

TR25 A Genomic Approach to the International Neuroblastoma Pathology Classification

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Background: To date, the International Neuroblastoma Pathology Classification (INPC: Favorable Histology-FH vs. Unfavorable Histology-UH) and MYCN status (Amplified-A vs. Non-amplified-NA) are the most reliable prognostic predictors in childhood neuroblastoma. By combining those two, four INPC/MYCN prognostic subsets: "FH&NA", "FH&A", "UH&NA", "UH&A", are distinguished.

Purpose: This was the first attempt to replicate the INPC/MYCN classification scheme by whole genome RNA expression profiling.

Methods: Human Exon arrays were used to profile 50 neuroblastoma cases (24 FH&NA, 7 UH&A, 19 UH&NA) from the CHLA Biorepository. The discriminative power was enhanced by building the classifier at probeset level that span 1.4 million of annotated and non-annotated RNA transcripts. Prediction analysis for microarrays (PAM) was applied to the data, and the accuracy was estimated by a nested cross validation loop to avoid biased estimators of classification performance.

Results: We developed a predictor model that correctly identified 42 of 50 (84%) cases based on RNA expression. FH&NA tumors were represented by SLC18A2, NTRK1, RPL7A, CACNA2D3, CD44, PMP22, HS6ST3, PRKCB1, ADCY1 and TFAP2B and enriched by transcripts on chromosomes 7, 5 and 17. UH&A signature was overrepresented by transcripts located on chromosome 2 and included well-known predictors like MYCN, MYCNOS, HOXD10, ST6GAL2. UH&NA tumors demonstrated a substantial molecular heterogeneity with all misclassification errors occurring within this group. Regardless of heterogeneity, however, SLITRK1, DCC and CACNG7 were found to be significantly down regulated in this UH&NA cohort.

Conclusions: Gene expression profiling discriminated groups of patients into three major INPC/MYCN prognostic subsets. Additional studies with a large sample are warranted to further improve this finding, and could lead to the discovery of molecular subclasses in the UH&NA tumors.

TR26 A Novel Minigene DNA Vaccine Encoding for Survivin-Derived Peptides with Superior MHC Class I Binding Affinities is Effective against Neuroblastoma

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Background: Survivin is a neuroblastoma-associated antigen recognized by specific cytotoxic T cells circulating in neuroblastoma patients. Here, we describe the rational design and the generation of a novel survivin minigene DNA vaccine encoding for antigenic peptides of survivin. This design avoids the potential hazard of using a full length anti-apoptotic gene for cancer therapy.

Methods: Identification of survivin-derived peptides 'WEPDDNPI', 'FEETAKTT' and 'EELTVSEFL' was based on a combined approach using predicted MHC class I binding affinities (*syfpeithi* and BIMAS binding scores) and computer modelling of peptides into the crystal structure of MHC class I (free energy). The plasmid DNA vaccine (pUS-high) was generated by cloning the sequences of survivin-peptides each separated by an AAY spacer into the ubiquitin-containing expression vector pCMV-F3Ub. Vaccination was performed by oral gavage of attenuated *Salmonella typhimurium* SL7207 carrying pUS-high. Results were compared to DNA vaccines encoding for the entire survivin protein.

Results: Mice receiving the plasmid vaccine (pUS-high) in a prophylactic setting presented with a 48% to 52% reduction in s.c. tumor volume, tumor weight and liver metastasis level in contrast to empty vector controls. This response was as effective as the survivin full-length vaccines and was associated with an increased tumor cell lysis and TNF-alpha and IFN-gamma production by systemic CD8+ T cells. CD8+ cells presented at higher levels at the primary tumor site in pUS-high vaccinated mice. CD8 but not CD4 depletion completely abrogated the pUS-high mediated primary tumor growth suppression. Therapeutic vaccination with pUS-high also led to a primary tumor growth suppression compared to the control group. Importantly, in 4/7 pUS-high vaccinated mice eradication of established neuroblastoma tumors was observed. Two of these survivor mice remained tumor-free after tumor re-challenge.

Conclusion: Survivin minigene DNA vaccination is effective against neuroblastoma and provides a promising survivin-based immunotherapeutic approach against a challenging childhood tumor.

TR27 Xenogeneic Immunization with a Human Tyrosine Hydroxylase DNA Vaccine Effectively Eradicates Established Neuroblastoma and Induces Long Lasting Protective Immunity in Mice

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Introduction: DNA vaccination against cancer antigens is considered to induce weak anti-tumor immune responses in humans. In order to enhance the effect, xenogeneic immunization is a promising approach, in particular for neuroblastoma. Neuroblastoma cells highly express the enzyme tyrosine hydroxylase (TH). Previous prophylactic vaccinations with human TH (hTH) DNA vaccines induced an effective CTL-mediated anti-neuroblastoma immune response in mice. Here, we report its efficacy in a therapeutic vaccination setting.

Methods: hTH cDNA was cloned into mammalian expression vector pCMV-F3Ub. A/J mice were challenged subcutaneously (s.c.) with a lethal dosage of syngeneic NXS2 neuroblastoma cells (day 0). Therapeutic vaccination was carried out on day 2 and 5 after tumor cell inoculation by oral gavage of attenuated *salmonella typhimurium* SL7207 bearing the hTH cDNA and empty vector controls. Primary tumor growth rate was monitored over time until tumor sizes reached a volume greater than 500mm^3. At that time, primary tumors were removed surgically and mice received a third immunization 14 days post surgery. Survival of mice was determined over time. Mice surviving longer than 90 days after tumor cell injection were re-challenged with a lethal dosage of wildtype NXS2 cells.

Results: Therapeutic vaccination with hTH cDNA induced long term survival in 50% of mice for up to 90 days. In 50% of the hTH cDNA vaccinated mice, established tumors were eradicated following first and second vaccination. Importantly, after re-challenge, tumor growth was suppressed in the hTH cDNA survivor mice compared to the naïve control group.

Conclusion: In summary, we report that an hTH cDNA vaccine effectively eradicates established s.c. tumors in mice, significantly prolongs their survival and suppresses tumor growth after a lethal re-challenge. Therefore, we think that xenogeneic vaccination with TH DNA vaccines is a promising concept for active immunotherapy in neuroblastoma.

TR28 Evaluation of Array-Based Outcome Predictors for Neuroblastoma

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Background: Using gene expression profiling studies we and others have reported the capability of predicting prognosis of neuroblastoma (NB) patients and have identified a number of prognosis signatures, but comparative analysis of these gene signatures revealed remarkably few overlapping genes.

Methods: We determined the prediction accuracy rate for each of the five published gene sets (Wei et al, Ohira et al, Schramn et al, Asgharzadeh et al and Oberthuer et al) using Artificial Neural Networks (ANN) and Prediction Analysis for Microarray (PAM) methods on three of the published NB data sets (Wei et al, Asgharzadeh et al and Oberthuer et al) and one independent data set of 52 samples.

Results: We found that the signatures from five different studies showed a great agreement in the outcome prediction for individual samples. The samples misclassified by one signature are usually misclassified by one or more other signatures as well. Although these five signatures were extracted from NB tumors of different stages and *MYCN* status, patients with good prognosis in different data sets were predicted well by all gene sets. However the prediction of patients with poor prognosis was various. For poor-outcome patients, the specificity ranges 76-97% and sensitivity ranges from 13% to 91% in different data sets of samples predicted by different gene sets. Both ANN and PAM methods showed the consistent results.

Conclusion: We found that all five published prognostic gene sets were able to accurately predict the NB patients with good outcome. However the performance for predicting poor outcome patients varied from random to high accuracy. Possible causes may include heterogeneity of sample quality and treatment. Further meta-analysis is under way to identify signatures to improve the predicting power for patients with poor outcome. In addition a larger definitive study is required to identify a robust gene expression signature.

Plenary Session: Clinical Study 1

C1 International Neuroblastoma Risk Group (INRG) Classification System

Andrew David John Pearson[1], Susan L. Cohn[2], Wendy B. London[3], Tom Monclair[4], Peter F. Ambros[5], Katherine K. Matthay[6] for the INRG Task Force
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Background: As current approaches to risk classification and treatment stratification for children with neuroblastoma (NB) vary greatly throughout the world, it is not possible to directly compare risk-based clinical trials. The International Neuroblastoma Risk Group (INRG) classification system was developed to establish a consensus approach for pre-treatment risk stratification.

Methods: The statistical and clinical significance of 13 potential prognostic factors were analyzed in a cohort of 8,800 children diagnosed with NB between 1990-2002 from North America and Australia (COG); Europe (SIOPEN and Germany), and Japan. Survival tree regression analyses were performed to test the prognostic significance of the 13 factors.

Results: Of the 13 prognostic factors analyzed, stage, age, histologic category, tumor grade, *MYCN* status, chromosome 11q status, and DNA ploidy were the most highly statistically significant and clinically relevant. A new staging system (INRG Staging System) based on clinical criteria and tumor imaging was developed for the INRG Classification System. The optimal age cut-off was determined to be between 15-19 months, and 18 months was selected for the classification system. Sixteen pre-treatment groups were defined based on clinical criteria and statistically significantly different EFS of the cohort stratified by the INRG criteria. Patients with 5-year EFS ≥85%, >75-<85%, ≥50-≤75%, or <50% were classified as very low-, low-, intermediate-, or high-risk, respectively.

Conclusion: By defining homogenous pre-treatment patient cohorts, the INRG classification system will greatly facilitate the comparison of risk-based clinical trials conducted in different regions of the world and the development of international collaborative studies.

C2 Data-Driven INPC (International Neuroblastoma Pathology Classification): Analysis of Age, Grade of Neuroblastic Differentiation, and MKI - A Report from the INRG Project -

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Background: INPC distinguishes Favorable (FH) and Unfavorable Histology (UH) group based on age-linked evaluation of histologic features.

Purpose: To determine optimum age cut-offs for maximizing the prognostic effects of histologic features, and to formulate Data-Driven INPC (DDINPC).

Materials and Methods: 4,078 cases with histologic variables (Neuroblastoma=NB, Ganglioneuroblastoma, Intermixed=GNBi, Ganglioneuroma=GN, Ganglioneuroblastoma, Nodular=GNBn; grade of neuroblastic differentiation (Grade): Undifferentiated=UD, Poorly differentiated=PD, Differentiating=D; Mitosis-Karyorrhexis Index: Low=L-MKI, Intermediate=I-MKI, High=H-MKI) from INRG database were analyzed. Cox proportional hazards model compared young vs. old for different age cut-offs within histologic subgroups: UD/PD, D, L-MKI, I-MKI, H-MKI. Optimum age cut-off had the maximum hazard ratio of statistically significant (p<0.0001) cut-offs. DDINPC was formulated based on survival-tree regression (5-year EFS cited).

Results: GNBi/GN had excellent prognosis regardless of age. As for NB/GNBn, older age indicated significantly worse EFS within each subgroup. Optimum cut-offs were: UD/PD: 15-19mo; L-MKI: 19mo; I-MKI: 24mo; H-MKI: 12mo. Risk continuously increased with increasing age for D (no cut-off). Three DDINPC risk groups were identified: **Low**: GNBi/GN (n=182, 92±5%EFS), NB/GNBn: Age<18mo, L-MKI (n=1,397, 89±3%EFS) or I-MKI (n=392, 84±5%EFS); **Intermediate**: NB/GNBn: Age≥18mo, D, L-MKI, (n=158, 80±6%EFS), **High**: NB/GNBn: H-MKI (n=393; 37+5%EFS); Age≥18mo, UD/PD, L-MKI, (n=418, 38±5%EFS); Age≥18mo, I-MKI (n=282, 30±6%EFS).

Conclusion: MKI had prognostic effect regardless of age. Within L-, I-, and H-MKI subgroups, risk for event increased with increasing age. The prognostic significance of Grade depended on age. Grade had predictive value within patients ≥18mo, but not in patients <18mo. DDINPC included a) combination of UD (extremely rare) and PD in one group; and b) elimination of the 60mo age cut-off and creation of Intermediate-Risk group "Age≥18mo, D, L-MKI". In INPC, a) UD was separated from PD and classified as UH regardless of age; and b) "Age≥18mo, D, L-MKI" was divided into FH (<60mo) and UH (≥60mo). Otherwise, DDINPC was the same as INPC.

C3 Significance of *MYCN* Amplification in INSS Stage 1 and 2 Neuroblastoma (NB): a Report from the INRG Project

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Background: Treatment of children with localized *MYCN* amplified NB tumors is controversial. Patient characteristics and outcomes in this subgroup were analyzed to aid development of an evidence-based treatment strategy.

Methods: The International Neuroblastoma Risk Group (INRG) database was used to analyze age, stage, histology, *MYCN* status, tumor cell ploidy, 1p aberration, initial treatment, and outcome of patients with INSS stage 1 and 2 NB. Event-free (EFS) and overall (OS) survival rates were assessed using the Kaplan-Meier method.

Results: Data regarding 2,660 patients with low stage NB and known *MYCN* status were analyzed. Amplification was detected in tumors from 87 patients (3%). Patients with *MYCN* amplified low stage tumors had less favorable 5-year EFS and OS than did patients with non-amplified tumors (53%±8% and 72%±7% vs 90%±1% and 98%±1%). Within the cohort of patients with *MYCN* amplification, EFS and OS were higher for patients whose tumors were hyperdiploid rather than diploid (82%±20% and 94%±11% vs 37%±21% and 54%±15%). 1p aberration did not have prognostic significance in this cohort. EFS and OS did not differ for those with stage 1 vs stage 2 disease (50%±12% and 76%±9% vs 57%±12% and 67%±11%) or for those greater or less than 18 months of age at diagnosis (58±11% and 77±9% vs 44±16% and 63±15%) . Initial therapy consisted of surgery alone in 29 of 87 patients. EFS and OS in this cohort were 50%±16% and 81%±11%, respectively. Nine patients with *MYCN* amplified, low stage NB underwent stem cell transplantation as part of upfront therapy. EFS and OS for these patients were 50%±25% and 62%±27%.

Conclusions: Patients with *MYCN* amplified low stage NB have a poorer prognosis than patients whose tumors are non-amplified. Surgery was curative for a portion of patients with *MYCN* amplified low stage tumors while transplantation did not appear to improve outcome.

C4 Prognostic Markers for Stage 3 Neuroblastoma: A Report from the International Neuroblastoma Risk Group (INRG) Project

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Background: There is lack of consensus on prognostic markers other than age and tumor *MYCN* amplification for children with INSS stage 3 neuroblastoma.

Methods: The INRG database (n=8800 patients) was utilized to assess whether age, elevated ferritin (≥ 96 ng/ml), tumor histology or somatically acquired chromosomes (ch) 1p or 11q aberrations impact EFS and OS from INSS Stage 3, *MYCN* not amplified (NA) neuroblastoma. Due to the confounding of Shimada histology with age, only tumor differentiation grade and MKI were tested in multivariate models.

Results: Of 1,483 patients with INSS stage 3 tumors, 1,013 had *MYCN*-NA tumors. Among these, 654 (64.6%) patients were age <547 days (<18 months) at diagnosis. The 5-year EFS and OS rates for patients with INSS stage 3 *MYCN*-NA tumors were 81%±2% and 89%±1%, respectively. Age ≥18 months at diagnosis was associated with significantly decreased EFS and OS compared to age <18 months (EFS 64%±3% vs. 90%±2% p<0.0001; OS 76%±3% vs. 95%±1%, p<0.0001). For patients <18 months of age, tumor ch11q aberrations had a significantly inferior EFS (64%±22%) that remained independently prognostic in multivariate analysis (HR 5.3, p=0.0351). The presence of tumor ch11q aberrations or elevated serum ferritin was associated with a significantly worse OS (73%±22% and 88%±4%, respectively) but neither was independently prognostic in the multivariate model. For patients age ≥18 months, poorly/undifferentiated histology and elevated serum ferritin led to a significantly inferior EFS and OS but only ferritin was independently prognostic in multivariate analyses (EFS: HR 2.5, p=0.0319; OS: HR 2.7 p=0.0052).

Conclusions: Patients <18 months of age at diagnosis of INSS Stage 3 *MYCN*-NA neuroblastoma with somatically acquired ch11q aberrations are at significantly higher risk for relapse. In older patients, elevated serum ferritin or poorly/undifferentiated tumor histology significantly increase risk for recurrence. These biomarkers should be considered in treatment algorithms.

Plenary Session: Clinical Study 2

C5 Adolescents and Young Adults with Neuroblastoma: A Report from the INRG Project

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Background: Neuroblastoma in adolescents and young adults (AYA) is a rare problem in an orphan disease. Reports suggest an indolent course with poor survival, but little is known about clinical and biological characteristics that distinguish a more indolent, difficult to cure phenotype.

Methods: We performed a retrospective analysis of the International Neuroblastoma Risk Group (INRG) database (n=8,800) to determine if the well-defined prognostic risk factors are applicable to older patients, and if transplant is effective in this subgroup. We defined two analytic cohorts for comparison: age ≥ 5yrs (n=930) and ≥10yrs (n=200). Uni- and multivariate analyses considered all prognostic factors from INRG, and outcome compared by log-rank test.

Results: Within all patients ≥5yrs, variables significantly predictive of event-free (EFS) and overall survival (OS) in univariate analyses were INSS stage, *MYCN* status, histology, and primary tumor site; with only stage, *MYCN* status, and histology independently significant for EFS in multivariate analyses. Within stage 4 patients ≥5yrs, only *MYCN* was significantly predictive of outcome. Within patients ≥10yrs, variables significantly predictive of EFS and OS were stage, *MYCN*, and histology. Despite only 9% of cases ≥10yrs had *MYCN*-amplified disease, outcome was poor in all patients ≥10yrs (5y EFS 32%±4%; 5y OS 46%± 5%). Patients ≥5yrs with stage 4 disease that underwent a transplant had significantly better EFS and OS than patients who did not (p<0.0001, p=0.0001). The same is true within the stage 4 subset who are ≥10yrs (p=0.0317, p=0.0468). Comparisons of OS by age group (≥18mo-<5yrs[A] versus ≥5yrs-<10yrs[B] versus ≥10yrs[C]) showed no difference. However, for relapsed patients only, older patients had significantly prolonged OS (p<0.0001: [A]vs[B], [A]vs[C]), but an ultimately dismal outcome.

Conclusions: AYAs with neuroblastoma have a distinct disease that is difficult to cure and requires novel therapeutic strategies. Autologous hematopoeitic stem cell transplantation does appear to provide benefit in this population.

C6 A Randomized Phase 3 Trial of Myeloablative Autologous Peripheral Blood Stem Cell (PBSC) Transplant (ASCT) for High-Risk Neuroblastoma (HR-NB) Employing Immunomagnetic Purged (P) versus Unpurged (UP) PBSC: A Children's Oncology Group (COG) Study

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Background: ASCT improves outcome for HR-NB, but potential risk of reinfusion of viable tumor in PBSC could affect event-free survival (EFS). We assessed the impact of ASCT with immunomagnetic purging of PBSC, compared to UP product, on EFS, PBSC tumor content, engraftment, and ASCT toxicity.

Methods: Between 2/2001 and 3/2006, 489 eligible newly diagnosed HR-NB pts were randomized at study entry to have P or UP PBSC obtained and cryopreserved after 2 cycles of chemotherapy. Purging was performed centrally using carbonyl iron depletion of phagocytes followed by 2 cycles of magnetic beads with 5 anti-NB monoclonal antibodies. After 6 cycles, pts received melphalan (180-210 mg/m^2), carboplatin (AUC 4.1 to max 1700 mg/m^2), and etoposide (800-1352 mg/m^2) based on GFR followed by PBSC. Post-ASCT radiation given to primary and MIBG+ sites, then six 13-*cis*-retinoic acid cycles. EFS and overall survival (OS) from enrollment were analyzed as intent-to-treat.

Results: Median age at entry was 3.1 yrs, 44% of 392 tumors tested had *MYCN* amplification, 424 were stage 4 and 368 were transplanted (178 P & 190 UP). Crossover from P to UP arm occurred in 30 pts and from UP to P in 5 pts. Only 5 pts (1 P & 4 UP) had NB detected in PBSC by immunocytology, none after purging. RT-PCR of tumor in PBSCs is ongoing. Median days to ANC>500 was 13 for P and 11 for UP (p=0.0001). Toxic death following ASCT was 3.2%, with no difference in toxicities between arms. Two-year EFS (SE) was 47 (+/-3%) for purged (N=244) and 49+/-3% for unpurged (N=245)(p=0.7881). Two-year OS +/- SE was 69% (+/-3%) and 73 (+/-3%)(p=0.2920) for purged and unpurged respectively.

Conclusions: Centralized immunomagnetic purging of PBSC in the context of dose- intensive chemoradiotherapy and biologic therapy in first response did not impact EFS or OS at 2 years.

C7 Excellent Outcome with Reduced Treatment for Infants with Disseminated Neuroblastoma without MYCN Gene Amplification. Results of Two Siopen Studies.

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Purpose: This co-operative study describes presenting features, treatment and outcome of a population of infants with disseminated neuroblastoma without *MYCN* amplification. Based on previous observations, a *wait and see* policy was extended to infants without relevant symptoms for having large primary or positive bone scan without X-ray/CT abnormalities.

Patients and Methods: Between 6.1999 and 6.2004 a total of 181 infants with disseminated neuroblastoma without *MYCN* amplification were eligible for INES trials. Of the 170 evaluable, 125 were enrolled in 99.2-Trial (86 with low symptom score [LSS] and 37 with high symptom score [HSS]) and 45 were enrolled in 99.3-Trial.

Results: Of 125 99.2-Trial infants, 86 had LSS of whom 56 did not receive chemotherapy (nine progression, 2 deaths) and 30 received chemotherapy (three progression, no deaths) and 37 had HSS (four progression, 3 deaths) for an 5-y EFS of 87.8% and a 5-y OS of 95.7%. None of the 23 infants with midline infiltration, none of the 17 with positive bone scan (± midline infiltration) died of disease. The 45 infants of 99.3-Trial had six progression and two deaths for a EFS of 86.7% and an OS of 95.6%. The only factor affecting both EFS and OS was chromosome 1p deletion.

Conclusion: the outcome of infants with disseminated Neuroblastoma without *MYCN* amplification is excellent. Infants with large primary and positive bone scan without X-ray/CT abnormalities may undergo an observational policy in absence of symptoms without compromising since of cure.

C8 Reduction of Treatment Intensity in Infants with Localized Neuroblastoma

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Background: We previously reported regression in localized infant neuroblastoma. Here we present the interims analysis of the subsequent trial NB2004 for infants with localized neuroblastoma, where the previous observation strategy was adopted, and interventional chemotherapy was further reduced.

Methods: Data of infants with localized, non-amplified neuroblastoma diagnosed October 2004 to April 2007 were analysed. Interventional chemotherapy (cyclophosphamide, doxorubicin, vincristin) was given to patients with serious symptoms only until the release of symptoms, regardless of the size of residual tumor.

Results: Eighty-eight infants with localized, non-amplified neuroblastoma were registered (31 stage 1, 31 stage 2, 27 stage 3). Tumors were resected in 38 patients, chemotherapy was applied in 15 patients, and 35 patients with unresected tumors were observed without cytotoxic treatment.
Local progression was seen in 15/88 patients (2 surgery; 2 chemotherapy; 11 observation group), progression to stage 4S in 4/88 patients (1 surgery, 3 observation group). Among these 19 patients with progression, 11 were treated with chemotherapy.
Regression occurred in 17/35 patients observed without treatment (49%; 7 patients finally achieved complete regression), thus confirming the results of the previous trial. Compared to the previous trial, less patients underwent initial resection (NB2004: 43%, NB95/97: 56%, p=0.04), and more were observed (NB2004: 40%, NB95/97: 27%, p=0.03).
The percentage of patients treated with chemotherapy (at diagnosis and for progression) was not different (NB2004: 30%, NB95/97: 23%, p=0.27), but number of cycles was reduced (median NB2004: 2, NB95/95 4 cycles, p<0.001). All but one patient treated with chemotherapy responded. Sixteen patients with partial remission at the end of chemotherapy showed regression later (6 CR, 10 VGPR). Overall survival was excellent. Only one patient died from progression (chemotherapy group).

Conclusions: Only 30% of infants with localized neuroblastoma needed interventional chemotherapy. The reduction of cycles did not impair the excellent prognosis.

C9 Consolidation Treatment with anti-GD2-Antibody ch14.18 in Children with Stage 4 Neuroblastoma Might Prevent Late Relapses

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Background: In trial NB97, children 1 year or older with stage 4 neuroblastoma underwent consolidation treatment with anti-GD2-antibody ch14.18 after high intensive induction chemotherapy. First analysis of this non-randomized trial showed no impact of ch14.18 on the event rate (J. Clin. Oncol. 2004; 22: 3549-57). Here, we report outcome data after further follow-up.

Methods: A total of 334 stage 4 patients >1 year were included. 166 patients completed initial treatment of trial NB97 successfully and were scheduled for six ch14.18 cycles (20 mg/m²xd over 5 days) given every 2 months (+AB-MT). Patients of the preceding trial NB90 who received oral low dose maintenance chemotherapy (-AB+MT, n=99) and those who did not receive any consolidation after induction chemotherapy (-AB-MT, n=69) served as controls.

Results: The median observation time was 9.2 years (2.3-16.2). Since last analysis, 36 patients in second remission had a second relapse and 25 of them finally died. Another 15 patients developed first relapse. Nine of them died. The three year event free survival rates were 49±4%, 44±5%, and 36±6% for +AB-MT, -AB+MT, and –AB-MT patients, respectively (logrank test p=0.101). In contrast to the earlier report, paired logrank test now clearly demonstrated an advantage of ch14.18 treatment (+AB-MT) compared to no additional therapy (-AB-MT, p=0.038). +AB-MT and –AB+MT treatment were very similar (p=0.152). As already published in 2004, the overall survival was better in the +AB-MT group (3-year-OS 68±4%) compared to –AB+MT (57±5%) and –AB-MT (45±6%, p=0.015). However, stepwise multivariate Cox regression analysis did not demonstrate an independent impact of ch14.18 consolidation therapy neither on EFS or OS.

Conclusions: Immunotherapy with anti-GD2-antibody ch14.18 as consolidation after high intensive chemotherapy may prevent late relapses. Further observation of the cohort is warranted.

C10 A Comprehensive Review of the Role of Primary Tumor Resection in Stages 3 and 4 Neuroblastoma

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Background: There are no prospective studies of the role of primary site resection in Stage 3 or 4 neuroblastoma. Published results evaluating the efficacy of gross total resection are inconsistent. Despite this, most protocols continue to recommend attempts at complete resection. We analyzed the world literature regarding surgical resection of the primary tumor in high stage neuroblastoma for an effect on survival and local control.

Methods: A comprehensive literature review was done using the keywords: neuroblastoma, ganglioneuroblastoma, esthesioneuroblastoma, surgery, surgical, resection, survival, local control, and primary tumor. Databases searched included: PubMed, Embase.com, the Cochrane Evidence Based Medicine database, and Scopus. The time period was all years and the search included all languages. All publications that included surgical data on stage 3 or 4 patients were included in analysis. This resulted in the identification of 32 non-redundant studies with adequate surgical data for inclusion in analysis with survival as the endpoint. Eight studies were identified with data adequate for analysis of an effect on local control. Data analysis was carried out using a random effect model in RevMan soft ware (Review Manager). Results are reported as odds ratios plus upper and lower limit and >90% resection is compared to less complete removal[1].

Results: Funnel plot analysis showed no evidence of publication bias. Results of meta-analysis are listed below.

Endpoint	Groups analyzed	N(Reports/ patients)	Odds ratio(range)	P<
Overall survival	Stage 3 and 4	32/2635	0.365(0.251-0.530)	0.0001
Overall survival	Stage 3	5/541	0.268(0.154-0.467)	0.0001
Overall survival	Stage 4	11/1357	0.400(0.219-0.728)	0.003
Local recurrence/progression	Stage 3 and 4	8/412	0.217(0.121-0.390)	0.0001

Conclusions: These data support complete removal of the primary tumor in these children.

1. Odds of endpoint with complete resection divided by the odds without.

Poster Discussion: Basic Research

B43 Identification and Manipulation of Genes Involved in the Differentiation of Neuroblastoma

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Background: Defects in differentiation are one of the major events present in highly malignant neuroblastoma tumours. Recent evidence suggests differentiated neuroblastomas are associated with improved patient outcome, but little is known regarding the molecular regulation of differentiation.

Methods: To investigate the mechanisms underlying the induction of differentiation in neuroblastoma, 3 differentiation models for IMR-32 neuroblastoma cells were established by whole chromosome 1 transfer, *MYCN* knockdown, and 9-cis retinoic acid treatment. Differentiation was classifed by neurite length > 2 x soma and expression of biochemical markers. Global gene expression was measured using Affymetrix microarrays to identify differentially expressed genes in the 3 models. The role of candidate genes in differentation was further investigated by RNA knockdown using RNA interference and stable transfection in IMR32 cells.

Results: Differentiated cells with neurite-like processes showed positive immunostaining with neuronal differentiation markers, including neurofilament, synaptophysin, and microtubule associated protein-2. Genome wide expression array studies revealed 128 genes related to ion and protein transport, G-protein signalling, cell death, and development, consistently changed > 2-fold during differentiation. Changes in expression of up-regulated genes related to G-protein signalling (*RGS4*), and neuronal/axonal genesis (*STMN4, ROBO2, GAP43*, and *ADM*), and down-regulated genes including *KLF4, SPRY2*, and *LATS2* were validated by Q-RT-PCR. The importance of selected genes in differentiation was confirmed by the induction of biochemical and morphological differentiation following stable over-expression of STMN4, ROBO1, and ROBO2 separately, and knockdown of KLF4 and SPRY2 separately in IMR-32 cells.

Conclusions: Differentiation of neuroblastoma can be induced by individually modulating the expression of a subset of genes, including *STMN4, ROBO1, ROBO2, KLF4,* and *SPRY2*. This provides an insight into mechanisms that can potentially be exploited to manipulate the differentiation status of neuroblastomas. The detailed mechanisms involved in the differentiation induced by modulating these candidate genes are the subject of further investigations.

B44 Sleeping Beauty Insertional Mutagenesis and Genetic Linkage Mapping Identify Loci Governing Tumor Susceptibility in a Mouse Model for Neuroblastoma

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Background: Mice carrying the TH-*MYCN* transgene (targeting MYCN expression to the neural crest via the rat tyrosine hydroxylase promoter) develop abdominal and thoracic paraspinous tumors that show histological and genetic features of neuroblastoma. Murine neuroblastoma displays strain-dependent susceptibility, indicating the presence of genetic modifiers of tumor development that may be relevant to human neuroblastoma. Mice in strain FVB/N are totally resistant to tumors, while mice in strain 129/SvJ are 100% susceptible by 4 months. F1 progeny show a 5% penetrance after a long (7-8m) latency, suggesting dominant resistant modifier genes. One-third of backcross mice (F1 x 129/SvJ) succumb to tumors.

Methods: We utilized two approaches to identify murine neuroblastoma susceptibility genes. First, we genotyped 239 backcross mice with 270 SNP and microsatellite markers and tested for genetic linkage to tumor incidence. Second, we employed the Sleeping Beauty (SB) transposon insertional mutagenesis system in TH-*MYCN* transgenic FVB/N mice. In this system, T2/Onc transposons are randomly inserted throughout the genome, activating and deactivating host genes. Cells harboring transposon-activated oncogenes or deactivated tumor suppressors are clonally expanded in developing tumors. Genes harboring insertions in multiple tumors (detected using the transposon as a genetic tag) are considered common insertion sites (CIS) and represent possible oncogenes or tumor suppressors.

Results: Backcross linkage analysis has identified a locus on mouse chromosome 10 with a LOD score of 5. Haplotype analysis of this region narrows the number of candidate genes to 10. Separately, mobile T2/Onc transposons in TH-*MYCN* FVB/N animals have to date generated five tumors in a genetic background that is normally 100% resistant, revealing three CIS (genes hit in 2 tumors). We are characterizing genes identified in these screens for involvement in tumor progression.

Conclusions: Identification of susceptibility genes in mice will illuminate pathways in human neuroblastoma and present novel drug targets.

B45 A Road to Elucidate Molecular Pathways Involved in Neuroblastoma Pathogenesis

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Background: Only few genes have been firmly implicated in neuroblastoma pathogenesis on basis of amplification, mutation or homozygous deletion. In contrast, many recurrent chromosomal gains and losses are known, which affect gene expression levels of hundreds of genes. Oncogenic pathways may be activated by combined copy number imbalances of activating and repressing genes and the consequent changes in expression level. Identification of pathways active in neuroblastoma is therefore important.

Methods: We constructed neuroblastoma cell lines with inducible expression or silencing (shRNAi mediated) of genes with a postulated role in neuroblastoma, like MYCN, CDK2, NOTCH, DLK1, MEIS1, PHOX2B, DKK3 etc. After induction of activation or silencing, we analyzed the subsequent changes in gene expression by microarray analysis of time-courses. Additionally, we generated microarray expression profiles of 110 neuroblastoma tumors. All data were entered in the R2 bioinformatic analysis tool.

Results: Manipulation of gene expression typically resulted in changes in expression of a few hundred genes, thus identifying the downstream pathways of the analyzed genes. When the expression levels of the genes in such individual pathways were analyzed in the tumor series, striking correlations were found (e.g. MYCN downstream pathway genes showed a correlation with MYCN expression in the tumor series). Combining the data from the cell lines and the tumor series thus identified core sets of genes in the different pathways. The pathways appeared to be strongly interconnected: many of the pathways consist of partly overlapping sets of downstream genes. This identified interdependencies in pathways, enabling a search for conditional lethal drug targets, e.g. in the MYCN and cell cycle pathways.

Conclusions: A dazzling complex network of gene expression regulation is emerging in neuroblastoma. This may form a basis to identify mechanisms of pathway activation as mediated by copy number changes of the hundreds of genes identified in this study.

B46 Gene Expression Profiling of *In Vivo* Resistance to Irinotecan in a Neuroblastoma Model

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Background: To study clinical resistance to camptothecins in neuroblastoma, we have recently developed an *in vivo* resistance model by prolonged exposure of a NB xenograft to irinotecan and showed that this model was of great interest because resistance mechanisms usually described *in vitro* were not observed. The objective of the present study was to characterize this model using high-throughput microarrays, to identify underlying resistance mechanisms.

Methods: The existence of 3 tumour phenotypes in our model (sensitive, resistant and reverted) was exploited to build a strong experimental design. Hybridization experiments were realised on Agilent 22K microarrays and data were processed using Resolver software. Genes were selected when having a fold change > |1.5| in resistant tumours compared to both sensitive and reverted ones ($p<0.001$). Validation of microarray data was realised using quantitative RT-PCR, Western-Blotting, immunohistochemistry and ELISA.

Results: The transcriptomes comparison of sensitive, resistant and reverted tumours led to the identification of 38 genes (out of 22.000) having a deregulated expression in accordance with a resistant phenotype to irinotecan. 5 genes were further validated using qPCR on an independent set of samples: the Corticotropin Releasing Hormone (CRH), the Anaplastic Lymphoma Kinase (ALK), the Transforming Growth Factor Beta 3 (TGFB3), the Interleukin 13 Receptor Alpha 2 (IL13RA2) and the Pleiotrophin (PTN) genes. Validations at the protein level showed that only 2 genes were always deregulated in accordance with CPT-resistance : PTN and ALK, ALK being up-regulated in resistant tumours while PTN was down-regulated. Interestingly, ALK and PTN have been reported to be receptor and ligand.

Conclusions: We described an original gene expression profile of *in vivo* resistance to irinotecan in neuroblastoma, and a strong association of the ALK-PTN pathway with this resistance. This pathway has been previously associated with both neuroblastoma progression and chemoresistance, and could thus lead to relevant resistance mechanisms.

B47 Direct and Coordinate Regulation of ABC Transporter Genes by N-Myc in Neuroblastoma

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Background: Resistance to chemotherapeutic agents is in part caused by the altered activity of a specific group of ATP-binding cassette transporters (ABC) responsible for the efflux of chemotherapeutic agents in cancer cells. In most cases, this is achieved by mis-regulation of their mRNA expression. Thus, establishing how ABC transporter genes are controlled at the transcription level and which transcription factors account for such control may help elucidate some aspects of how drug resistance is established in cancer. Here we have investigated how N-Myc can regulate transcription of the ABC gene family in neuroblastoma

Methods: We have employed both transcription profiling and chromatin immunoprecipitation (ChIP) analyses. Specifically we have developed a new ChIP technique named dual crosslinking ChIP, in which two crosslinking agents are used to stabilize the N-Myc complex in vivo. Finally, to confirm the direct role of N-Myc on ABC gene transcription, ABC promoters were cloned into a luciferase vector and tested in neuroblastoma cells as a function of N-Myc expression

Results: Our results show that N-Myc regulates ABC gene transcription as either an activator or a repressor. In particular N-Myc activates transcription of ABCC1/MRP1, and ABCC4/MRP4 genes by direct binding to E-box sites present within their promoters. N-Myc can also repress transcription of ABCC3/MRP3. In this case repression results from the interaction of N-Myc with the Sp1 transcription factor bound to the ABCC3 promoter but not through recognition of typical E-box sites. Notably, c-Myc can also affect transcription of the same set of ABC genes in leukemia and breast cancer cells through similar dynamics.

Conclusions: Overall, our findings demonstrate a general role of the Myc oncoproteins in the coordinate regulation of several ABC transporter genes and support a model in which Myc over-expression may contribute to the appearance of drug resistance in cancer.

B48 Exploiting Gene Expression Profiling to Identify Novel Markers of Neuroblastoma

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Background: Despite achieving clinical remission, many patients with metastatic neuroblastoma (NB) relapse and die, due in part to the presence of subclinical minimal residual disease (MRD). Immunotherapy and biologic therapy directed against MRD can improve outcome. The ability to measure MRD is critical for gauging the success of these targeting approaches, especially in the key metastatic compartments of marrow and blood. However, no single MRD marker will be adequate because of tumor heterogeneity. Genome-wide expression profiling can uncover novel genes differentially expressed in NB tumors over normal marrow/blood with potential as MRD markers.

Methods: Gene expression array was carried out on 48 stage 4 NB tumors and 9 remission marrows using the Affymetrix U-95 gene chip. 34 genes with a tumor-to-marrow expression ratio higher than tyrosine hydroxylase, the most widely used NB marker, were identified. Quantitative RT-PCR was performed on all 34 genes to study the sensitivity range of tumor cell detection and the expression of these genes in normal marrow/blood samples and in stage 4 NB tumors. Top ranking markers were then tested for prognostic significance in the marrows of stage 4 patients collected from the same treatment protocol after 2 cycles of immunotherapy (n=116).

Results: Based on sensitivity assays, 8 top-ranking markers were identified: CCND1, CRMP1, DDC, GABRB3, ISL1, KIF1A, PHOX2B, and TACC2. They were also highly expressed in stage 4 NB tumors (n=20) and had low to no detection in normal marrow/blood samples (n=20). Moreover, expression of CCND1, DDC, GABRB3, ISL1, KIF1A, and PHOX2B in post-treatment marrows had prognostic impact on the progression-free survival of these stage 4 patients with a median followup of 5.9 years ($p<0.001$).

Conclusions: Marker discovery based on differential gene expression profiling, stringent sensitivity and specificity assays, and well-annotated patient samples, can rapidly prioritize and identify novel MRD markers of neuroblastoma.

B49 MYC Overexpression and MYCN Pathway Activation in MYCN-Nonamplified Unfavorable Neuroblastoma

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Background: It is well-known that MYCN gene amplification resulting in its over-expression is associated with poor prognosis of neuroblastoma. However, the molecular pathways activated by MYCN overexpression and the genetic alterations leading to poor prognosis in MYCN-nonamplified neuroblastoma are largely unclear.

Methods: MYCN expression was suppressed using two independent siRNAs in MYCN-amplified neuroblastoma cell lines NB39 and TGW, and the alterations of gene expression were analyzed with an Affymetrix DNA microarray HG-U133_plus_2.0. Genes up- or down-regulated by MYCN were identified, and their expressions in neuroblastoma tumors were studied using gene expression data of 10 MYCN-amplified tumors and 7 unfavorable (death within 4 years) and 37 favorable (survinal of ≥4 years and mass screening) MYCN-nonamplified tumors.

Results: The microarray analysis of siRNA-treated neuroblastoma cell lines identified >300 genes as MYCN downstream candidates. Then, 23 genes (6 up-regulated and 17 down-regulated) were selected as representative MYCN downstream genes in actual tumors, by considering their expressions in MYCN-amplified and MYCN-nonamplified favorable tumors. DDC, NTRK1, and PRPH were included in the down-regulated genes. The average normalized expression value of those 23 genes was calculated as an index indicating the status of whole MYCN downstream genes, and used to evaluate the MYCN pathway activation level of each tumor. Expectedly, MYCN-amplified tumors exhibited high indices (median 0.993), and MYCN-nonamplified favorable tumors exhibited low indices (median -1.012). Interestingly, indices of MYCN-nonamplified unfavorable tumors were varied, and two of them displayed especially high indices (1.346 and 1.507), indicating MYCN pathway activation. In those tumors, MYC instead of MYCN was highly expressed. In addition, MYC suppression in MYCN-nonamplified neuroblastoma cell lines affected the expression of MYCN downstream genes similarly as MYCN suppression in MYCN-amplified cell lines.

Conclusions: In some MYCN-nonamplified neuroblastoma, overexpression of MYC contributes to poor prognosis through MYCN pathway activation by regulating MYCN downstream genes.

B50 Molecular Allelo-Karyotyping of Neuroblastoma Using High-Resolution Single Nucleotide Polymorphism Genomic Microarrays

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Background: Neuroblastoma (NB) is one of the most common pediatrcic cancers arising from undifferentiated neural crest cells that are committed to sympathetic neurons. Although highly variable clinical and biological features suggest the considerable heterogeneity in NB cases, the genetic basis of this heterogeneity has not been fully understood. Therefore, to identify the genetic changes underlying molecular pathogenesis of NB, we performed comprehensive analysis of copy number alterations as well as allelic imbalances in NB genomes using high-density SNP-genotyping microarrays.

Methods: Previously, we developed a novel algorithm, CNAG/AsCNAR, that enables accurate determination of allele-specific copy numbers as well as sensitive detection of LOH even in the face of up to 70-80% normal cell contamination by sensing subtle distortions in allele-specific signals caused by allelic imbalance using anonymous controls (Am J Hum Genet 2007). Using Affymetrix® GeneChip® Human Mapping 250K array and the CNAG/AsCNAR algorithm, we analyzed a total of 215 primary NB samples.

Results: In our analysis, a number of common homozygous deletions, LOH and high-grade amplifications were identified not only in the previously-described regions such as 1p, 11q and 14q, but also in regions that have never been reported before including 3p, 3q, 6q, 7q, 16p, and 18p. While individual genomic profiles are variable among different cases, they collectively showed a characteristic genomic profile in each stage. Advanced stages of NB genomes are characterized by common 17q gain with or without 1p LOH/*MYCN* amplification, and gains in 1q, 2p, and 7q, loss of 3p, 10q and 11q, in varying combinations.

Conclusions: Copy number analysis using high-density SNP-genotyping microarrays is a powerful tool for genome-wide detection of genetic abnormalities in NB and expected to facilitate identification of novel genes relevant to development of NB.

B51 Gene Expression Profile in the Precancerous and Initial Stage of Neuroblastoma in MYCN Tg Mice

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Background: MYCN transgenic (Tg) mouse is a model for neuroblastoma. Because the celiac ganglion is the tumor origin in most cases, and the period of tumor onset is restricted (around 2-week old in homozygous mice), these mice are profitable to address gene expression profiles in the precancerous and initial stages of neuroblastoma.

Methods: To examine gene expression profiles, we dissected following tissues: 1) normal celiac ganglion (wild-type mice, 2-week old), 2) celiac ganglion at hyperplasia stage (homozygous mice, 2-week old), 3) neuroblastoma at initial stage (homozygous mice, 3-week old), 4) neuroblastoma at initial stage (hemizygous mice, 9-10-week old). cDNAs were synthesized and hybridized to MG 430 2.0 Array (Affymetrix).

Results: 626 genes were upregurated in hyperplasia ganglia comparing to normal ones. Among them, 17 genes, containing 9 transcription factors, were upregulated more than 10 times. 1676 genes were downregulated in initial tumors comparing to hyperplasia ganglia. Most of those up- and downregulated genes showed intermediate expression levels in the hyperplasia ganglia comparing to normal ganglia and initial tumors. These results might indicate the precancerous character of the hyperplasia ganglia. We next addressed the chromosomal location of those genes. 9 upregulated genes were positioned in human 17q (17q21-qter). Survivin, which has been already reported as a tumor promoter, was included in these 9 genes. 11 downregurated genes and 14 downregurated genes were positioned in human 1p (1p36.1-pter) and 11q (11q23), respectively. We also examined expression profiles of some stemness-related genes to address the involvement of cancer stem cells in tumorigenesis. As a result, we found that a few genes were upregulated in hyperplasia ganglia and initial tumors.

Conclusions: We identified some candidate genes involved in the tumorigenesis of neuroblastoma. Some of them may be related to chromosomal aberrations. Among them, there should be good targets for molecular therapy of neuroblastoma.

B52 Molecular Phenotyping of True Positive and False Negative Primary Neuroblastomas from the German Mass Screening Program

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Background: The molecular phenotypes of neuroblastoma patients with true positive (TP) and false negative (FN) mass screening results were analyzed and compared by means of gene-expression profiling.

Methods: A cohort of 80 patients participating in German mass screening programs between 1991 and 2000 was analyzed using a customized oligonucleotide microarray (TP stages 1/2/3/4/all: n=31/12/4/8/55, FN stages 1/2/3/4/all: n=3/5/5/12/25). Differentially expressed genes were identified using the *Significance Analysis for Microarrays* (SAM) algorithm and categorized using the Gene Ontology (GO) database. Hierarchical cluster analyses using differentially expressed genes and unsupervised principal component analysis (PCA) were performed to elucidate underlying biological subgroups and phenotypes of TP and FN tumors. In addition, patients were classified using the *Prediction Analysis of Microarrays* (PAM) algorithm.

Results: Between TP and FN tumors, 1873 differentially expressed genes were identified by SAM (FDR<0.05), most of which were involved in cell cycle regulation or cell growth and division. Hierarchical clustering analysis using these 1873 genes grouped screening patients in two dominant subcohorts: 1. FN patients with either stage 4 or *MYCN*-amplified disease and 2. TP patients (stage 1-4) and FN patients with non *MYCN*-amplified stage 1-3 disease. The same subgroups were also separated by unsupervised PCA. Furthermore, little to no difference was found between TP patients with metastatic stage 4 and TP patients with localized stage 1-3 disease neither by PCA nor SAM analyses. Likewise, classification of screening patients grouped patients rather according to biological tumor behavior than according to screening status.

Conclusions: Gene-expression profiling reveals a similar biology of stage 1-3 not detected by mass screening and all mass screening detected stages. Large differences of gene expression profiles were found to MYCN amplified and screening negative stage 4 neuroblastomas.

B53 Disentangling MYCN Downstream Signaling via Meta-Analysis of Targets in the MYCNot Database

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Background: Amplification and subsequent overexpression of the *MYCN* transcription factor is the hallmark of an aggressive subgroup of neuroblastomas. While this member of the MYC-family was one of the first genetic markers for therapy stratification, profound insights into its role in neuroblastoma pathogenesis are lacking. The identification of downstream genes and pathways involved in the tumor phenotype therefore remains a major challenge and could offer the basis for development of new molecular therapies.

Methods: In addition to our experimental approaches for identifying mRNA and miRNA target genes, we also performed extensive literature data mining in order to document all reported primary and secondary targets of MYCN. Gene lists with literature annotations and relevant information on the experimental methods, the nature of the relationship with MYCN, and putative MYCN-binding motifs in the target gene promoter region, are stored in the MYCN oncogene targets database MYCNot.

Results: Currently, the database consists of 1062 coding gene and 27 non-coding gene records from 880 unique mRNAs and 25 unique miRNAs extracted from 113 publications. Gene Ontology analysis showed that a significant part of the MYCN upregulated proteins are involved in ribosome function or are active in DNA processing or cell metabolism. To further expand the limited number of currently validated direct MYCN targets, we performed *in silico* promoter analysis of the MYCN downstream genes. MYCNot is clearly enriched with genes having MYCN binding sites in their proximal promoter regions. For a representative set of genes, predicted MYCN-promoter binding was confirmed using chromatin immunoprecipitation.

Conclusions: MYCNot provides the most complete list of protein coding genes and miRNAs directly or indirectly regulated by MYCN. A meta-analysis of the literature in combination with gene ontology and pathway analysis and experimental validation affirms the widespread mode of action of MYCN in aggressive neuroblastoma. The database is available at: http://medgen.ugent.be/MYCNot/.

B54 Genome-Wide Analysis of Epigenetic Abnormalities in Neuroblastoma Using Oligonucleotide Tiling Array

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Background: Neuroblastoma (NB) is the most common solid tumor in childhood arising from embryonal neural crest tissues. The etiology of NB has not been fully understood although previous studies have shown that genetic abnormalities, such as *MYCN* amplification, gain of 17q and loss of heterozygosity of 1p, are relevant to its development. In addition, it has recently been postulated that "epigenetic" abnormalities such as hypermethylation of tumor suppressor genes often may also play important roles in the pathogenesis of NB. Thus, to better elucidate the role of epigenetic aberrations in the pathogenesis of NB, we performed genome-wide methylation analysis using methylated DNA immunoprecipitation (MeDIP) method in combination with oligonucleotide tiling array analysis.

Methods: In our assay, genomic DNA from NB samples is fragmented by ultrasonication, and immunoprecipitated using anti-methyl-cytidine antibody, followed by PCR amplification. Amplified DNA was labeled, and subjected to hybridization to high-density oligonulceotide tiling arrays (MeDIP-on-chip). We analyzed 20 samples of NB (3 cell lines, 17 primary samples) using Affymetrix Human Promoter 1.0R array which comprised over 4.6million probes tiled through over 25,500 promoter regions and at an average resolution of 35 bp.

Results: We identified a number of candidate methylation sites (CMS) in NB genome and the CMS were subsequently verified by bisulfate sequencing. As previously reported, hypermethylation of *CASP8*, *MDR1* and *RASSF1A* were confirmed in the present study. Furthermore, we also found a number of novel hypermethylated regions in NB.

Conclusions: Our study suggested that epigenetic abnormalities were also involved in pathogenesis of NB as well as genetic abnormalities. The MeDIP-on-chip assay is an excellent and useful tool for comprehensive high-resolution analysis of DNA methylation, and providing new findings in the epigenomics in oncogenesis

B55 Hypoxia Signature of Neuroblastoma Cells: Role Of N-myc Overexpression

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Background: High N-myc expression is associated with advanced neuroblastoma stage and poor prognosis, but the relationship between N-myc and immunity has remained obscure. Hypoxia, a local decrease of oxygen tension, is a critical determinant of tumor progression. Transcriptional activation by hypoxia is mediated primarily by the hypoxia-inducible factor-1 (HIF-1). N-myc shares with HIF the ability to bind the E-box and a crosstalk between hypoxia and N-myc has been suggested.

Methods: Using Affymetrix GeneChip we studied the changes in gene expression caused by hypoxia exposure in 11 neuroblastoma cell lines with or without N-myc overexpression. In addition, we used the SHEP21N cell line with N-myc expression inducible by tetracycline. Cells were exposed to hypoxia (1% oxygen) or normoxia (20% oxygen) for 18 hours. The gene expression data were analyzed using GeneSpring GX 7.3 and new algorithms based on sparsity-based regularization and spectral clustering techniques.

Results: We found that the neuroblastoma cell lines responded to hypoxia with modulation of the gene expression profile involving 300 to 750 genes but only 31 genes were concomitantly upregulated in all cell lines. Gene ontology analysis revealed that similar pathways were modulated by hypoxia in the various cell lines (e.g. glycolysis, angiogensis, apoptosis) despite the fact that different genes were represented in the same pathway. Interestingly, we found that N-myc overexpression (whether achieved by cell engineering or by spontaneous mutations) caused a major change in the response of the cells to hypoxia and let us define N-myc dependent and independent hypoxia signatures. Experiments with neuroblastoma spheroids demonstrate that these myc-dependent changes in hypoxic response alter the influx/recruitment of leukocytes to the hypoxic tumor site and inhibit the macrophage influx.

Conclusions: We demonstrated the existence of a crosstalk between N-myc and hypoxia in neuroblastoma cells and we speculate that this crosstalk could be instrumental in favoring the expression of tumor promoting genes accounting the poor prognosis of N-myc overexpressing neuroblastoma tumors.

B56 Genome-Wide Copy Number Variation Analysis of Neuroblastoma Using High Density Oligonucleotide Genotyping Array

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Background: Genomic aberrations such as gain, loss, and LOH (loss of heterozygosity) of genomic region are frequently observed in cancer cell genome. These alterations are known to be involved in onset and/or development of tumor. By rapidly advancing densification of the DNA microarray, now it became possible to examine the whole genome aberration in tumor with the resolution of the several kb in detail. To unveil the underling molecular mechanisms of onset and/or development of neuroblastoma, we couducted a genome-wide copy number variation (CNV) analysis of 152 sporadic cases of neuroblastoma.

Methods: A genome-wide CNV analysis was performed using high density oligonucleotide genotyping array (Affymetrix GeneChip Mapping 250K EA Nsp). We have developed our own algorithms and data viewer for this analysis.

Results: Genome aberration patterns of 62 cases out of 152 had been settled previously by BAC array based CGH analysis. These samples were equally classified by this analysis. In addition, we analyzed 90 unclassified cases. We analyzed allelic losses of chromosome arms 1p and amplifications of 2p including *MYCN* especially in detail. In 1p losses, there were small amplifications which include a candidate tumor suppressor gene of neuroblastoma in some cases. Some *MYCN* including 2p amplifications were accompanied with an adjacent small losses.

Conclusions: Because the high-density SNP typing array used in this analysis have much higher resolution compared to the BAC array, more detailed analysis was possible.

B57 DNA-Repair Gene Expression at Different Stages of Neural Crest Cells in Mouse Embryos: A Study to Search Genes Associated to Neuroblastoma Pathogenesis

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Background: Neuroblastoma (NB), a paediatric tumour deriving from neural crest cells (NCCs), has been detected in foetus and in neonatal period indicating its onset during foetal life. Some NCCs migrate and form the dorsal root ganglia. Other migrate more ventrally to form sympathetic ganglia, the adrenal medulla (AM) and nerves surrounding the aorta. Although molecular aberrations as *MYCN* amplification, 1p36 deletion and 17q gain are frequently observed; NB carcinogenesis remains unclear. Since some cancers are due to inappropriate or defective DNA-repair machinery, we studied DNA-repair genes during mouse embryos development and in adult tissues.

Methods: We identified and isolated mouse NCCs during 3 developmental stages (E8.5, E13.5 and adult life) by using an *in vivo* mouse model. Migrating NCCs were laser capture microdissected and used for total RNA purification, mRNA linear amplification and cRNA synthesis. Gene expression profiling was evaluated for each stage by using high-density oligonucleotide microarrays Affymetrix GeneChip Mouse-Genome 430 2.0 with biological triplicates. dChip and SAM were used for microarray data analysis.

Results: We observed over-expression of 11 genes (*Apex1, Cdt1, Chaf1b, Chek1, Clspn, Eme1, Ercc6l, Exo1, Rad18, Recc1 and Uhrf*) involved in DNA-repair pathways at E8.5. At E13.5 these genes were less expressed and their expression was undetectable in adult life.

Conclusions: High expression of 6 out 11 genes was never been described in mouse embryology. DNA-repair gene expression indicates a high NCCs activity in repairing error-prone DNA or damage possibly induced by xenobiotics insults. DNA-repair genes has been shown involved in both DNA instability and drugs resistance in NB. Our data suggests that defective DNA-repair machinery during early stages of NCCs may be critical for NB pathogenesis.
We are expanding our study using transgenic TH-*MYCN* model developing neuroblastoma. In addition, we will compare gene expression profiles of NCCs in transgenic and non transgenic mouse models.

B58 Topotecan Antagonizes Hypoxia Effects on Gene Expression of Neuroblastoma Tumors

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Background: Angiogenesis is essential for neuroblastoma tumors development and correlates with their aggressive behavior, metastatic spread, and poor clinical outcome. One of the major drives to tumor angiogenesis is hypoxia, a local decrease in oxygen tension that characterizes solid tumors. Transcriptional activation by hypoxia is mediated primarily by the hypoxia-inducible factor-1 (HIF-1), a heterodimeric transcription factor that transactivates the hypoxia responsive elements (HRE) in the promoter sequence of many genes. We investigated the effects of topoisomerase-1 inhibitor, topotecan, on neuroblastoma cell lines exposed to hypoxia.

Methods: Using Affymetrix GeneChip we studied the changes in gene expression profile of neuroblastoma cell line LAN-5 caused by exposure to hypoxia and by topotecan treatment. Cells were exposed to hypoxia (1% oxygen) or normoxia (20% oxygen) and treated with 500 nM topotecan for 6 hours. The gene expression data and the presence of the HRE in the promoter sequences were analyzed using GeneSpring GX 7.3 software.

Results: We found that 60% of the genes upregulated by hypoxia are not regulated when the cells are treated with topotecan in hypoxic condition and 39% of the genes are even downregulated. This means that 99% of the genes generally induced by hypoxia are affected by topotecan treatment. A gene ontology analysis reveals that most of the genes upregulated by hypoxia but downregulated by topotecan are belonging to the pathways of apoptosis, angiogenesis, cell cycle regulation, and signal transduction. Furthermore, we found a statistical significant enrichment of the presence of HRE in the promoter region of the genes downregulated by topotecan. In fact, 80% of the genes show the consensus sequence of HRE in their promoter region confirming the regulation of HIF-1 function by topotecan.

Conclusions: Our data indicate that topotecan prevents the hypoxia effects on gene expression and that pharmacological inhibition of HIF-1 is a promising therapeutic approach for the reduction of neuroblastoma angiogenic potential.

B59 Gene Expression Analysis of MYCN Downstream Genes by Expulsion of Extrachomosomally Amplified MYCN Copies in Neuroblastoma Cell Lines

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Background: The gene expression web regulated by the MYCN transcription factor has long been studied by comparing the mRNA expression levels of MYCN amplified (MNA) versus MYCN single copy neuroblastoma (NB) cells. The quantification of MYCN targets in panels of primary tumours and cell lines is complicated due to the cellular and genetic hereogeneity of the tumor cells. Therefore, new model systems based on MYCN overexpression in NB single copy cells emerged but might lack the same physiological levels and context present in MNA NB cells. Recently, controlled MYCN knockdown through antisense oligonucleotides or RNA interference has been evaluated. These approaches often hardly achieve MYCN silencing down to levels found in MYCN single copy cells. We explored the utility of a new approach of MYCN downregulation by analyzing cells with expulsed extrachromosomally amplified MYCN copies through micronuclei formation.

Methods: Micronuclei formation in MYCN amplified NB cells was induced by hydroxyurea treatment or occurred spontaneously in a process called F-cell selection. The decrease of MYCN copies was monitored by fluorescence *in situ* hybridization and quantitative PCR. Gene expression of NB cells before and after micronuclei formation was evaluated with microarrays and the expression of a subset of genes known to be induced or repressed by MYCN was measured by quantative RT-PCR.

Results: Both hydroxyurea treatment and spontaneous expulsion resulted in a substantial reduction of MYCN expression ranging from 96% to 98%, respectively. The expression of known MYCN regulated genes changed significantly upon hydroxyurea treatment and F-cell selection.

Conclusions: The strong reduction in MYCN expression, proper cellular environment and the ability to discriminate between true and false MYCN regulated genes suggests that the proposed model meets the required criteria to serve as a platform for new candidate MYCN target gene selection.

B60 Low Expression of miRNAs miR-30e, miR-135 and miR-338 Correlate to Poor Clinical Outcome in Primary MYCN Amplified Neuroblastoma Tumors

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Background: Micro-RNA (miRNA) are small non-coding RNA molecules that regulate gene expression by hybridizing to the 3'UTR of specific mRNAs, thereby preventing translation, or promoting mRNA degradation. They are key players in proliferation, apoptosis, development and differentiation. Erroneous miRNA expression has been linked to cancer development and different miRNAs may function as tumor suppressor genes or oncogenes. We here present data on the expression of miRNAs mapped on chromosome 1 and chromosome 17 and their correlation to NBL development.

Methods: Structural chromosomal aberrations involving chromosome 1 and 17 are common features of unfavorable NBL tumors. We analyzed the expression of all known miRNAs located in these two chromosomes in a set of 46 primary NBL tumors using real-time PCR. Tumors genomes were characterized by array-CND using high-density SNP arrays. The Taqman expression data of 41 miRNAs was confirmed with RNAse protection assay also on SH-SY5Y, SH-SY5Y +RA, SHEP, LAN5, thus selecting 24 miRNAs differentially expressed. Statistical analyses on miRNA expression vs. survival, presence/absence of 1p-deletion, MYCN amplification etc., were performed.

Results: MicroRNAs miR-30e (1p34.2), miR-135b (1q32.1) and miR-338 (17q25.3) showed significantly higher expression in tumors with favorable outcome, as shown by Kaplan-Meier analyses (p=0.021, p=0.038, p=0,03 respectively). The expression patterns of these miRNAs correlated with MYCN-amplification (p<0.001) and 1p-status (p<0.003). Interestingly, miR-34a, earlier suggested as a tumor suppressor gene in NBL tumors, showed no outcome correlated expression. Potential target genes for the three significant miRNAs were identified by prediction software using miRANDA and PITA algorithms. The study of these genes and their regulation in vitro is ongoing.

Conclusions: Low expression of a small set of miRNAs correlate to worse prognosis, thus indicating miR-30e, miR-135 and miR-338 but not miR-34a acting as oncosuppressor in NBL. Their expression correlate to poor clinical outcome in primary MYCN amplified NBL tumors.

B61 Bayesian Detection of Genome Copy Number Alterations from Microarray Analysis of Neuroblastoma Cell Lines

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Background: DNA Copy number alterations (CNA) characterize specific types of cancer. Microarray technology allows large scale evaluation of CNA across multiple samples. However, there is a need for accurate and computationally efficient algorithms to detect CNA.

Methods: A new CNA detection method (GADA, Genome Alteration Detection Analysis) that can efficiently analyze high resolution SNP arrays for large cohorts of samples was developed. GADA is founded in compact linear algebra representation of the array probe intensities as a piece-wise constant (PWC) vector and uses a two-step detection approach. The first step uses sparse Bayesian learning (SBL) to identify breakpoints that delimitate the CNA. The second step uses a backward elimination (BE) procedure to statistically rank the identified breakpoints, and a cut-off point can be set to control for the false discovery.

Results: GADA was compared to existing techniques (DNAcopy, GLAD, HMM, RJaCGH, GEMCA and RHMM) using simulated and real datasets. In the simulated dataset where CNA are known, GADA achieved the highest accuracy and lowest FDR. Four neuroblastoma cell lines (SK-N-BE-2, LAN-6, SMS-KAN, CHLA-20) were analyzed with Affymetrix (50K, 500K, SNP 6.0) and Illumina (550K) arrays. GADA identified all known alterations and achieved a high concordance in detecting CNA by more than two array platforms. Of the 105 breakpoints identified, 68 (65%) were identified on all platforms using GADA. The lowest density platform detected 78 (75%) of the 105 breakpoints, while the highest density platforms detected all (100%). The detected alterations include MYCN amplification and other common alterations: loss of proximal 1p, gain of 17q, loss of distal 11q. The computational speed of GADA is 100 times greater than the second best algorithm (DNAcopy).

Conclusion: GADA improves flexibility in controlling the significance level of discovered CNA and facilitates comparison across different samples and platforms.

B62 Discovering Hidden Significance in Neuroblastoma Gene Expression Studies Using the Neuroblastoma Gene Server

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Background: Microarray based gene expression profiling has become an invaluable tool for outcome prediction, tumor subclassification and gene interaction network dissection. Microarray studies typically result in the generation of one or more lists of genes, leaving the task of extracting meaningful and relevant information. In most cases, the following step is a Gene Ontology annotation analysis to further analyze a gene list. However, even after functional annotation, uncertainty remains as to whether the results help explain why the gene expression changes occurred. An alternative approach to interprete gene expression data is to compare it with existing expression data. To facilitate this we developed the Neuroblastoma Gene Server (NBGS, http://medgen.ugent.be/nbgs).

Methods: NBGS is a web application that enables the identification of hidden significance in user's own microarray expression data by comparing a custom list of differentially expressed genes or the genes located in a genomic region of interest with gene lists from published neuroblastoma microarray studies. We reviewed all literature on neuroblastoma microarray gene expression profiling, selected more than 30 publications and extracted and curated 92 gene lists.

Results: Currently, NBGS contains 4800 records of 3400 differentially expressed genes, and allows substantial better prioritization of gene lists and accelerated identification of critical neuroblastoma genes. The matching results of an uploaded list are summarized in a grid of genes vs. annotated publications in which they are identified, with hyperlinks to more detailed information and reference databases.

Conclusions: The comparison of a gene list with published microarray data enables better identification of the 'usual suspect' genes and improves delineation of common gene patterns that can help to generate new hypotheses in neuroblastoma research.

Poster

B63 Two Distinct Gene Expression Signatures Drive the Separation between Neuroblastic and Schwannian Stromal Cells in Neuroblastic Tumours Indicating a Different Cell Lineage

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Background. Tumour tissue heterogeneity is a well known feature of several solid cancers. Neuroblastic Tumours (NTs) are paediatric cancers with great tissue heterogeneity. Stroma-poor NTs (SP-NTs) are mainly composed of undifferentiated neuroblastic cells (Nbc), whereas stroma-rich NTs (SR-NTs) show abundant Schwann stromal cells (Ssc) with nets of neuroblasts. We previously found that Ssc lack genetic abnormalities observed in Nbc indicating that Ssc are reactive cells.

Methods. We analyzed gene expression profiles of 10 SP-NTs and 9 SR-NTs by high density oligo-microarrays. Nbc and Ssc were isolated by laser capture microdissection from 2 SP-NTs and 2 SR-NTs and probed with microarrays. Gene expression data were analyzed by Significance Analysis of Microarrays (SAM) and Game Theory (GT) methods.

Results. SAM and GT identified 84 and 50 genes differentially expressed between Nbc and Ssc, respectively. Nbc mainly express genes associated with cell replication, nervous system development and anti-apoptotic pathways, whereas Ssc component expresses genes of cell-cell communication and apoptotic pathways. Sixteen genes driving the separation between Nbc and Ssc were identified by SAM and GT together. Five genes over expressed in neuroblasts encode nuclear proteins (*TOP2A*, *CENPF*, *EYA1*, *PBK* and *TFAP2B*), whereas only 1 gene up-regulated in Ssc encodes a nuclear receptor (*NR4A2*).

Conclusions. We identified SP-NT and SR-NT gene expression signatures and Nbc and Ssc populations can be distinguished by the two signatures. According to our previous results of DNA imbalance and present gene expression data we suggest that Ssc and Nbc although have a common origin show different cell lineages development. Moreover, we found low intratumoral gene expression heterogeneity both in SP-NTs and SR-NTs, differently from genetic heterogeneity usually observed in NTs. Based on chromosome map, function and pathway, candidate genes associated to Nbc and Ssc has been selected for further studies.

B64 Genome-Wide Analysis of Neuroblastoma Cells with Differential Expression of the Tumor Suppressor SPARC

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Background: SPARC functions as a tumor suppressor in neuroblastoma (NB) and other types of cancers, and it is silenced in many neoplasms. In NB cells, tumorigenicity inversely correlates with SPARC expression. To investigate the mechanisms contributing to the anti-tumor activity of SPARC, we compared the gene expression profiles of 293 and NBL-S cells with forced expression of SPARC, and in SHEP cells, in which endogenous expression of SPARC was blocked by siRNA.

Methods: The tumorigenic SPARC-negative NB cell line NBL-S and HEK 293 cells were transfected with a SPARC-expressing construct. In non-tumorigenic SPARC-positive SHEP cells expression was blocked with siRNA. Gene expression profiling on Affymetrix U133a or U133Plus chips was analyzed using R and BioConductor with probe intensities background-corrected and normalized with the RMA algorithm. Analysis of differential gene expression was performed using the RankProd package. Gene set enrichment analysis was performed with permutation testing after using KEGG to assign genes to pathways.

Results: Using the rank product and the estimated percentage of false positive predictions, we found 33, 360, and 947 genes differentially expressed in 293, NBLS and SHEP cells, respectively (adjusted $p < 0.05$). GSEA revealed the enrichment for genes in the focal adhesion (FA) pathway in SPARC-expressing cells in all comparisons. Secreted extracellular matrix (ECM) proteins laminin, parvin, and thrombospondin were present among the differentially expressed genes regulated by the FA pathway, as were protein kinases AKT3 and PTK2.

Conclusions: Focal adhesions connect the intracellular cytoskeleton with the ECM and transmit cell adhesion signals regulating various physiological processes. SPARC can induce restructuring of FA and facilitate transition to an intermediate state of cell-matrix adhesion. The identified genes in FA pathway form the basis for validation and further study of the tumor suppressor activity of SPARC in NB.

B65 *MYCN* Gain Cases Analyzed by Neuroblastoma Specific Multiplex Ligation-Dependent Probe Amplification (MLPA)

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Background: Currently, the *MYCN* copy number is usually determined by fluorescence based in situ hybridization (FISH) as routine technique. *MYCN* gain (MNG), corresponding to 1- to 4-fold excess of *MYCN* copies in relation to the reference probe on chromosome 2, have been poorly characterized. In fact, the tendency is to consider that MNG cases are homologous to non-amplified *MYCN* so they are usually included in the same group. The aim of this study was to determine the molecular profiling of genetic changes by a multi-genomic approach in our MNG cases diagnosed by FISH.

Methods: In our series of tumour material, derived from non-treated neuroblastoma patients, analyzed by FISH from 2000 to 2007 (n=483) we found, 30 cases with more than 50% of neuroblasts and more than 40% of tumour cells with MNG. We report in 21 MNG cases the status of gains or losses of up to 115 different genomic loci using Multiplex Ligation-dependent Probe Amplification (MLPA) technique.

Results: The patients present a mean age of 3.5y (1m-17y); INSS stages were 1 (n=3), 2 (n=1), 3 (n=1), 4 (n=14) and 4s (n=2) and INPC was unfavourable in 15/17. DNA index was diploid-tetraploid in 15/19 cases. Segmental chromosome alterations were observed in 18 cases; the other 3 cases did not show any genomic alteration. MNG was detected in 15 cases plus other 2p gain regions. Chromosome 1p loss (n =8), 11q loss (n=14), and 17q gain (n=18) were associated with variable entire and segmental chromosome imbalances.

Conclusions: Structural aberrations are associated with MNG, independently of clinical parameters. FISH technique will be essential for the mandatory study of *MYCN* status. The use of MLPA as routine technique will be a very interesting procedure to detect the implication of the common genetic alterations.

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B66 High-Resolution Mapping of Single Nucleotide Polymorphisms and DNA Methylation in Human Neuroblastoma Using Oligonucleotide Microarray

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Background: Neuroblastoma is a biologically and genetically heterogeneous tumor and demonstrates favorable or unfavorable outcomes. Genome-wide single nucleotide polymorphism (SNP) arrays could frequently identify chromosomal aberrations and allelic imbalances in neuroblastoma. Recently, DNA methylation is an epigenetic mark crucial in neuroblastoma. In this study, we examined chromosome aberration and DNA methylation patterns in the representative cases using genome-wide microarray.

Methods: Genomic DNA and total RNA were extracted from 198 neuroblastoma samples and 10 cell lines SNP array was used to determine genome-wide aberrations. Moreover, the DNA methylation in promoter regions of whole genome was examined ChIP on chip assay in promoter regions using anti-methylcytosine antibodies. Expression profile profiles were also examined using whole genome oligonucleotide microarrays and RT-PCR.

Results: SNP array classified the chromosome aberrations in neuroblastoma into four types: whole gain/loss (n = 55), partial gain/loss type (n = 52), *MYCN* amplified (n = 32), and silent (n = 49). Almost all *MYCN* amplified tumors showed poor prognosis and 28 showed large 1p deletion. In partial gain/loss type tumors, most tumors with 2p gain or 11q unbalanced loss showed poor prognosis. Hypermethylation status in promoter regions were detected in *MYCN* amplified type, some partial gain/loss or silent tumors but rare in whole gain/loss type tumors. Seven of 10 neuroblastoma cell lines also showed hypermethylation status in whole genome. The expression profiles of the unfavorable tumors and cell lines revealed high expression of several genes in aberrant loci and low expression of several genes due to methylation of promoter lesions.

Conclusions: Genome-wide genetic analyses are useful to predict the outcome of patients. The whole genome hypermethylation was also correlated with poor prognosis. In the silent type and partial gain/loss type, differential regulation of several genes due to methylation or alteration of gene was observed only in unfavorable tumors. These microarray analyses provided important biological behaviors and therapeutic targets for unfavorable neuroblastoma.

B67 The POU Transcription Factor Brn-3b; A Tumor Suppressor in High-Stage or a Survival Factor in Low-Stage Neuroblastoma Tumors?

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Background: In the present study we sought potential tumor suppressor genes by exploring gene expression differences between primary neuroblastoma with favorable and unfavorable biology from a Swedish patient group.

Methods: We used expression profiles (analyzed by Affymetrix HU133A) to guide our selection of 90 transcripts with low expression in unfavorable (UF) compared to favorable (F) tumors for high throughput real-time PCR analysis with microfluid cards (MFC) in 18 cases. Next, 14 significant transcripts from the MFC study were picked out for further verification in a second set of 13 cases.

Results: By real-time PCR with MFCs we found 51 out of 90 transcripts to show significantly lower expression in unfavorable NB tumors compared to favorable tumor types ($p<0,05$, 2-tailed t-test). By the second verification we could verify four out of 14 transcripts. However, the overall analysis of both verification groups (31 NB cases) showed all these 14 transcripts to be significantly lower in unfavorable tumors ($p<0,05$). The two most differentially expressed transcripts were the very large gene CNTNAP2 (also called CASPR2) and the POU transcription factor BRB-3B showing overall fold changes of 15 and a 75, respectively. The *CNTNAP2* gene spanning 2,3 Mbs has recently been shown to be located in a fragile site in 7q35. The Brn-3b transcription factor has been reported to have ambiguous functions, both as an apoptosis inducer as well as a survival and progression factor. The current study confirms the differential expression of five earlier reported transcripts: DPYSL3, GNB1, BRN-3B, RAPGEF6, and SLC35E.

Conclusions: In the current study 51 transcripts was found to show significantly lower expression in high stage versus low stage NB tumors by real-time PCR with TaqMan. These transcripts are potential down-regulated tumor suppressor of unfavorable NB tumors, alternatively they may act as survival factors in the more favorable ones.

B68 Asymmetrical Activity of a Bidirectional Promoter between the Divergently Oriented Genes *HAND2* and *DEIN* in Neuroblastoma

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Background: *DEIN*, a recently identified novel gene with stage specific expression in primary neuroblastoma (NB), is located on chromosome 4q33 in a head-to-head orientation with *HAND2*, a key regulator for the development of the sympathetic nervous system. Both genes show high expression in primary NB as well as most NB cell lines and are separated by a putative promoter region of 228 bp. The similar qualitative expression profile of both genes suggests a common transcriptional regulation that may be mediated by a bidirectionally active promoter.

Methods: The transcriptional start sites of *HAND2* and *DEIN* were confirmed by 5' -RACE. The putative promoter alone or together with either of two other highly conserved sequence fragments that may represent additional regulatory elements was cloned in both orientations into a *luciferase* reporter vector. Six different constructs were transfected into NB cell lines SH-SY5Y, SH-EP, SK-N-AS and KELLY, and *luciferase* activity was measured. Expression levels of *DEIN* and *HAND2* were analyzed in 77 primary NB by quantitative PCR.

Results: While the bidirectional promoter alone displayed weak activity in *DEIN* orientation, it strongly activated transcription in *HAND2* orientation. The presence of a regulatory element that has been described to enhance *HAND2*-expression in branchial arches intensified *luciferase* activity in *DEIN* but not in *HAND2* orientation. Another highly conserved sequence element displayed weak repressor activity for both genes. Unlike *DEIN*, *HAND2* expression was not associated with stage, age or genetic alterations in primary NB. Moreover, expression levels of the major transcripts of *DEIN* and *HAND2* were not correlated.

Conclusions: *HAND2* and *DEIN* represent a gene pair that is tightly linked by a bidirectional promoter in an evolutionary conserved manner. Expression of the genes in NB is regulated by asymmetrical activity of the bidirectional promoter and the branchial arches enhancer.

B69 Prognostic Significance of *NTRK1* Gene Polymorphisms in Human Neuroblastoma

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Background: Expression of TrkA high-affinity tyrosine kinase receptor for neurotrophins (encoded by *NTRK1* gene) has been reported to be associated with favorable prognosis in neuroblastoma. Therefore, mutational analysis of the entire coding sequence of *NTRK1* gene has been performed in order to search for somatic mutations and/or for polymorphisms which may alter TrkA expression and to identify correlation of their occurrence with clinical and biological prognostic factors.

Methods: DNA extracted from neuroblastoma tumors of 55 Polish and 114 Italian patients and DNA from leukocytes of 158 healthy controls were included in the study. Denaturing High-Performance Liquid Chromatography (DHPLC) and Single-Strand Conformation Polymorphism (SSCP) analysis were used to screen for sequence variants of all the exons of the *NTRK1* gene. Genetic changes were confirmed by direct sequencing and correlated with biological and clinical data.

Results: Four previously reported and nine new single nucleotide polymorphisms (SNPs) were detected. No difference of SNP frequency in tumors with respect to healthy controls was found. No haplotype blocks were observed within *NTRK1* sequence, yet a linkage disequilibrium between G1730A, C1866T, and G1894T was found (D'=0.57; LOD=4,74; r^2=0.094). Multifactoral analysis revealed that the C1866T (rs6336) polymorphism was significantly related with the risk of disease recurrence and of decreased survival rates ($p=0,009$ OR=13,3 and $p=0,041$ respectively), yet it did not cluster to any of the known clinical subtypes of the disease.

Conclusions: The C1866T polymorphism appears to be an independent marker of poor prognosis in neuroblastoma patients. Substitution of the corresponding amino acid within the conservative region of the tyrosine kinase domain might interfere with the catalytic function of TrkA protein.

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B70 Investigation of the Function of 'S-Type' Cells in Neuroblastoma

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Background: Low stage neuroblastomas comprise neuronal cell types as well as a characteristic Schwannian stroma. In contrast, advanced stage neuroblastomas are typically 'Schwannian stroma poor', but may contain a prominant fibrovascular stroma. In chemotherapy treated tumours this stroma may predominate and evidence suggests that these stromal cells are critical to disease pathogenesis and may facilitate repopulation of the tumour with a chemoresistant clone.
Neuroblastoma cell lines and primary cultures, similar to tumour tissue, contain neuronal (N) and stromal (S) cell types. The relationship between S-type cells observed in culture, and tumour components *in vivo*, is unclear however, as is the function of S-type/stromal cells in tumorigenesis.

Methods: Gene expression profiling of N- and S-type neuroblastoma cell lines, as well as analysis of expression data from 200 tumours was carried out in order to investigate the functional significance of S-type/stromal cells in neuroblastoma cell lines and tumours. Data analysis was carried out using the 'DAVID' functional annotation tool and Gene Set Enrichment Analysis (GSEA).

Results: S-type cell lines showed upregulation of genes involved in cell communication, in the regulation of extracellular matrix and adhesion and in TGFβ receptor signaling. A very similar S-type gene expression signature was also identified in approximately 35% of neuroblastoma tumours, in parallel with the expression of neuronal genes. In both cell lines and tumours the gene expression profile was consistent with cells having a myofibroblast or carcinoma associated fibroblast (CAF)-like phenotype, with upregulated genesets having roles in tumour-microenvironment interactions, in the process of epithelial-mesenchymal transition (EMT) and in tumour invasion and metastasis.

Conclusions: S-type neuroblastoma cell lines may represent an *in vitro* equivalent of the fibrovascular stroma *in vivo*. These cells are likely to play a similar role to myofibroblasts/CAFs observed in adult cancers, providing a supportive environment for tumour growth and facilitating tumour invasion and metastasis.

Poster

B71 Presence of 1p Loss and 1q Gain and Absence of 7p Gain Predict Risk of Metastatic Relapse But not Overall Survival Probability in Localized Resectable Neuroblastoma.

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Background: We have addressed the search of novel genetic prognostic markers in a selected cohort of patients with localized resectable neuroblastoma (NB), stroma poor, who underwent relapse or progression (group 1) or complete remission (group 2) over a minimum follow-up of 32 months from diagnosis.

Methods: Twenty three Italian patients with localized resectable NB (stages 1 and 2) diagnosed from 1994 to 2005 were studied. All patients received surgical treatment. Chemotherapy was administered only to the three stage 2 patient who had *MYCN* amplified tumors. Age at diagnosis ranged from 2 to 116 months. Tumors were stage 1 in 11 cases and stage 2 in 12 cases. After surgical resection, nine patients suffered from local recurrence and/or tumor progression (from now onwards referred to as group 1), whereas 14 patients remained disease-free (from now onwards referred to as group 2) over a minimum follow-up of 32 months. High-resolution array-comparative genomic hybridization (CGH) DNA copy-number analysis technology was used in order to identify novel prognostic markers.

Results: Chromosome 1p36.22p36.32 loss and 1q gain, detected almost exclusively in group 1 patients, were significantly associated with poor event-free survival (EFS) (P = 0.0024 and P = 0.024, respectively). In contrast, patients with 7p21.1 gain, who belonged predominantly to group 2, had a significantly better EFS (P = 0.015). The frequency of 17q gain or 3 and 11q losses did not differ significantly in group 1 *vs* group 2 NBs. The sensitive technique used allowed us to define the position of 1p and 7p breakpoints.

Conclusions: Chromosome 1p36.22p36.32 status should be routinely assessed in NB patients with localized resectable NB to predict the risk of recurrence or progression and to start early adjuvant treatment.

B72 Three New Targets of "Unknown Function" are Associated to Tumoral Progression in Neuroblastoma

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Background: The use of retinoic acid (RA) in clinical trials in leukemias and NB has been found valueble expecially for its ability to induce benign differentiation. We decided to investigate the expression profile of cellular lines of NB induced to differentiation by RA in order to identify "new master genes" of this process.

Methods: Affymetrix chip in two cellular lines of NB (LAN-5 and SH-SY5Y) treated with RA for different experimental times. Stable clones, RNAi technology, Cell cycle and apoptosis scan FACS.

Results: 477 and 705 genes were found reg
ulated in LAN-5 and SH-SY5Y cell lines respectively while 47 are commonly expressed. Of these, 6 genes map in genomic regions known to be altered in NB. Protein expression show an increase during neuronal differentiation. Three genes show high expression in human and murine tissues of neural origin and are found to be regulated during embryonic murine development.
RTPRC on 60 cohorths shows that two out of three genes have a greater expression in stages 1 and 2 compared to latest stage of cancer development was found. Kaplan Meyers survival analyses show that loss of expression in advanced stages, sign of worse prognosis (p value <0.05).
Increase in the population G0/G1 phase and a significant decrease in S phase compared to the control population was observed with FACs analysis on stable clones of each independ gene, thus being in agreement with their potential oncosuppressor role. RNAi analyses control experiments confirm our previous results.

Conclusions: The data presented suggest that the three proteins (KIAA0495, MPPED2, PRR16) might function througth a block of G0/G1 phase and could represent new genes with oncosuppressor activities in NB. In agreement with the in vitro studies, the expression of genes in vivo decreases during tumor progression in patients with NB, identifying new potential molecular target for future therapeutic approaches.

B73 Protein-Protein Interaction Network of AHR and MYCN Involved in Neuroblastoma

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Background: Neuroblastoma, a heterogeneous tumor with variable clinical and biological behavior, is one of the most common pediatric cancers that derived from sympathetic nervous system. To explore the heterogeneous behavior of neuroblastoma, many prognostic biological markers have been discovered, especially v-myc myelocytomatosis viral related oncogene (MYCN) amplification predict a poor prognosis. However, the regulatory mechanism by MYCN expression still has been unclear.

Methods: The microarray technique is a powerful and high-throughput method for accurately determining changes in global gene expressions. In this study, we performed microarray experiment to measure gene expression profiles of 20 Taiwanese neuroblastoma patients. Furthermore, we used pathway mapping and *in silico* interaction prediction to elucidate and construct the protein interaction network.

Results: We showed that 2718 genes are differentially expressed between neuroblastoma and control samples using 3-fold change as cut-off. By using unsupervised hierarchical clustering, we found that MYCN-amplified samples separated from not-amplified samples. By pathway mapping, the Aryl Hydrocarbon Receptor (AHR) pathway was significant ($p<0.05$) and MYCN and AHR have highly correlation in the expression profile. In addition, we constructed the protein network which MCYN connect through 2 nodes such as B-cell CLL/lymphoma 6 and androgen receptor with AHR network and the gene expressions of MYCN- and AHR- interacted proteins are highly relevant.

Conclusions: Our findings indicate that MYCN and AHR have highly correlation and AHR may also play an important role in neuroblastoma development. The protein-protein interaction network of AHR and MYCN involved in neuroblastoma is useful to know the regulatory mechanism by MYCN expression. This study shed light on neuroblosatoma therapy.

B74 Alternative Splicing of *PIK3CD* is Common in Aggressive Neuroblastoma

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Background: Alterations of the PI3K-Akt pathway are common in human cancers. *PIK3CD* encodes p110δ, a catalytic subunit of type I phosphatidyl-inositol-3' kinase. *PIK3CD* resides in chromosome region 1p36.2, commonly deleted in neuroblastoma and we have previously shown that *PIK3CD* is significantly lower expressed in unfavourable neuroblastomas compared to favourable.

Methods: Total RNA and protein was extracted from primary tumors using standard protocols. Expression analysis of RNA from patients and a tissue panel were performed using TaqMan. Primers and probes were designed and tested for specific detection of either wild type or alternative spliced *PIK3CD*. Western blot was performed by standard procedures using commercially available antibodies.

Results: We have discovered an alternative splicesite of *PIK3CD*, resulting in an extra insertion in the mature mRNA causing a frame shift and an early termination of the protein. This splice variant encodes a protein that comprises a regulatory p85-binding domain but no catalytic domain.
PIK3CD is strongly expressed in blood and the alternative spliced variant comprises on average 10% of all *PIK3CD* transcripts in this tissue. A panel of 20 different tissues were also tested but none showed high abundance of this alternative *PIK3CD*. Intriguingly we can show, on both mRNA and protein level that this alternatively spliced variant of *PIK3CD* is abundant in neuroblastoma. TaqMan specific for either wild type or spliced *PIK3CD* show significant higher amounts of alternative spliced product in aggressive neuroblastoma tumours (63% splice variant) compared to tumours from patients that was cured from disease (35% splice variant).

Conclusions: This alternatively spliced variant of *PIK3CD* could have a regulatory function in the PI3-pathway, leading to a possible binding of p85 without resulting in a functional PI3-kinase complex. The fact that this alternative product of *PIK3CD* is common in neuroblastoma is intriguing, implicating involvement in neuroblastoma biology.

B75 Status and Function of the Tumour Suppressor p14ARF in Neuroblastoma

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Background: p14ARF plays a key role in cell cycle regulation and is reported to counteract oncogenic signalling by MDM2, MYC and E2F proteins. To determine the role of this pleiotropic tumour suppressor in neuroblastoma (NB) we analyzed its genomic, epigenetic and expression status in primary tumours. Furthermore, its regulation and function was assessed in NB cell lines.

Methods: Array-CGH was used to determine the genomic status of *p14ARF* in 194 primary NBs. Methylation specific PCR (MSP) and bisulfite sequencing served to assess *p14ARF* promoter methylation. Expressional levels of p14ARF were determined via quantitative Realtime RT-PCR in a cohort of 117 primary NBs. Functional characterizations of p14ARF protein were carried out by tetracycline-inducible expression in NB cell lines.

Results: CGH analysis revealed that heterozygous losses of the *CDKN2A* locus (encoding p14ARF and p16INK4a), either by whole chromosome 9 loss (28 cases) or by partial chromosome 9 loss (14 cases) occurred in 22% (42 cases) of tumours. A homozygous deletion affecting the *CDKN2A* locus was observed only in one case. Gain of this locus occurred only in 2% (5 cases, including 4 patients with whole chromosome 9 gain). p14ARF mRNA-expression was generally low in primary NBs with no significant differences between tumour subtypes. MSP screening of 60 primary NB DNA samples suggested that the *p14ARF* promoter is partially methylated only in a subset of tumours. Conditional overexpression experiments depicted p14ARF's ability to attenuate proliferation in NB cell lines.

Conclusions: Low expressions of p14ARF in almost all primary NB samples indicate that this tumour inhibitory branch is generally suppressed in NB. Inactivation by genomic loss of the locus or by epigenetic silencing might contribute to the low expression of p14ARF observed in primary NBs. However, additional mechanisms that suppress p14ARF expression are likely to contribute.

B76 Regulation of BIRC5/survivin at the mRNA and Protein Level by E2Fs in Neuroblastoma

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Background: Expression of the inhibitor of apoptosis protein BIRC5/survivin is associated with advanced tumour stage and poor overall survival in a large set of cancer types, including neuroblastoma. A prerequisite for the development of new therapeutic approaches that are based on the inhibition of BIRC5/survivin is to understand its regulation at the mRNA and protein level in neuroblastoma cells.

Methods: In this study, we investigated the expression and the regulation of BIRC5/survivin in NB specimens and NB model systems. mRNA expression of BIRC5/survivin, and E2Fs was analyzed in two cohorts by QPCR (n=117) and Oligo-microarray (n=251). Transcriptional regulation of BIRC5/survivin by E2Fs was assessed by promoter assays, ChIP, and conditional overexpression of E2F1 in different NB cell lines. BIRC5/survivin protein stability was analyzed by cycloheximide chase experiments. siRNA technology was used to specifically target CDC2. Knock down efficiency was assessed by QPCR and Western blot. Microscopy readout, Alamar Blue assay and TUNEL staining was used to analyze proliferation and apoptosis after targeted CDC2 knock down.

Results: In primary neuroblastoma tumors, mRNA expression of BIRC5/survivin is strongly associated with the expression of activating E2Fs, such as E2F1. Using different experimental approaches, we identified BIRC5/survivin as a direct transcriptional target of activating E2Fs. We also found that BIRC5/survivin protein stability is increased in NB cells with high E2F activity. BIRC5/survivin was co-expressed in vitro and in vivo with CDC2, another E2F target that is involved in BIRC5/survivin phosphorylation and stabilization. Targeted knock down of CDC2 inhibits phosphorylation and abundance of BIRC5/survivin and reduces viability of neuroblastoma cells in vitro

Conclusions: BIRC5/survivin expression and stability is controlled via different E2F-dependent mechanisms. Transcriptional regulation is controlled by E2Fs directly, whereas protein stability is controlled indirectly via induction of CDC2 by E2Fs.

B77 Optimizing Tumor-Based Proteome Profiling Platforms for Neuroblastoma Investigation

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Background: Genomic copy-number alterations (CNAs) strongly correlate with NB outcomes. Still, the causal suppressors and oncogenes targeted by distinct CNAs remain largely unknown. As cell behavior is governed by proteins, a systematic view of cancer requires proteome information (from platforms with desirable coverage and sensitivity) that can be integrated with genomic and transcriptomic data-sets.

Methods: We developed a novel 3D platform using 2D-liquid chromatographic separation (17x24) to complexity-reduce lysates, followed by MALDI-TOF detection of intact proteins from each fraction ("top-down" approach). This generates a "fingerprint" consisting of 408 mass spectra (of m/z peaks) for each input. We used SK-N-AS and NGP lysates and compared performance against four alternative strategies: 2DGE, off-line MudPit (SCX chromatography followed by LCMSMS protein detection), 1DGE/Gel pixelation followed by LCMSMS detection, and 2DLC followed by LCMSMS detection. Of these, only 2DGE is similarly "top-down" in that intact proteins are detected. The remainder are "bottom-up", relying on high-resolution MSMS of tryptic digests to sequence peptides and infer proteins.

Results: The 3D platform had sub-femtomole sensitivity and resolved 5,575 and 5,972 protein peaks from SK-N-AS and NGP, respectively, which is 2-to-10-fold coverage improvement over alternatives. M/z peaks provide semiquantitative information on intact protein species, and liquid-phase aliquots remain for validation assays, however, each m/z peak is anonymous in feature space until identified off-line. We plan to use our 3D platform to acquire proteome profiles (unique fingerprint of m/z peaks in feature space) from highly annotated NBs clustered by genomic CNAs. Supervised analyses with proprietary pattern discovery algorithms (CIRA Discovery) are employed to define features (proteins) correlating with NB subtypes that can subsequently be identified and validated.

Conclusions: We hypothesize that the systematic addition of high coverage non-biased proteomics data to the NB profile will identify pathways targeted by discrete genomic alterations and identify potential therapeutic targets.

B78 Functional Analysis of Neuroblastoma Phox2b Missense and Frameshift Mutations in Immature Sympathetic Neurons

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Background: The paired homeodomain transcription factor Phox2b is essential for the development of autonomic neurons. Heterozygous mutations in PHOX2b account for a series of disorders of the autonomic nervous system, including neuroblastoma (NB), congenital central hypoventilation syndrome (CCHS) and Hirschsprung disease (HD). Whereas polyalanine expansion accounts for CCHS, NB patients harbor either a missense or frameshift heterozygous mutation of the PHOX2b gene. PHOX2b mutations discovered in familial NB represent the first genetic predisposition to NB.

Methods: To determine the molecular basis of altered PHOX2b functions that may be involved in the formation of NB we have ectopically expressed wild-type and NB Phox2b variants in proliferating sympathetic neurons from E7 chick embryos. Effects on proliferation, differentiation and survival were analysed.

Results: Overexpression of wild-type Phox2b results in a strong reduction in sympathetic neuron proliferation. The antiproliferative effect is lost in all missense and frameshift Phox2b mutations analysed. The NB Phox2b mutation nt463 results in a Phox2b variant with dominant negative effects on sympathetic neuron differentiation (TH/DBH-expression). The dominant- negative Phox2b variant also increases the cell death of sympathetic neurons. All other NB Phox2b variants analysed do not affect differentiation and sympathetic neuron survival.

Conclusions: These results indicate that NB mutations lead to both loss- and gain-of-function Phox2b variants, affecting differentiation, proliferation and apoptosis of immature sympathetic neurons. We propose that the loss of antiproliferative functions and/or the acquisition of de-differentiating function of Phox2b variants may predispose immature sympathetic neurons to NB.

Poster

B79 Control of the Skp2 Ubiquitin Ligase as a New Therapeutic Concept for High-Risk Neuroblastomas

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Background: The E3 ubiquitin ligase Skp2 plays a central role in controlling p27 function during cell cycle progression. We have previously shown that high *Skp2* expression together with low p27 levels characterizes high-risk neuroblastomas (NB). Aim of this study is to investigate the regulation of Skp2 in NB cells in order to develop therapeutic concepts for high-risk NBs that are based on Skp2 inhibition.

Methods: We analyzed the expression of *Skp2* in relation to *MYCN* and *E2F1* in primary NBs by QPCR (n=117) and oligo microarray (n=251). To define and characterize key regulatory elements in the *Skp2* promoter, we used luciferase reporter assays and *Skp2* promoter mutants in different NB cell lines. Additionally, we used chromatin immunoprecipitation (ChIP) to assess MYCN, E2F, and pocket protein (Rb, p107, p130) binding to the *Skp2* promoter. Using a small compound library screening, we searched for compounds that inhibit Skp2 transactivation in NB cells.

Results: *Skp2* mRNA levels correlated closely with *E2F1* mRNA levels in *MYCN* single-copy and amplified tumors (Pearson's correlation coefficient=0.70; 95% confidence interval, 0.60-0.79). A core promoter region at -133 to +248 of the transcriptional start site of *Skp2* was defined that contains activator and repressor elements. The regulation of these elements was impaired in *MYCN* amplified cell lines. ChIP analyses revealed that the activator and repressor elements differentially bind E2F1/4/5 and Rb/p107/p130 in *MYCN* single-copy and amplified NB cell lines. We found that the repressor mechanism was restored in *MYCN* amplified cell lines by CDK4/6 inhibitors but not by CDK2 inhibitors or chemotherapeutic drugs such as doxorubicin.

Conclusions: High *Skp2* expression is found in high-risk NBs. E2Fs and pocket proteins control the regulation of *Skp2* in NB cells. Specific CDK4/6 inhibition abrogates *Skp2* expression in NB cells and might serve as a new therapeutic concept for high-risk NBs.

B80 TSLC1 Mapped to Chromosome 11q23 is a Candidate Tumor Suppressor in Neuroblastoma

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Background: We previously performed array-CGH analysis for 236 primary neuroblastomas to search for the genomic alterations. In this study, we defined a smallest region of overlap of chromosomal deletion at 11q23. In this region, we found the presence of *TSLC1/ CADM1 (tumor suppressor in lung cancer 1/ cell adhesion molecule 1)* which had been identified as a tumor suppressor gene in some cancers including lung cancer.

Results and Discussion: Since 11q23 was frequently deleted in neuroblastoma, we performed LOH analysis of *TSLC1* locus and found that *TSLC1* LOH was observed in 25 of 72 (35%) of primary neuroblastomas. We therefore investigated whether the inactivation of *TSLC1* differs in the favorable and unfavorable neuroblastoma subsets. Semi-quantitative RT-PCR showed that *TSLC1* was expressed at lower levels in unfavorable neuroblastomas compared to favorable tumors. Furthermore, quantitative real-time RT-PCR in 108 neuroblastomas showed that low expression of *TSLC1* was significantly ($p<0.05$) correlated with advanced stages, Shimada unfavorable pathology, *MYCN* amplification, and DNA diploidy, but not allelic loss of *TSLC1*. More importantly, decreased expression of *TSLC1* was significantly associated with poor prognosis in patients with neuroblastoma. Interestingly, the immunohistolgical analysis in 11 neuroblastomas showed that TSLC1 was detectable even in unfavorable tumors without *MYCN* amplification. Previous reports have indicated that promoter methylation of *TSLC1* gene was frequently observed in various cancers, we therefore performed bisulfite sequencing in 27 cell lines and 115 primary neuroblastomas. However, no hypermethylation was observed in its promoter regions. Finally, colony formation and cell growth assays in SH-SY5Y cells transfected with the plasmid carrying *TSLC1* or the siRNA against *TSLC1* provided functional evidence that TSLC1 has growth inhibitory ability in SH-SY5Y cells. In conclusion, *TSLC1* is one of the candidate tumor suppressor genes in neuroblastoma, which is not correlated with chromosome 11q aberration and promoter hypermethylation.

B81 Expression of the Tumor Suppressor Gene *CADM1* is Associated with Favorable Outcome and Inhibits Cell Survival in Neuroblastoma

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Background: The cell adhesion molecule 1 (*CADM1*), a putative tumor suppressor gene, is down-regulated in many solid tumors. *CADM1* maps to chromosome 11q23.2, a region frequently deleted in aggressive neuroblastoma.

Methods: We previously found high *CADM1* expression in stage 4S neuroblastomas comparing SAGE expression profiles of primary tumors of stage 4 and stage 4S disease. Oligonucleotide-microarray analysis of 251 neuroblastoma specimens was performed and *CADM1* levels were correlated to known risk predictors. The nature of *CADM1* down-regulation was investigated using bisulfite sequencing (n=18) and mutational analysis (n=25). *CADM1* was re-expressed in four neuroblastoma cell lines to study its effect on cell proliferation, viability and colony formation in soft agar.

Results: *CADM1* transcript levels were defined as high (>75th percentile; n=62/251), intermediate (>25th and <75th percentiles; n=127/251) and low (<25th percentile; n=62/251). 22/153 of stage 1-3 tumors, 34/67 of stage 4 tumors and 6/31 of stage 4S tumors had low *CADM1* expression. 29 patients with chromosome 11q deletion and 5 patients with 11q imbalance had low *CADM1* levels, but also 23 patients with normal chromosome 11q status. *CADM1* down-regulation was associated with unfavorable prognostic markers like disseminated stage 4, age >18 months and *MYCN* amplification ($p<0.001$ each). Moreover, low *CADM1* expression was correlated with unfavorable gene expression-based classification and adverse patient outcome ($p<0.001$ each). Bisulfite sequencing and genetic analysis suggested that neither haploinsufficiency nor hypermethylation is regularly involved in *CADM1* gene silencing in neuroblastoma. Additionally, no mutations disrupting the *CADM1* reading frame were found. Re-expression of *CADM1* in four neuroblastoma cell lines significantly reduced of cell proliferation, viability and colony formation in soft agar.

Conclusions: Down-regulation of *CADM1* appears to be a feature of unfavorable neuroblastoma in general rather than of neuroblastoma with loss of chromosome 11q material. It occurs as a critical event in neuroblastoma pathogenesis resulting in tumor progression and unfavorable patient outcome.

B82 Frequent SLC16A5 Promoter Methylation in Progressive Neuroblastomas

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Background: The identification of tissue-specific differentially methylated regions (T-DMRs) is indispensable for understanding mammalian development. The variation of DMRs reveals a distinct genomic character. It is suggested that T-DMR is aberrantly methylated in cancer and may regulate tumor-suppressor genes and/or oncogenes. We confirmed that the SLC16A5 promoter is a testicular-specific differentially methylated region in mice and humans. The SLC16A5 gene is located on chromosome 17q 25.3 and express Monocarbxylate transporter 6 (MCT6). In this report, we analyzed an aberrant methylated region of the SLC16A5 promoter in neuroblastic tumors and neuroblastoma cell lines.

Methods: We used the MASSARRAY Epityper method to determine the quantitative level of methylation at the CpG sites of the SLC16A5 promoter in 21 neuroblastic tumors and two cell lines of neuroblastoma. The expression levels of SLC16A5 in those tumors were analyzed by immunohistochemical staining. Correlations between methylation status and Shimada's classification, or MYCN amplification, were compared.

Results: SLC16A5 promoter methylation status was hypermethylated in the two neuroblastoma cell lines. Among 21 neuroblastic tumors, 11 were Favorable and 10 were in the Unfavorable group. The frequency of SLC16A5 promoter methylation between two groups was different. The promoter methylation and loss of MCT6 protein expression occurred preferentially in the Unfavorable group. MYCN amplification was seen in three neuroblastic tumors, and 18 tumors were in the non-amplification group. Although the number is low, there was no difference in methylation between the MYCN amplification group and the non-amplification group.

Conclusions: There was an inverse correlation between methylation level and Shimada's classification and no correlation between methylation status and MYCN amplification. This report suggests that hypermethylation in the CpG sites of the SLC16A5 promoter and expression of MCT6 might be implicated in the pathological development of at least a part of neuroblastoma cases, and SLC16A5 might be a candidate oncogene in neuroblastomas.

B83 Identification of Candidate Genes Involving in Neuroblastoma Using a New Mathematical Model for GeneChip Data

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Background: The Affymetrix *GeneChip (PM. MM)* probe pair is designed with the intention of measuring non-specific binding. Although the rationale behind the design is that the *PM* probe is expected to have a larger value than that of the *MM* probe, there are many exceptions in actual data. We provide a mathematical explanation for this inconsistency based on the functional states of a gene-'*ON/OFF*'- where both *PM* and *MM* values are assumed to have the same distribution when a gene is in the 'OFF' state. This means that the probability that *MM>PM* is equal to that of *MM<PM* for 'OFF' genes. Using this algorism, we selected 5 top 'ON/OFF' genes in the comparison between favorable and unfavorable neuroblastomas, and analyzed.

Methods: We selected 10 neuroblastoma cell lines and 50 untreated neuroblastoma specimens including 40 favorable (alive with disease-free) and 10 unfavorable tumors (died of disease). All samples were analyzed by Affymetrix U133 Plus 2.0 microarray. Top-ranked genes switching 'ON/OFF' between favorable and unfavorable tumors according to our mathematical explanation were quantified by real-time RT-PCR.

Results: The expression levels of top selected 5 genes were low so that none of these genes could be nominated as candidate genes by usual algorisms such as MAS5 or RMA. The quantitative RT-PCR revealed the significant difference of expression in all 5 genes between favorable and unfavorable tumors. Among these 5 genes, siRNAs against one gene which was ON in unfavorable and OFF in favorable tumors were effective to repress the growth of cell lines.

Conclusions: These data suggest that our new 'ON/OFF' algorism might be a promising approach to screen candidate genes discriminating unfavorable tumors from favorable ones from whole-genome array experiments. The selected genes might be candidate for risk assessment as well as molecular-targeted therapy.

B84 Apitd1, a 16kDa Protein with a Histone-Fold Superfamily Domain Induces Apoptosis in Neuroblastoma Cells

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Background: *APITD1* is a novel gene in the neuroblastoma tumor suppressor candidate region on chromosome 1p36.2. It has been proposed to play a role in neuroblastoma tumorigenesis based on previous studies showing transcriptional down-regulation of *APITD1* in primary neuroblastoma tumors from high-risk patients and cell growth reduction following *APITD1* overexpression. Furthermore, the predicted protein contains a Histone-fold superfamily domain with similarity to the TATA box-binding protein-associated factor, $TAF_{II}31$, required for p53 mediated transcription activation. Here, we wanted to study the expression of the predicted Apitd1 protein and its effect on p53.

Methods: Custom made Apitd1 antibodies were used in Western blot analysis to detect proteins of the predicted molecular weight, 16 kDa in cells from various tissues and primary neuroblastoma tumors. Mass spectrometry (MS/MS) analysis was performed on immunoprecipitated proteins separated by SDS-PAGE to confirm the identity of the 16 kDa proteins. The levels of p53 and the p53 responsive genes Mdm2, $p21^{WAF/CIP1}$ and Bax were determined by gene expression analysis or Western blot analysis after *APITD1* overexpression or silencing by siRNA transfection. Induction of apoptosis in *APITD1* transfected cells was studied by FACS analysis of TUNEL labeled cells and by Annexin V staining.

Results: The identity of the purified Apitd1 protein was confirmed. Protein expression levels of Apitd1 were low in primary tumors from patients with both favorable and unfavorable outcome. *APITD1* overexpression induced apoptosis with increased levels of p53 but unchanged levels of the p53 target genes Mdm2, $p21^{WAF/CIP1}$ and Bax.

Conclusions: Apitd1 expression is low in primary neuroblastoma tumors, both at the genetic and protein levels and this may be one of the factors which prevent cell death in neuroblastomas. It could be involved in p53 dependent cell cycle regulation or apoptosis independent of Mdm2, $p21^{WAF/CIP1}$ and Bax.

B85 Simultaneous Multifocal Neuroblastoma and Embryonal Rhabdomyosarcoma in a Patient with Combined Germline *TP53* R248W Mutation and Turner Syndrome

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Background: Germline *TP53* mutations underly the Li-Fraumeni multi-tumor syndrome (LFS). Several childhood cancers are associated with LFS, in particular adreno-cortical cancer and rhabdomyosarcoma. Neuroblastoma (NB) is, however, only weakly associated with LFS and there is presently very little evidence for a role of the p53 pathway in NB tumorigenesis. There are four reports on simultaneous tumors in children with LFS. Two of these included NB and both had a germline missense pointmutation in codon 248 of *TP53,* which is a well-known hot spot for LFS and for LFS-associated tumor types. In one of these two cases a Turner syndrome (TS) was found in addition. TS has been associated with an increased risk for localized NB/ganglioneuroma, whereas Klinefelter and triple X syndromes are suggested to have a lower risk than normal for NB. The genetics underlying multifocal NB appears oligogenic. Implied loci include *PHOX2B* and other undefined neurocristopathy-causing germline mutations. *TP53*- or X-linked NB multifocality has not been reported previously.
We present here genetic data of a patient with simultaneous trifocal (ganglio)neuroblastoma and stage 1 embryonal rhabdomyosarcoma of the chest wall presenting at an age of 3.5 years. There were no major clinical signs of TS at diagnosis.

Methods: DNA sequencing, 36K whole-genomic array comparative genomic hybridization (aCGH), QPCR, cytogenetic karyotyping.

Results: Constitutional DNA showed a germline heterozygous *TP53* R248W missense mutation. No copy number abnormalities were seen in ganglioneuroblastoma DNA, but samples were most likely not representative because of low tumor cell contents in the specimens. Amplifications on the short arms of chromosomes 1 and 11, were found in rhabdomyosarcoma DNA. Peripheral lymphocyte karyotype was 45 X0. aCGH verified haploidy for the entire X chromosome in constitutional and tumor DNA.

Conclusions: This patient highlights possible roles for *TP53* and/or X-linked tumor suppressor loci in NB tumorigenesis.

B86 Screening of Candidate Differentially Methylated Regions (DMRs) to Identify New Candidate Tumor-Related Genes in Neuroblastic Tumors

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Background: Methylation of promoter CpG islands is indispensable for understanding gene regulation. The tumor genome is generally hypomethylated. However, specific hypermethylation of the tumor-suppressor gene-promoter occurs during tumorigenesis. We confirmed 32 tissue-specific differentially methylated regions (T-DMRs) and 25 tumor-specific methylated regions in mouse models of human tumors (skin, lung and hepato-cellular carcinoma models) by using the Restriction Landmark Genomic Scanning (RLGS) method. In this report, we search for new candidate tumor –suppressor or oncogenes in neuroblastic tumors using these regions.

Methods: We have set up the MASSARRAY Epityper system to determine the quantitative level of methylation at the CpG sites of 52 conserved DMRs in 21 neuroblastic tumors and two cell lines of neuroblastoma. Aberrantly methylated regions in neuroblastomas are subjected to correlation studies between methylation status and clinical manifestations. The expression levels of the target downstream genes are also analyzed by real-time PCR and/or immunohistochemical staining.

Results: In the initial screening of 27 DMRs, frequent aberrant methyaltion in neuroblastic tumors has been observed in at least two genomic regions (Pvu2 and 5D52) (Spot ID referred in PNAS 2005,102 3336–41, Genomics 2004, 84: 647-60). Pvu2 is preferentially methylated in the Unfavorable group of neuroblastic tumors compared with those in the Favorable group (P=0.017). 5D52 was aberrantly methylated in most of the neuroblastic tumors.

Conclusions: DMRs in the mouse genome are well conserved in the human genome and also differentially methylated among human tissues and tumor development. So far, we have analyzed 27 out of 52 candidate DMRs in this study and identified at least two regions with frequent aberrant methylation. By the date of the meeting, all 52 regions will be screened in the same fashion and will present more aberrant methylated regions involved in neuroblastoma development. Downstream genes or transcripts will confer candidate neuroblastic tumor-related epigenetic factors.

Poster

B87 *EMP3* may be a Tumor Suppressor Gene in Late Stage of Neuroblastoma Development

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Background: It is well-known that biological heterogeneity of neuroblastoma results in favorable or unfavorable outcomes. Recently, the EMP3, an epithelial membrane protein was reported to be a tumor suppressor gene in neuroblastomas. However, the relationship between this gene and other prognostic factors, such as *MYCN* amplification, remains to be elucidated.

Methods: A total of 49 neuroblastoma tissue specimens collected at surgery before chemotherapy and 10 neuroblastoma cell lines were used. quantitative methylation specific PCR of bisulfite-treated genomic DNA using fluorescent primers and fragment analysis was carried out to evaluate the methylation status of CpG island in the *EMP3* promoter region. The expression levels of *EMP3* were examined by quantitative TaqMan RT-PCR method. CpG island methylation status and expression levels of *EMP3* were compared to clinical profiles and other biological factors in neuroblastomas.

Results: Hypermethylation of *EMP3* CpG island was detected in all 10 cell lines but rarely in 49 neuroblastoma tissue samples. In all hypermethylated samples, EMP3 was strongly repressed. Small number of clinical samples showed methylation of *EMP3* CpG island at low levels, but they were solely in advanced stages and showed poor outocomes. Interestingly, the tumors with *MYCN* amplifiction showed the repression of *EMP3* expression levels regardless of the methylation status of CpG island. Thus, although methylation status was apparently low in clinical samples compared to cell lines, the repression of *EMP3* was significantly correlated with poor prognosis in patients with neuroblastomas.

Conclusions: The *EMP3* was repressed commonly in unfavorable neuroblastoma with or without *MYCN* amplification as well as in neuroblastoma cell lines, but methylation frequency was still low in the former, suggesting that *EMP3* may be one of tumor suppressor genes in neuroblastoma that play a role in relatively late stage of carcinogenesis.

B88 Low Expression of the 1p36 Tumor Suppressor Candidate *CAMTA1* is an Independent Predictor of Poor Outcome in Neuroblastoma Patients

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Background: Distal 1p is frequently deleted in human neuroblastomas, and it is generally assumed that 1p35-36 harbors at least one gene relevant for neuroblastoma development. However, thus far, extensive sequence analysis of genes mapping to this region revealed only rare mutations. Reduced expression of a large portion of 1p35-36 genes in 1p-deleted tumors is in line with a haploinsufficiency model where 1p deletion would result in lower transcript levels of gene(s) relevant for neuroblastoma suppression.

Methods: Expression of all 1p35-36 genes was measured in 251 primary neuroblastomas applying an oligonucleotide microarray platform. In a first step we identified all genes with significantly lower expression in 1p-deleted tumors. Of these, a large portion is likely to act as innocent bystanders, genes that are not causally linked to neuroblastoma development but mirror 1p deletion due to dosage compensation failure. To focus on transcripts not simply representing surrogate markers for 1p deletion, in a second step we applied multivariate survival analysis adjusting for established risk markers including 1p deletion. Genes selected by these two steps were mapped in relation to previously described 1p SROs. The expression pattern of candidate genes was confirmed via real time PCR in a second cohort of 102 neuroblastomas.

Results: For twelve of 425 1p35-36 genes, low expression was significantly associated with i) 1p-deletion and ii) poor outcome, the predictive value being independent of established risk markers. One of these twelve, *CAMTA1*, maps to a 1p36.3 commonly deleted region, bordered by *D1S2731* and *D1S214*. The correlation of *CAMTA1* expression with 1p deletion and its independent prognostic value was confirmed in a second cohort of 102 patients.

Conclusions: Our data suggest that assessment of *CAMTA1* expression may improve the prognostic models for neuroblastoma and that it will be important to define the biological function of *CAMTA1* in this disease.

B89 Identification of Neuroblastoma Specific Stem Cells Markers by Micro-Array Time-Course Analysis of Neurospheres

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Background: Recent studies in breast, brain and colon cancers suggest that solid tumors may arise from a unique and minor population of so-called cancer stem cells (CSCs), which possess the exclusive ability to initiate and maintain the tumour. The identification of CSCs as efficient therapeutic targets is thus urgently needed.
Neuroblastoma (NB) originates from neural crest-derived malignant sympatho-adrenal cells. We have indeed identified within primary NB tissue and cell lines rare cells that express neural crest stem cells markers, or markers of the more differentiated neural crest stem cells progeny, suggesting the existence of CSCs in NB tumor (NBSC) that recapitulate the properties of sympathetic precursor cells.

Methods & Results: In the absence of specific markers to identify CSC in solid tumours, NBSCs isolation and analysis was attempted by serial passage neurospheres (NS) formation and micro-array time-course analysis. Serial passages NS generated from a stage 4 patient tumour cells resulted in the selection of a subset of cells, which gene expression profile was further identified by micro-array time-course analysis. GeneOntology analysis revealed that differentially expressed genes in NS were enriched in development-related genes and known CSCs markers (e.g. CD133 and NOTCH3). Moreover, a homogeneous population of cells expressing CSCs markers was selected by propagation of the same patient tumour cells in a neural basic medium preventing differentiation (NBM cell line). Several differentially regulated genes identified in the high passages NS and NBM cell line overlapped with genes reported in neural crest stem and other types of stem cells, suggesting that NS and NBM cell line may be highly enriched in NBSC.

Conclusions: The described strategy successfully identifies putative CSC markers and selects for CSC-like cells in NB. The stem cell-related tumorigenic and multipotent properties of the selected population are currently investigated by in vivo tumourigenic and in vitro differentiation induction analyses.

B90 Identification of Neural-Specific MicroRNAs that Contribute to the Pathogenesis of Neuroblastoma

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Background: Neuroblastoma cells show complex patterns of acquired genetic defects, including specific chromosomal aberrations and *MYCN* amplification. Despite intense efforts, the fundamental role of these features in initiation and progression in neuroblastoma remains unclear. The aim of this project is to investigate differential miRNA expression patterns in a neuroblastoma cell line with inducible MYCN expression in order to identify MYCN-regulated miRNAs.

Methods: We have analyzed differential miRNA expression patterns in a neuroblastoma cell line with regulated MYCN expression using a human miRNA oligonucleotide array. To validate the array-identified miRNA expression pattern obtained, we have carried out a combination of miRNA Northern Blotting and miRNA-specific qPCR assays.

Results: To date, we have verified the regulation of several miRNA clusters associated with cancer, including miRs from the *C13orf25* cluster, previously shown to be regulated by c-Myc. In addition, we have identified novel microRNA candidates that are downregulated following MYCN induction. To ascertain the direct regulation of these miR species/clusters, we have demonstrated binding of MYCN upstream our candidate miRs using chromatin precipitation assays.

Using an intersection of microRNA-target prediction programs we have identified putative target genes potentially regulated by our miR candidates. By fusing the 3' UTRs of our targets to a *luciferase* reporter and co-transfecting these with specific miRNA hairpin oligonucleotides, we have showed the direct regulation of target genes downstream the MYCN-regulted miRs. Interestingly, many of these genes are either cancer-associated or involved in processes linked to neural development and differentitaion. We are currently performing miR knock-out/knock-in experiments in neuroblastoma cell lines with MYCN amplification to determine phenotypic outcomes following miR inhibition/overexpression.

Conclusions: We have identified several up- and downregulated miRNAs in neuroblastoma cells with MYCN overexpression in comparison to non-expressing cells. Their further characterization will give insights to the contribution of MYCN-regulated miRNAs to the genesis of neuroblastoma.

B91 A Novel p53-Target Gene *UNC5A* Induces Caspase-Dependent Apoptosis in Neuroblastoma Cells

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Background: The *UNC5* genes, including *UNC5A*, *B*, *C* and *D*, encode the netrin-receptors that mediate the signaling for axonal extension and cell death. It has been reported that *UNC5B* (also designated as *p53RDL1*) is directly regulated by tumor suppressor p53, which mediates p53-dependent apoptosis.

Methods: To evaluate the role of UNC5A as a p53-target gene, we examined the expression of *UNC5A in vitro* or *in vivo* and the activity of a potential p53-binding sequence (p53BS) in the promoter region of *UNC5A* gene. Furthermore we investigated apoptotic induction of SH-SY5Y neuroblastoma cells by UNC5A. In addition we compared the effect of UNC5A to that of UNC5B

Results: The expression of *UNC5A* mRNA was induced by exogenous or endogenous p53. Furthermore, *UNC5A* mRNA was increased in the spleen and colon of the irradiated p53 (+/+) mice, but not in those of the irradiated p53 (-/-) mice. A chromatin immunoprecipitation (ChIP) assay indicated that a potential p53BS in the promoter region interacts with p53. A heterologous reporter assay showed that p53BS has a p53-dependent transcriptional activity. Ectopic expression of UNC5A induced caspase-dependent apoptosis in several cancer cells including SH-SY5Y cells. The effect seemed to be stronger than UNC5B.

Conclusion: *UNC5A* is a novel p53-target gene and regulates caspase-dependent apoptosis in neuroblastoma cells. Therefore, UNC5A might become a promising molecule for new therapy against neuroblastoma.

B92 Significance of TrkA Isoform Expression in Neuroblastomas

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Background: Neuroblastomas (NBs) are characterized by heterogeneous clinical behavior. TrkA expression is generally associated with favorable clinical outcome, but some tumors with high TrkA expression have an unfavorable outcome. We investigated the expression of TrkA isoforms to determine if this could explain the unfavorable outcome in some NBs.

Methods: Case analysis of 163 patients identified 49 with high TrkA expression in their tumors. Of these, 23 were clinically and biologically favorable (stage 1, 2), and 26 were unfavorable (stage 3 or 4, >1 year of age). We analyzed the expression of TrkA-I/II and TrkA-III in 49 NBs using RT-PCR with exon-specific primers. We used the Pearson correlation coefficient to assess total TrkA and relative TrkA-III expression between favorable and unfavorable groups. We also examined the expression of TrkA isoforms in three NB cell lines (SY5Y, KCNR, CHP-134) *in vitro* under normoxic and hypoxic (1% O2) conditions. We determined the expression of hypoxia inducible factor (HIF1-alpha), VEGF, TrkA-I/II and TrkA-III by semi-quantitative RT-PCR. Densitometry was used to quantitate relative gene expression.

Results: There was no significant difference in the level of total TrkA expression in favorable or unfavorable neuroblastomas. However, the relative expression of TrkA-III was significantly higher in the unfavorable group (p=0.0008). The SY5Y line (nonamplified *MYCN*) exhibited stable expression of TrkA-I under hypoxic conditions, but TrkA-III and HIF1-alpha expression were upregulated. In the *MYCN* amplified cell lines, SMS-KCNR and CHP-134, we observed a marked increase in the relative expression of TrkA-III with hypoxia, along with an increase in the level of HIF1-alpha and VEGF.

Conclusions: We conclude that high expression of TrkA-III is associated with poor outcome in high-risk neuroblastomas. The relative expression of TrkA-III in neuroblastoma lines is induced *in vitro* under hypoxic conditions, supporting TrkA-III as a target for kinase inhibitor therapy in these tumors.

B93 The Dependence Receptor TrkC Regulates Apoptosis in Neuroblastoma

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Background: We have recently shown that the neurotrophin receptor TrkC is a dependence receptor and, as such, it induces apoptotic death in the absence of its ligand, neurotrophin-3 (NT-3). This activity relies on the caspase-mediated cleavage of the intracellular domain of TrkC, which allows the release of a proapoptotic fragment. Dependence receptors have been proposed to act as tumour suppressors by inducing apoptosis of tumour cells that grow or migrate beyond the regions of ligand availability. In parallel, TrkC expression has been correlated with good prognosis of neuroblastoma (NB). However, there is little information about NT-3 involvement in tumor progression. We propose that TrkC proapototic activity could regulate tumorigenesis of NB.

Methods: We measured TrkC and NT-3 expression in 26 human neuroblastoma cell lines by Q-RT-PCR. We selected two cell lines with high NT-3 levels, as verified by immunocytochemistry. We incubated them in presence of an antibody antagonizing TrkC-NT3 bound (AF1404) and we measured cell death induction by trypan bleu exclusion and caspase 3 activation. We set up a model of neuroblastoma on chicken embryos to evaluate primary tumor growth and spontaneous metastasis. In this model, we treated tumors with TrkC-blocking antibody. We thus analyzed TrkC proapoptotic activity as a mechanism of control of neuroblastoma, when it can no longer interact with its ligand.

Results: TrkC induces apoptosis in NT-3 expressing neuroblastoma cell lines, when incubated in presence of TrkC-blocking antibody. Preliminary results showed reduced primary tumor development, and suppression of metastasis *in vivo*, upon antibody treatment, while no effects where observed with control antibody treatment. Similar results where obtained on patients samples.

Conclusions: Our results suggest that the dependence receptor TrkC could act as a conditional tumor suppressor that regulates survival and invasive capacity of neuroblastoma cells.

B94 E2F-1-Mediated Modulation of p73 Isoforms by COX-2 Inhibitor in Neuroblastoma

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Background: The *p73* gene encodes both pro-apoptotic TAp73 and anti-apoptotic DNp73 isoforms, which lack the TA (transactivation) domain and confers poor prognosis in neuroblastoma and other tumors. Thus, drugs that upregulate TAp73 or downregulate DNp73 represent an important potential therapeutic strategy.

Methods: Immunoblotting, immunoprecipitation, chromatin immunoprecipiaton (ChIP), RT-PCR, MTT, pulse-chase, luciferase reporter assays, xenograft studies.

Results: The cyclooxygenase-2 (COX-2) inhibitor celecoxib inhibits cell growth, induces apoptosis, and differentially modulates endogenous p73 isoforms in neuroblastoma with different p53 and *MYCN* status both *in vitro* and *in vivo*, as well as in primary neuroblastoma tumor-initiating cells with properties characteristic of neuroblastoma stem cells. Celecoxib increases the expression of TAp73beta and its targets $p57^{kip2}$, PUMA, NOXA, and p21, independent of p53 status. In contrast, $DNp73^{AS}$ (alternative splice) isoforms, which are transcribed from the *p73 P1 promoter*, are diminshed following celecoxib treatment. We have previously demonstrated that the transcription factor E2F-1 transcriptionally activates the *p73 P1 promoter.* In addition, $DNp73^{AS}$ overexpression correlates with high E2F-1 levels in primary tumors. Here, we show that downregulation of $DNp73^{AS}$ by celecoxib is mediated by E2F-1 inhibition. Celecoxib induces dose-dependent inhibition of the *p73 P1 promoter* transactivation and decrease in E2F-1 levels, as well as inhibiting recruitment of E2F-1 to the *p73 promoter* as detected by ChIP. We have also evaluated the celecoxib analogue OSU03012 that lacks COX-2 inhibitory function. OSU03012 induces apotosis in the nanomolar range and, like celecoxib, downregulates E2F-1 and $DNp73^{AS}$, suggesting that modulation of p73 is independent of COX-2 enzyme inhibition.

Conclusions: COX-2 inhibitors are the first agent reported to differentially modulate pro- and anti-apoptotic p73 isoforms. The relative potency and lack of COX-2 inhibition of OSU03012 suggest that newer celecoxib analogues may have improved efficacy and safety profiles for the treatment of neuroblastoma and tumors with aberrant E2F-1 and/or DNp73 expression.

Poster

B95 Opposite Transcriptional and Post-Transcriptional Regulation of MYCN by p73

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Background: *MYCN* gene activation, mainly by amplification, is one of the most frequent molecular events in neuroblastoma (NB) and is associated with a decreased propensity for neuronal differentiation and increased malignancy. Concomitantly with *MYCN* amplification, NBs frequently display LOH at the 1p36.3 locus, harboring the *p53* tumor suppressor gene homologue *p73*. Hence, *MYCN* and *p73* alterations might cooperate in the pathogenesis of NB. The *p73* gene encodes multiple isoforms that differ in their N- and C-termini and are known to play crucial roles in neurogenesis, immune response, and potentially carcinogenesis.

Methods: *MYCN* overexpressing NB cells were transfected with p73 isoforms expression vectors (TAp73α, ΔNp73α and TAp73β) or TAp73 siRNA to ectopically express or inactivate p73. RNA and protein levels were monitored by real-time RT-PCR and western blotting. *MYCN* promoter activity was assessed using a Luciferase reporter construct. *MYCN* mRNA stability was evaluated by measuring transcript levels at different time points following actinomycin D treatment.

Results: Ectopic expression of p73α isoforms in p73-deficient *MYCN*-overexpressing NB cells strongly down-regulated *MYCN* expression, at both transcript and protein levels. Consistently, siRNA-mediated depletion of endogenous TAp73 led to an increase in *MYCN* expression. As *MYCN* promoter reporter analysis showed that p73α stimulates *MYCN* transcription, we thought that p73 also negatively regulates *MYCN* expression at the post-transcriptional level. Indeed, we found that *MYCN* mRNA half-life was decreased or increased upon transfection with p73α expression vectors or TAp73 siRNA, respectively. *MYCN* transcriptional activation and post-transcriptional inhibition was observed with both TAp73α and ΔNp73α isoforms, suggesting that this regulation is independent of p73 N-terminal domain. Contrarily to α isoforms, TAp73β lacking the C-terminal SAM (sterile alpha motif) domain significantly inhibited *MYCN* promoter.

Conclusions: P73α isoforms activate *MYCN* transcription but inhibit its expression at the post-transcriptional level, the latter effect being dominant. We are currently investigating the mechanisms involved.

B96 Hypoxia Abrogates NGF-Induced Differentiation by Inhibition of MAPK Signaling in Human Neuroblastoma Cells

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Background: Neuroblastoma is a tumor originating from the sympathetic nervous system. The tumors display a high degree of heterogeneity and a large proportion of the affected children die from their disease. There is an established negative correlation between grade of differention and degree of aggressiveness among these tumors and we have earlier shown that neuroblastoma cells loose some of their differentiation markers, become more stem cell-like and possibly more aggressive when cultured at hypoxic conditions.

Methods: In order to further expand our previously accumulated data we wanted to investigate the effect of hypoxia on a panel of neuroblastoma cell lines considering differentiation status. Furthermore, we wanted to know if hypoxia could inhibit induced neuroblastoma cell differentiation. We used microarray analyses, Western blot and Q-PCR to detect and quantify differences in gene and protein expression of known neuronal differentiation markers and of scaffold proteins involved in regulating MAPK signaling.

Results: We here show that growth of neuroblastoma cells at hypoxia changed the expression of differentiation-associated genes and proteins such as *β-III tubulin*, *islet-1* and *vimentin*, suggesting a more dedifferentiated cell phenotype. Furthermore, severe hypoxia (0.1% O2) markedly reduced NGF-induced differentiation, measured both as reduced neurite outgrowth and expression of sympathetic neuronal markers. In addition, we observed a hypoxia-induced abrogation of TRKA-mediated signaling as well as of ERK/MAPK-signaling.

Conclusions: The results above support our previous conclusion that hypoxia pushes neuroblastoma cells toward a stem cell-like phenotype and indicates that hypoxia abrogates NGF-induced differentiation by promoting the expression of MAPK signaling-inhibiting scaffold proteins, such as *TNIP1* and *RGS4*.

B97 Role of the Plasminogen-Plasminogen Activator System in Neuroblastoma Angiogenesis

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Background: The activation of plasminogen into plasmin by urokinase plasminogen activator (uPA) plays an important role in the progression of many cancers, including neuroblastoma. It is controlled by the PA inhibitor-1 (PAI-1). We have previously shown that in human neuroblastoma tumors, PAI-1 is expressed by endothelial cells (EC) and that high PAI-1 expression paradoxically correlates with metastatic stage and tumor recurrence. Here we provide evidence for a mechanism by which PAI-1 promotes angiogenesis.

Methods: Using PAI-1 null immunodeficient mice orthotopically implanted with human neuroblastoma tumors, we demonstrated that in the absence of host-derived PAI-1, neuroblastoma formed smaller and poorly vascularized tumors that contained a higher number of apoptotic EC. We then used human microvascular EC in which PAI-1 was knocked down by siRNA to investigate the effect on survival.

Results: Knockdown of PAI-1 in human EC increased spontaneous apoptosis, reduced angiogenesis *in vitro*, and enhanced cell-associated plasmin activity. It also increased the activities of caspase-3 and 8 but not caspase-9. The increase in apoptosis in EC upon PAI-1 knockdown was dependent on FasL because downregulation of FasL in EC or treatment with an anti-Fas antibody prevented apoptosis in PAI-$1^{-/-}$ EC. We documented that plasmin cleaved FasL at Arg^{144}-Lys^{145}, releasing a soluble FasL pro-apoptotic fragment. Consistently, overexpression of FasL but not of a Val^{144}-Ala^{145} mutant FasL resistant to plasmin increased apoptosis in PAI-1 knockdown EC whereas treatment of EC with a recombinant soluble FasL fragment stimulated apoptosis and inhibited tube formation *in vitro*.

Conclusions: The data thus provide a new mechanism for the pro-angiogenic activity of PAI-1 in which PAI-1 plays a critical regulatory role in protecting EC from FasL-mediated apoptosis. Considering that inhibitors of PAI-1 such as monoclonal antibodies and small molecules have been developed and tested in pre-clinical models of thrombosis, the data suggest that PAI-1 could be a therapeutic target in neuroblastoma.

B98 Favorable Neuroblastoma Genes are MIZ-1 Targets

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Background: We have recently identified *MIZ-1* encoding a MYC-interacting zinger protein as a new favorable neuroblastoma gene. Favorable neuroblastoma genes are genes whose high-level expression predicts good neuroblastoma disease outcome and forced expression of these genes in neuroblastoma cell lines results in growth suppression. *MYCN* amplified neuroblastomas express little or no expression of favorable neuroblastoma genes. The attenuated expression of these genes in unfavorable neuroblastomas (*MYCN* amplified and non-amplified) is due to epigenetic silencing. Known favorable neuroblastoma genes include *EPHB6*, *EFNB2*, *EFNB3*, *TrkA*, *CD44*, and *MIZ-1*. Among these, *MIZ-1* is the first to encode a transcription factor. Forced expression of MIZ-1 in neuroblastoma cell lines induces the expression of other favorable neuroblastoma genes. This study was undertaken to determine if favorable neuroblastoma genes are MIZ-1 targets and to identify specific modifications of MIZ-1 protein that modulate its activity.

Methods: Mouse xenografts, RT-PCR, luciferase assay, Western blot, and 2D gel analysis were used in this study.

Results: In mouse therapeutic models of neuroblastoma, high *MIZ-1* expression was found in the small size xenografts. Moreover, *MIZ-1* expression was correlated with the expression of other favorable neuroblastoma genes in these xenografts. MIZ-1 activated transcription from proximal promoters of *TrkA*, *CD44*, and *EFNB3*. Similar experiments are in progress for *EPHB6* and *EFNB2*. MIZ-1 was modified in neuroblastoma cells via ubiquitination and phosphorylation, and phosphorylation likely stabilized MIZ-1 protein. In addition, DNA damage resulted in hyper-phosphorylation of MIZ-1.

Conclusions: Favorable neuroblastoma genes are MIZ-1 targets. The expression of favorable neuroblastoma genes may serve as a molecular indicator for drug responsiveness in therapeutic models. Phosphorylation of MIZ-1 and regulation of the phosphorylation process are important to MIZ-1 function. Lacking MIZ-1 expression is a key factor leading to the aggressive behavior of neuroblastoma.

B99 Identification of Cells Coexpressing Glial and Neuroblastic Markers in Both Neuroblastic Primary Tumors and Neuroblastoma Bone Marrow Metastasis

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Background: Neuroblastic tumors (NBT) are derived from neural crest stem cells. Double staining cells by neurofilament and calcyclin have recently been proposed as NBT precursor cells. However, in our hands, neurofilaments are expressed in both glial and neuroblastic cells. Instead, we found specific neuroblastic and glial markers in the GD2 membrane staining and nuclear calcyclin immunostaining, respectively. In this study, we searched for bilineage cells in primary NBT and bone marrow metastasis.

Methods: Immunofluorescence (IF) and flow cytometry (FCM) for membrane GD2 (neuroblastic lineage) and nuclear calcyclin (glial lineage) was used independently and simultaneously looking for GD2/calcyclin double stained cells. Fresh frozen sections (n=12) and bone marrow metastasis specimens (n=5) were investigated.

Results: By IF, GD2 staining was detected in all neuroblastic cells. Calcyclin was detected in the stromal-glial bundles and endothelial cells. Nine of the 12 primary tumors were evaluated for double staining and 3 different populations were identified: GD2$^+$/calcyclin$^-$ neuroblastic cells, GD2$^-$/calcyclin$^+$ Schwannian-like cells and some GD2$^+$/calcyclin$^+$ population, which included neuroblastic-like cells but also some of cells within the stromal bundles. The double staining neuroblastic subpopulation did not form clusters and was surrounded by GD2$^+$/calcyclin$^-$ neuroblasts. All metastatic bone marrow specimens analyzed showed double stained cells in the neuroblastic aggregates. Most cells in such aggregates were GD2$^+$/calcyclin$^-$ and only few double staining cells were present.
By FCM, GD2$^+$/calcyclin$^+$ cells were investigated in 7 tumors and the percentages varied from 10-60% of cells analyzed. A significant increase in the amount of GD2$^+$/calcyclin$^+$ cells was detected in one pair of samples, pre (18%) and post-chemotherapy (53%).

Conclusions: The presence of neuroblastic cells which coexpress glial and neuronal lineage markers in neuroblastic primary tumors and metastasis shows their bipotential capacity. The degree of differentiation of double stained cells is the subject of our ongoing work.

B100 Slug (SNAI2) Silencing by RNA Interference Affects Apoptosis and Inhibits Invasion of Neuroblastoma Cells in Vitro and in Vivo

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Background: About 50% of patients with neuroblastoma (NB) present at diagnosis with metastatic disease associated with very poor outcome despite intensive treatment. However, the mechanisms of NB invasion are still poorly understood.

Methods: We evaluated the effect of Imatinib Mesylate (IM) on invasion and analyzed the genes modulated by IM treatment in human NB cell lines. Slug expression, inhibited by IM treatment, was then knocked-down by RNA interference and the effects on invasion and apoptosis were evaluated *in vitro*. A pseudometastatic model of NB in SCID mice was used to assess the effects of Slug silencing alone or in combination with IM treatment on metastasis development.

Results Microarray analysis revealed that several genes, including Slug, were down-regulated by IM. Slug expression was detectable in 8/10 NB cell lines. Two Slug-expressing cell lines were infected with a vector encoding a miRNA to Slug mRNA. Infected cells with reduced levels of Slug were tested for the expression of apoptosis-related genes (p53, Bax, Bcl-2), previously identified as Slug targets. Bcl-2 was down-regulated in Slug-interfered cells. Slug down-regulation increased sensitivity to apoptosis induced by IM treatment. Invasion of Slug-silenced cells was reduced *in vitro*. Animals injected with Slug-silenced cells had fewer tumors than controls and the inhibition of tumor growth was even higher in animals treated with IM.

Conclusions: Slug silencing stimulates apoptosis induced by IM in NB cells and decreases their invasion potential *in vitro* and *in vivo*. Slug inhibition, possibly combined with IM, may represent a novel strategy for treatment of metastatic NB.

B101 Retinoids Decrease Expression of BDNF in Neuroblastoma (NB) Cells

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Background: BDNF activation of TrkB modulates survival and differentiation in normal neurons. BDNF is poor prognostic indicator in NB and stimulates metastasis and attenuates chemosensitivity of TrkB expressing tumor cells. Retinoids arrest the growth and induce differentiation of NB cells. In this study we assessed the role of BDNF and TrkB in retinoid mediated differentiation.

Methods: The kinetics and levels of BDNF, TrkB and N-myc mRNA and protein were assessed by qPCR, western blot and FACS. The function of BDNF and TrkB was evaluated using siRNAs targeted to BDNF and TrkB.

Results:Surprisingly RA decreased BDNF mRNA expression (50% decrease, $p<0.01$) and secretion(83% decrease, $p<0.01$) despite increasing levels of TrkB mRNA (80-fold, $p<0.01$) and protein (38-fold, $p<0.01$). RA mediated decreases in BDNF levels temporally followed decreases in N-myc. Although transfection of N-myc siRNA (60-80% reduction) significantly reduced secretion of BDNF (30-40% decrease) yet over-expression of N-myc did not alter BDNF levels indicating N-myc affects BDNF production indirectly. To understand the function of BDNF in RA mediated differentiation of NB cells, siRNAs targeted to BDNF were transfected into NB cells prior to RA treatment or after RA differentiation. BDNF siRNA caused a 50-90% decrease in BDNF and blocked RA induced initiation of neurites (63% decrease, $p<0.01$) but had no effect on established neurites. In contrast transfection of siRNAs targeted to TrkB (40-90% reduction) blocked both initiation of neurites (59% decrease, $p<0.01$) as well as inhibited the ability of differentiatied NB cells to maintain neuritic extensions (25% decrease, $p<0.05$).

Conclusions: 1) Retinoids decrease BDNF levels. 2) BDNF affects initiation but not maintenence of retinoid induced differentiation. These studies indicate that suppression of BDNF expression by RA may be an important factor in RA suppression of NB tumorigenicity.

B102 Differences Between TrkA vs. TrkB-Mediated Signal Transduction and Inhibition in Neuroblastoma

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Background: The clinical heterogeneity of neuroblastoma (NB) may depend on the differential expression and signaling properties of the neurotrophin (NT) receptors, TrkA and TrkB. These receptors utilize homologous intracellular signaling pathways, but downstream signaling events may diverge to promote biological and clinical differences observed in this disease.

Methods: We characterized the effects of NT-mediated receptor activation and pathway inhibition through TrkA and TrkB on cell morphology, signaling effector protein activation, cell proliferation, and apoptosis. The NB cell line, SY5Y was engineered to express full-length TrkA or TrkB. Cells were grown in serum-depleted medium and treated with an inhibitor to PI3K, LY294002 (10-40 uM) or MEK1/2 (Erk1/2), U0126 (5-20 uM), prior to the addition of nerve growth factor (NGF) for TrkA or brain-derived neurotrophic factor (BDNF) for TrkB. Morphology was assessed by phase-contrast light microscopy, proliferation by BrdU incorporation, and apoptosis by PARP cleavage.

Results: Addition of NGF to TrkA-NB cells produced neurite outgrowth, protracted TrkA receptor phosphorylation, and PI3K/Akt and MEK/Erk1/2 signaling pathway activation. In contrast, BDNF addition to TrkB-NB cells produced marked cell proliferation without morphological differentiation, and transient phosphorylation of TrkB, PI3K/Akt and Erk1/2. Inhibition of PI3K and Erk1/2 resulted in marked decreased proliferation in both TrkA and TrkB NB cells, but TrkB-NB cells were more sensitive to Erk1/2 inhibition than TrkA-NB cells at lower U0126 concentrations ($P<0.05$). Additionally, Erk1/2 signal inhibition in TrkB-NB cells resulted in morphologic changes and PARP cleavage consistent with apoptosis. TrkA-NB cells had decreased neurite outgrowth after PI3K inhibition, whereas TrkB cells demonstrated a modest increase in neurite growth and length.

Conclusions: These findings suggest that the distinct temporal and cellular responses to signaling through the TrkA or TrkB receptors in the SY5Y NB cell line may underlie the diverse clinical behaviors of NB and may provide more targeted therapeutic approaches.

Poster

B103 Potent Barriers to *MYCN* Deregulation are Retained by Malignant Neuroblasts without *MYCN* Amplification

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Background: *MYCN* amplification correlates with aggressive neuroblastoma (NB) behavior. However, in non-malignant cells enforced *MYC* activates potent barriers against transformation. We showed enforced MycN synergizes with cellular stressors to engage apoptosis in human neural cells (ANR 2006). We now extend these studies to malignant neuroblasts without *MYCN* amplification.

Methods: *MYCN*-non-amplified (SK-N-AS, SHEP, LHN, SK-N-SH, SY5Y, LAN6, NB69 and SK-N-FI) and non-malignant RPE1 cells were studied. Inducible *MYCN* constructs included retroviral MycN:ER and Tet-regulatable *MYCN* (RevTet; Clontech). Transfectants were characterized for phenotypic responses to deprivational and cytotoxic stressors in vitro.

Results: Up to 8 retroviral selection passes were attempted for each cell line, yet only SKNAS-MYCN:ER and SHEP-MYCN:ER were derived. In all others, non-induced basal low-level MycN activity was sufficient to engage apoptosis, differentiation (extensive neurites) or senescence (SA-Bgal+) programs. Attempts with the RevTet system are ongoing. Unlike RPE1-MYCN:ER cells, SKNAS-MYCN:ER and SHEP-MYCN:ER cells did not increase proliferation with enforced *MYCN* under non-stress conditions, and *MYCN*-induced apoptosis during serum deprivation was modest. *MYCN*-amplified NBs demonstrate glucose-deprivation sensitivity, and MycN:ER activation in non-amplified cells confered a similar glycolytic phenotype with enhanced glucose depletion-induced death. MycN:ER also synergized with cisplatin (4-fold lowered IC50), doxorubicin, VP16 and topotecan. This pattern differs from RPE1-MYCN:ER cells where only cisplatin synergized with *MYCN*. Of note, SK-N-AS (p53 homozygous del-ex10/11) and SHEP (Ink4a/Arf null) are the only NB cell lines we are aware of in which inducible MycN is tolerated. Affymetrix-based transcriptome profiles are being studied to identify operative *MYCN*-induced apoptosis pathways.

Conclusions: Robust barriers to deregulated *MYCN* are retained by malignant NBs unaccommodated to *MYCN* amplification. Those accepting deregulated MycN may be predisposed through abrogation of p53 signaling. By extension, *MYCN*-amplified NBs must circumvent *MYCN*-imposed barriers to oncogenicity and these biopathways (including engagement of p53) remain of interest.

B104 High-Resolution Copy Number Analysis and Identification of Target Genes in Neuroblastoma Using High-Density SNP-Genotyping Microarrays

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Background: Neuroblastoma (NB) is particularly noted for extensive heterogeneity in clinical behavior, ranging from spontaneous regression to death from disease. Although age, stage, and several genetic abnormalities, such as *MYCN* amplification, 1p LOH and 17q gain are known to be important factors that influence clinical outcome, the target genes and genetic pathways involved in the pathogenesis of NB have remained elusive.

Methods: To explore the genetic alterations underlying the pathogenesis of NB, we analyzed 55 advanced NB specimens using high-density single nucleotide polymorphism (SNP)-genotyping microarrays (Affymetrx® 50K/250K GeneChip ®). The CNAG/AsCNAR algorithms were used to calculate allele-specific CNs without depending on the availability of matched control DNA, enabling sensitive detection of LOH as well as CN alterations in a wide spectrum of primary tumor specimens, even in the face of up to 70-80% normal cell contamination.

Results: Analysis of 55 NB samples disclosed a number of previously unrecognized regions of high-grade amplifications including 1p36, 7q21, 7q31, 11q13 and 15q13. Furthermore, common homozygous deletions such as 1p31, 3q13, 9p24, 15q11 and 16p13 were observed in several cases. A number of candidate gene targets of common deletions and amplifications were identified, including *NEGR1* in common 1p31 deletions and, and *MYEOV* found in overlapping critical amplicons in 11p13. When expressed in NB-19 cell, NEGR1 inhibits cell growth. On the other hand, siRNA-mediated downregulation of *MYEOV* expression in NB-19 and CHP-134 cells resulted in a significant decrease in cell proliferation.

Conclusions: These results indicated that copy number analysis using high-density SNP-genotyping microarrays is a powerful tool for genome-wide detection of genetic abnormalities in NB and expected to facilitate identification of novel genes relevant to development of NB.

B105 Deregulated Cytochrome C Oxidase Activity Promotes Drug-Induced Apoptosis in Neuroblastoma Cells

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Background: Despite the recent advances in the diagnosis and treatment of neuroblastoma, high incidence of chemotherapy resistance remains the primary problem in the treatment of high-risk tumors. Using TKO functional gene cloning strategy we have previously identified *COX8A* as a mediator of doxorubicin sensitivity in neuroblastoma cells.

Methods: *COX8A* was ectopically overexpressed or downregulated by siRNA in Tet21N cells and drug-induced apoptosis was analyzed by FACS of PI stained cells. The intracellular superoxide levels were analyzed by FACS in MitoSOX™ stained cells. Cytosolic proteins were selectively isolated using digitionin and analyzed by Western blotting. Cytochrome c oxidase activity was measured on isolated mitochondria using colorimetric assay. Expression of *COX8A* was analyzed in 251 primary tumors using oligonucleotide microarray.

Results: Transient downregulation of *COX8A* decreased sensitivity of Tet21N cells to doxorubicin, while its ectopic overexpression significantly sensitized these cells to doxorubicin-induced apoptosis. *COX8A* overexpression deregulated enzymatic activity of cytochrome c oxidase. Doxorubicin treatment of Tet21N cells overexpressing *COX8A* (Tet21N-COX8A) resulted in elevated levels of the intracellular superoxide, accelerated mitochondria fission, and increased phosphorylation of c-Jun, suggesting involvement of oxidative stress. Increased sensitivity of Tet21N-COX8A cells to doxorubicin did not involve enhanced caspases activity but was associated with cytosolic translocation of Apoptosis-inducing factor (AIF) from mitochondria and cathepsin D from lysosomes, suggesting involvement of caspase-independent forms of cell death. In primary neuroblastomas, high *COX8A* expression together with low levels of *AIFM1* mRNA was found in high-risk tumors.

Conclusions: Sensitivity of neuroblastoma cells to cytotoxic drugs may be modulated by the redox state and caspase-independent cell death pathways. In high-risk tumors upregulated expression of *COX8A* together with downregulation of *AIFM1* suggests resistance to oxidative stress-induced cell death.

B106 N-Myc is a Novel Regulator of PI3K–Mediated VEGF Expression in Neuroblastoma

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Background: Angiogenesis in neuroblastoma (NB) correlates with increased expression of vascular endothelial growth factor (VEGF) and a worse clinical outcome. Other cellular markers, such as Akt activation and *MYCN* amplification, are also associated with poor prognosis in NB; therefore, we sought to determine the role of N-myc in the regulation of the PI3K/Akt/VEGF pathway.

Methods: *In vivo*, human NB SK-N-SH xenografts were established in athymic nude mice and treated with vehicle or wortmannin (1.5 mg/kg/day). Tumors and plasma were collected for protein analysis and VEGF ELISA, respectively. *In vitro,* human NB cell lines, BE(2)-C, SK-N-SH, LAN-1 and JF were treated with small molecular inhibitors of PI3K, rapamycin (an mTOR inhibitor), p110α siRNA, PTEN adenovirus, *MYCN* siRNA, or *MYCN* vector, in the absence or presence of IGF-1. VEGF secretion and protein expression of p-Akt, Akt, p-mTOR, mTOR, HIF-1α, p110α, N-myc or PTEN were determined.

Results: Tumor growth, vascularization, and VEGF levels were significantly inhibited in wortmannin-treated mice when compared to the control group. PI3K inhibition, using small-molecule inhibitors, p110α siRNA, or PTEN adenovirus, and mTOR inhibition using rapamycin led to decreased levels of VEGF secretion in the presence or absence of IGF-1 *in vitro*. Moreover, IGF-1 increased N-myc expression; this effect was attenuated by LY294002 or PTEN overexpression. Blocking N-myc expression with siMYCN significantly reduced VEGF secretion in high N-myc expressing NB cell lines. Moreover, *MYCN* overexpression or PI3K stimulation led to attenuation of VEGF secretion by siMYCN in low N-myc expressing cells. The inhibition of VEGF secretion with *MYCN* knockdown was further enhanced when combined with rapamycin.

Conclusions: Our results, for the first time, show that N-myc plays an important role in the PI3K-mediated VEGF regulation in NB cells. Targeting *MYCN*, as a novel effector of PI3K-mediated angiogenesis, has significant potential for the treatment of highly vascularized, malignant NB.

B107 A Novel Neuronal Receptor, NLRR3, Induces Differentiation of Neuroblastoma and Is a Direct Transcriptional Target of MYCN for Its Repression

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Introduction: We have previously reported that the NLRR family genes, which encode orphan receptors with unknown function, are differentially expressed in primary neuroblastomas. They may regulate growth, differentiation and apoptosis in neuroblasotma. However, their precise molecular mechanisms are elusive. In the present study, we have identified that *NLRR3*, the novel human gene whose expression is predictive of favorable neuroblastoma, induces neuronal differentiation and that it is a direct target of MYCN for its repression.

Results ad Discussion: Human *NLRR* family genes have previously been identified from the screening of >2000 novel genes selected from primary neuroblastoma cDNA libraries for differentially expressed genes between favorable (stages 1 or 2 tumors with a single copy of *MYCN* and high expression of *TrkA*) and unfavorable (stages 3 or 4 tumors with amplification of *MYCN*) subsets. Expression of *NLRR3* was high in favorable subsets as compared to unfavorable ones, whereas another family member NLRR1 was highly expressed in unfavorable tumors. Interestingly, *NLRR2* and *NLRR6* were not correlated with the prognosis of neuroblastoma. We found that expression of *NLRR3* was downregulated by enforced expression of *MYCN* and was upregulated by knockdown of *MYCN* at both mRNA and protein levels. *NLRR3* was also upregulated during retinoic acid-induced differentiation of neuroblastoma cells, that might be mediated by downregulation of endogenous MYCN. We identified three putative E-boxes which showed repressor activity. The chromatin immunoprecipitation (ChIP) analysis demonstrated that MYCN is recruited on to the putative E-boxes within the promoter region of *NLRR3* gene. Interestingly, c-MYC oppositely regulated *NLRR3* expression. Thus, MYCN specifically inhibited both NLRR3 transcription and the following differentiation phenotype in neuroblasotma, which could lead us to find out new therapeutic strategy to cure the patient with aggressive neuroblastoma.

B108 EF1A1 Physically Interacts with BMCC1 and Regulates Differentiation and Survival of Neuroblastoma Cells via TrkA Signaling Pathway

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Background: To unveil the molecular mechanism of neuroblastoma (NBL) tumorigenesis, we have previously performed screening for the genes differentially expressed between favorable and unfavorable subsets by using the neuroblastoma proper cDNA libraries we generated, and identified a novel gene BMCC1. BMCC1 is expressed significantly at high levels in favorable neuroblastomas and regulates their differentiation and apoptosis (Machida *et al.*, *Oncogene*, 2006). However, its precise molecular mechanisms remain to be elucidated.

Results and Discussion: The yeast two-hybrid screening was performed to identify proteins that interact with BMCC1. The C-terminal portion of BMCC1 which includes BCH domain was used as bait for screening. Subsequent analysis identified the translation elongation factor 1 alpha 1 (EF1A1) as a BMCC1-interacting molecule. It is well known that EF1A1 promotes the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes during protein biosynthesis. Moreover, previous report indicated that both of EF1A1 and EF1A2 may play an important role in mouse neuronal development (Khalyfa et al, JBC 2001). Both GST pull-down and immunoprecipitation assays using the EF1A1 and/or BMCC1-transfected cell lysates confirmed the physical interaction between BMCC1 and EF1A1. The immunofluorescence analysis revealed that EF1A1 co-localizes with BMCC1 in cellular cytosol, that was further supported by cell fractionation and Western blot analysis. In PC12 cells treated with NGF, EF1A1 knockdown induced neurites extension and overexpression of EF1A1 clearly inhibited the differenciation. Interestingly, the knockdown of BMCC1 promoted the NGF-induced neurites outgrowth even in the EF1A1-overexpressing PC12 cells, suggesting that function of EF1A1 in the neuronal differentiation is dependent on BMCC1. In TrkA signaling pathway, of note, EF1A1 overexpression modified ERK phosphorylation in the NGF-treated PC12 cells. Thus, the BMCC1/EF1A1 complex may play an important role in regulating differentiation and cell survival in neuroblastoma cells through a NGF/TrkA signaling pathway.

B109 BH3 Response Profiles from Isolated Neuroblastoma Mitochondria Predict Sensitivity to Discrete Small Molecule BH Antagonists

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Background: Bcl2-homology (BH) proteins govern mitochondrial apoptosis. Hematolymphoid malignancies frequently overexpress a single BH protein and are sensitive to its selective antagonism, which is predicted by profiling tumor mitochondria for responses to diverse BH3 stimuli. We applied this approach to the more heterogeneous neuroblastoma (NB).

Methods: We optimized solid tumor BH3 response profiling: isolated mitochondria from NB cell lines were assayed for cyto-c release following exposure to BH3-mimetic peptides representing distinct death stimuli.

Results: Most NB mitochondria released cyto-c following BimBH3 or BidBH3 supporting intact Bak/Bax signaling. Responses to peptides incapable of directly activating Bak/Bax suggest activator-BH3 proteins are tonically sequestered by pro-survival proteins but can be competitively displaced to initiate cyto-c release. We demonstrated Bim bound to Mcl1 and Bcl2 in NB at steady state (coIP). Most cell lines showed NoxaBH3-induced release, supporting Mcl1 addiction and/or BadBH3 release, supporting Bcl2 addiction. Moreover, cell line immunoblot (IB) profiles and primary Tumor Microarray IHC demonstrated heterogeneity and redundancy with both Mcl1 and Bcl2 commonly co-expressed. However, IBs from isolated mitochondria showed increased Mcl1:Bcl2 ratio compared to whole cell expression. We confirmed selective knock-down of survival proteins using siRNA and noted increased apoptosis following Mcl1 compared to Bcl2 reduction, even in Bcl2-overexpressing cells (SK-NAS, NLF, IMR5). Validating specific pro-survival protein dependence, we tested cells in vitro with small molecules having Mcl1 (AT-101, Ascenta) or Bcl2, -xl, -w affinity (ABT-737, Abbott). Most NB cell lines tested were more sensitive to AT-101 (IC50 2-5 uM) while demonstrating variable sensitivity to ABT-737 (IC50 > 20 mcM). Nonmalignant neural cells were insensitive to AT-101 or ABT-737 consistent with their mitochondrial profiling.

Conclusions: Mitochondrial studies support a functional Mcl1-dependence in NB despite redundant Mcl1/Bcl2 expression. Mining tumor-specific mitochondria for BH protein addiction patterns in NB can prioritize pro-death therapeutics for these chemorefractory tumors.

B110 Identification of Neuroblastoma Stem Cells in Cell Lines

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Background: Better understanding of neuroblastoma stem cells and how they respond to retinoic acid may advance future treatments. Such cell populations have been identified using different methods but it is unclear whether different techniques isolate the same cell populations.

Methods: We used microscopy and flow cytometry to assess phenotypic heterogeneity and stem cell marker expression in SH-SY5Y, SH-EP and IMR32 cells. N-type markers Chromogranin A, beta-tubulin III, GD2, S-type: Vimentin and stem cell markers: CD117 and CD133 were used. Aldehyde dehydrogenase (ALDH) activity was also measured by flow cytometry in live neuroblastoma cells as a stem cell marker. Retinoic acid (1 microM, 4 days) was used to differentiate the cells.

Results: SH-SY5Y, IMR32 and SH-EP neuroblastoma cell lines comprised a range of morphologically distinct cell types. Chromogranin A expression identified two distinct populations in SH-SY5Y cells, but not in IMR32 or SH-EP cultures. GD2 expression identified two distinct populations in IMR32 cultures but not in SH-SY5Y or SH-EP cultures. This raised concerns about the classification of neuroblastoma cells into N-, I- or S-type using conventional N- and S-type markers. Retinoic acid treatment led to morphological differentiation of SH-SY5Y cells and down-regulation of beta-tubulin III in a sub-population. This suggests retinoic acid is involved in the differentiation of non-neuronally commited neuroblastoma cells.SH-EP cells were negative for stem cell markers CD117 and CD133 whereas SH-SY5Y cells contained 0.6%(CD117) and 13.9%(CD133) positive cells. ALDH activity was found in SH-SY5Y(1.39-1.98%) and IMR32(0.06%) but not in SH-EP cells.

Conclusions: Estabilised neuroblastoma cell lines comprise heterogeneous cell populations with variable expression of stem cell markers. Down-regulation of an N-type marker in a sub-population of SH-SY5Y cells by retinoic acid is an observation that warrants further study and more work is required to establish the effect of retinoic acid on stem cell markers.

Poster

Poster

B111 High-Resolution ArrayCGH and MLPA Detect Regions of Deletion, Gain, Amplification and Copy-Neutral LOH in Primary Neuroblastoma Tumors; *CDKN2A* in 9p is Subjected to Homozygous Deletions in Four Cases

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Background: Neuroblastoma is a highly heterogeneous pediatric tumor. Favourable tumors are likely to have near-triploid karyotypes with few structural rearrangements. Aggressive stage 4 tumors often have near-diploid or near-tetraploid karyotypes and structural rearrangements. We have used Affymetrix oligonucleotide microarrays to analyze the chromosomal structure of a large number of neuroblastoma tumors.

Methods: 92 neuroblastoma tumors were analyzed with 50K and/or 250K SNP arrays from Affymetrix and the CNAG3.0 software.

Results: Of the 92 tumors, 30% harbored 1p-deletion, 22% deletion of 11q, 26% had *MYCN*-amplification and 45% 17q-gain. Loss of 11q was with three exceptions only found in tumors without *MYCN*-amplification. 1p-deletion were found both in tumors with *MYCN*-amplification and those with 11q-loss, while in the 11q loss, tumors the 1p-deletions tended to be smaller. In regards to the *MYCN*-amplification, two types were identified. One type displayed amplification with a simple continuous region in and around *MYCN*, while another group harbored more complex rearrangements of different distinct regions. No other genes beside *MYCN* were found in all cases with amplification. Complex amplification of regions on chromosome 12 was detected in two tumors; three different overlapping regions of amplification were identified, one of which contained the *MDM2* gene. Two regions with homozygous deletions in primary neuroblastoma tumors were detected in the study; four cases of homozygous deletions (and five heterozygous cases) of the *CDKN2A* region in 9p and one case with deletion on 3p (the gene *RBMS3*).

Conclusions: SNP arrays are a very useful tool for analyzes of chromosomal rearrangements in neuroblastoma tumors. The mapping arrays from Affymetrix provide both copy number and allele-specific information where breakpoints can be defined with an accuracy of 10-12 kb. The gene *CDKN2A*, is subject to homozygous (four cases) and heterozygous deletions (five cases) in neuroblastoma tumors.

B112 An Unexpected Role for Caspase-10 in TRAIL-Induced Neuroblastoma Apoptosis

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Background: Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) selectively induces apoptosis in most tumour cells. The silencing of caspase-8 expression in invasive NB cells was shown to be responsible for their resistance to TRAIL-induced apoptosis. We have previously demonstrated that stable restoration of caspase-8 expression fully restored TRAIL sensitivity in the caspase-8/-10 silenced IGRN-91 cell line.
Caspase-10 is also frequently downregulated in aggressive NB cell lines. As the role of caspase-10 and its ability to substitute for caspase-8 in TRAIL-induced apoptosis is still controversial, we have analysed the particular contribution of caspase-10 in the mechanisms of TRAIL-induced cell death in NB cells.

Methods: Stable re-expression of caspase-10 was performed by retroviral infections in the caspases-8/-10 negative IGR-N91 cells. Caspases-8 and/or caspase-10 were silenced in the SH-EP, CA-2-E and SK-N-AS cell lines by stable shRNA lentiviral infections or transient siRNA transfections. mRNA quantification was performed by real time PCR (Lightcycler).

Results: In contrast to caspase-8, stable re-expression of caspase-10 in the TRAIL-resistant IGR-N91 cells was not able to restore TRAIL sensitivity. Reverse experiments were performed in TRAIL sensitive caspases-8/-10 positive NB cells using RNA interference strategy. Caspase-8 silencing resulted in complete resistance to TRAIL-induced apoptosis, indicating that caspase-10 on its own was unable to substitute for caspase-8 to activate downstream caspases, Bid and ultimately cell death. Interestingly, caspase-10 silencing did not increase NB cells resistance to TRAIL, but in contrast enhanced their sensitivity to TRAIL-induced apoptosis.

Conclusions: Caspase-10 is not able to substitute caspase-8 in NB cells to initiate a full apoptotic cascade in response to TRAIL. The downregulation of caspase-10 in NB cells does not contribute to TRAIL resistance, but in contrast sensitises NB cells to TRAIL in the presence of caspase-8. The relative roles of caspases-10/-8 in the initiation of NB apoptosis may be tumour-type specific and is currently explored.

B113 Bmi-1 Is a Key Oncogene in Neuroblastoma Development

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Background: Activation of oncogenes underlies the pathogenesis of most human cancers. Identification of oncogenes that are consistently mutated or overexpressed in neuroblastoma is crucial for a molecular understanding of the pathogenic process. We investigated the role of Bmi-1 in the pathogenesis of neuroblastoma. Bmi-1 is a member of the Polycomb Group family of transcription repressors and has an essential role in maintaining sympathetic neural crest stem cells from which neuroblastoma may originate.

Methods: We examined Bmi-1 expression by immunoblotting and immunohistochemistry in human neuroblastoma cell lines, primary tumor specimens, and during neuroblastoma development in *MYCN* transgenic mice, an animal model for the human disease. Retroviral systems were used to overexpress or knockdown Bmi-1 in neuroblastoma cell lines, followed by tumorigenic assays. Finally, Bmi-1-deficient mice were crossed with *MYCN* transgenic mice to investigate the effect of Bmi-1 deficiency on neuroblastoma development in vivo.

Results: We found that Bmi-1 is highly expressed in human neuroblastoma cell lines and primary tumors. Also, neuroblastoma development in MYCN transgenic mice is associated with a marked increase in the levels of Bmi-1 expression. Bmi-1 cooperates with MYCN in transformation of benign S-type neuroblastoma cells and avian neural crest cells by inhibiting the apoptotic activity of MYCN. Importantly, downregulation of Bmi-1 impairs the ability of neuroblastoma cells to grow in soft agar and to induce tumors in immunodeficient mice, and Bmi-1 deficiency significantly decreases the tumor incidence in *MYCN* transgenic mice.

Conclusions: Bmi-1 is an important oncogene the pathogenesis of neuroblastoma.

B114 Mechanisms of PKCε-Induced Morphological Changes of Neuroblastoma Cells

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Background: Neuroblastoma, deriving from the sympathetic nervous system, is one of the most common primary tumors in childhood and causes up to 15 % of all childhood cancer deaths. The fatal cases result from tumors that are poorly differentiated and consist of cells resembling immature sympathetic neuroblasts lacking morphological differentiation markers.
We have previously seen that the protein kinase C ε isoform (PKCε), independently of its catalytic activity, induces profound morphological changes of cultured neuroblastoma cells such as outgrowth of neurites, a trait characteristic for mature, differentiated neurons. This study focuses on the mechanisms mediating this morphological differentiation and investigates if PKCε also influences other cytoskeleton-driven processes such as migration.

Methods: Immunoprecipitation assays; Migration assay (using Neuroprobe); Scratch assays; Immunofluorescence microscopy.

Results: By using the most potent neurite-inducing part of PKCε in an immunoprecipitation assay, we could identify several PKCε-associating proteins, including the intermediate filament protein peripherin and G3BP2, a putative RNA-binding protein with several suggested functions. The association between full-length PKCε and the individual proteins has been confirmed with immunoprecipitation and the domains in PKCε that are crucial for interaction coincides with domains critical for neurite induction. We also have preliminary data indicating that downregulation of PKCε, but not of PKCα or PKCδ, suppresses the migration of neuroblastoma cells while downregulation of peripherin enhances the migratory capacity.

Conclusions: The data highlight two previously unrecognized PKCε-interacting proteins, peripherin and G3BP2, as potential mediators of PKCε induction of neurite outgrowth of neuroblastoma cells. It is also conceivable that they have important functions in the migratory capacity of these cells.

B115 Uncovering the Biological Function of Caspase-8 in NB

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Background: Caspase-8 expression is decrease or absent in a large percentage of human neuroblastoma patients. Because early neural progenetitor cell lack caspase-8 this observation reflect the origin of the tumor cells. However, the absence of caspase-8 may also alter the development, growth or metastasis of tumors. Our laboratories have been examining the biological consequences of diminished caspase-8 expression using a variety of model systems and in vitro assays.

Methods: The effects of caspase-8 on tumor cell growth, metastasis and attachement and migration were assayed using human neuroblastoma cell lines with and without caspase-8 expression in SCID mice, chick embryos and cell culture. Caspase-8 expression was monitored by immunoblotting and immunostaining. Tumor cell attachment and migration were assasyed with using standard in vitro assays. Metastatsis was assessed in the chck embryo model sytem by PCR using primers specific fro human Alu repeats and in the mouse models by histological staining. The role of caspase-8 in vivo was also assessed in caspase-8 conditional knockout mice in the presence of absence of overexpressed MYCN.

Results: Deletion of caspase-8 did not cause tumor formation in knockout mice. However, loss of caspase-8 did increase the ability of NB cells to metastasize in SCID mouse and chick model systems. This is due at least in part to the lack of caspase-8 dependent integrin mediated death. Paradoxically, we also found that the presence of caspase-8 increased tumor cell attachment and migration on ECM substrates. Interestingly the catalytic activity of caspase-8 was not required for the effects of caspase-8 on attachment and migration. These data are consistent with the SCID mouse studies which demonstrated that NB cells expressing caspase-8 formed larger metastatic tumors however, these tumors in longer expressed caspase-8.

Conclusions: These data support the conclusion that caspase-8 plays multiple roles in NB tumorigenesis.

B116 ShcC Protein Controls Differentiation of Neuroblastoma Cells

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Background: Shc family docking proteins possess two different phosphotyrosines-binding motifs and conduct signals of various receptor tyrosine kinases such as ALK, TRK and RET. We have shown that ShcC, a neuro-specific member of Shc family proteins, is hyperphosphorylated in some of the neuroblastoma cell lines which has activation of ALK protein and is playing the essential roles in the proliferation of these cell lines. Many other neuronlastoma cell lines show overexpression of ShcC protein which is moderately phosphorylated. In this study, the roles of ShcC protein in wide range of neuroblastoma cell lines and tissues are analyzed.

Methods: The biological effects of suppression of ShcC expression by siRNA and overexpression of ShcC protein in neuroblastoma cells were analyzed as for cell proliferation, survival and differentiation. Expression and phosphorylation of ShcC protein were examined in human neuroblastoma tissues and their involvement in various aspects of neuroblastoma was analyzed.

Results: The suppression of the expression of ShcC by specific siRNA induced the neurite outgrowth of neuroblastoma cells such as TNB-1 which overexpress ShcC. At the same tme, appearance of several neuronal markers and sustained activation of ERK was observed, suggesting the suppression of ShcC induced differentiation of neuroblastoma cells. Supression of ShcC protein in TNB-1 cells significantly inhibited tumor formation in the nude mice. By overexpression of wildtype and mutant ShcC proteins, it was revealed that ShcC negatively regulate the ShcA pathway in a phosphorylation-independent manner. Preliminary results of the expression analysis indicate that the ShcC protein is preferentially expressed in the unfavorable type of neuroblastoma.

Conclusions: It was suggested that the expression of ShcC protein is regulating the prognosis of neuroblastoma by suppression of the differentiation of neuroblastoma cells. Potential value of ShcC as a predicting tool of prognosis and as a therapuitic target of neuroblastoma will be further investigated.

B117 Evidence that MYCN May Regulate p53 Expression and Transcriptional Activity in Neuroblastoma

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Background: We previously observed an association between nuclear p53 expression and MYCN expression by immunocytochemistry and with *MYCN* amplification ($p<0.05$) in neuroblastoma tumours (unpublished). We hypothesised that MYCN regulates p53 expression. **Aims:** a) To determine if MYCN and p53 expression correlate in neuroblastoma cell lines b) To investigate whether MYCN regulates p53 expression using MYCN conditionally expressing Tet21N cells.

Methods: p53 mRNA and protein levels were analysed in 5 *MYCN* amplified and 5 *MYCN* non-amplified cell lines, and Tet21N MYCN+/- cells, using Western blotting and quantitative reverse transcription PCR (qRT-PCR). p53 transcriptional activity was investigated using a p53 dependent luciferase gene assay. Microarray analysis of Tet21N MYCN+/- cells was used to identify whether p53 or any p53 regulated genes showed higher transcript levels in the presence of MYCN. Expression changes in selected genes were validated using qRT-PCR. p53 siRNA was used to knock down p53 in Tet21N MYCN+ cells to validate if the changes in expression of p53 regulated genes were p53 dependent.

Results: p53 protein expression in cell lines was associated with *MYCN* amplification ($p<0.05$) and MYCN protein expression ($r=0.977$). However p53 mRNA expression, and transcriptional activity was not associated with either *MYCN* amplification or MYCN protein expression. Tet21N MYCN+ cells expressed higher p53 mRNA ($p<0.05$) and protein ($p<0.05$), and had higher p53 transcriptional activity ($p<0.05$), compared with Tet21N MYCN- cells. Modulation of MYCN expression correlated with p53 expression. Microarray analysis of Tet21N MYCN+/- cells showed several p53 regulated genes were upregulated by MYCN e.g. MDM2 (2-fold) and BBC3 (3-fold). Knock down of p53 using siRNA confirmed that the increased expression of p53 regulatable genes in the presence of MYCN was p53 dependent.

Conclusions: These results suggest that p53 expression and consequent transcriptional activity may be regulated by MYCN. Chromatin immunoprecipitation is currently being performed to determine whether the p53 gene is a direct transcriptional target of MYCN.

B118 Epigenetic Study of Disseminated Tumoral Cells of Neuroblastoma

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Background: Epigenetic changes, as DNA hypermethylation, are heritable changes in gene expression that do not involve alterations in DNA sequence. Most tumours including neuroblastoma have aberrant DNA methylation leading to epigenetic silencing.

Bone marrow dissemination in neuroblastoma is frequent but not well-understood. Study of epigenetic alterations confined to disseminated disease might reveal a specific mechanism of spreading capacity, when disseminated cells are compared to cells from primary tumor.

Methods: Methylation patterns of genes involved in matrix adhesion, signal transduction, cell cycle regulation, apoptosis, DNA repair and drug resistance were studied by methylation-specific-PCR of matched samples from primary tumour, two samples of highly infiltrated bone marrow (BM) and/or peripheral blood (PB) of 17 neuroblastoma patients. Infiltration levels were measured by real-time quantitative PCR (DCX and TH gene expression) and cytology.

Results: Almost all paired samples showed epigenetic alterations in the disseminated tumoral cells partially different to the primary tumour in genes of DNA repair (26,7%), drug resistance (15,8%), signal transduction (18%), and apoptosis (16%).

All these alterations were seen in BM, and hypermethylation pattern was similar in the paired BM samples. In tumors we found more evidence of hypermethylation than in BM. PB samples, despite showing similar levels of DCX/TH expression than BM, they showed a lower degree of epigenetic alterations.

CFTR1, hMLH1, APAF1 and RB genes showed different methylation pattern in BM and tumor samples, with lack of correlation between them (R<0,1).

Conclusions:
- Most epigenetic alterations present in disseminated disease were also present in primary tumors.
- Epigenetic alterations seen in BM but not in tumor mainly belong to genes involved in DNA repair, drug resistance, apoptosis and signal transduction, which are mechanisms involved in tumoral cell spreading.
- Almost all alterations are seen in BM but not in circulating tumoral cells from PB.

Poster

B119 Transcription Factor AP2alpha is Involved in the Regulation of Differentiation in Neuroblastoma Cells

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Background: Neuroblastoma, the most common extracranial tumor of childhood, is derived from neural crest progenitor cells that fail to differentiate along their predefined route to sympathetic neurons or sympatho-adrenergic adrenal cells. The AP-2 family of transcription factors is not only involved in basal programs of development and differentiation, but their dysregulation also contributes to the pathogenesis of various malignancies. As TFAP2a is a central factor in the patterning of the neural crest, we here explore its role in neuroblastoma.

Methods: TFAP2a protein expression was immunohistochemically analyzed on a tissue microarray including 97 primary neuroblastic tumors. TFAP2 expression in neuroblastoma cell lines was analyzed using real-time PCR. The functional consequence of siRNA-induced TFAP2a knock-down was analyzed in cell-based assays *in vitro*.

Results: We report TFAP2a to be highly expressed in a subset of neuroblastomas, which is characterized by an undifferentiated histology. Vice versa, neuroblastic tumors with a differentiated phenotype demonstrated very low or no expression of the TFAP2a protein. In addition, we found TFAP2a protein to be expressed in nine established neuroblastoma cell lines with undifferentiated morphology, which were all derived from aggressive, high-stage neuroblastomas. Knock-down of TFAP2a by siRNA in human neuroblastoma SH-EP cells resulted in a morphological change towards a more differentiated phenotype concomitant with induction of neurotensin as a marker of neuronal differentiation. Further functional analyses of the SH-EP siRNA transfectants revealed a significant decrease in proliferation upon knock-down of TFAP2a.

Conclusions: Taken together, our data suggest that expression of the TFAP2a transcription factor is involved in the regulation of differentiation in neuroblastoma cells.

B120 Lack of a Transcription Factor Binding to a Site Located at the p21 Promoter in Human Neuroblastoma Cell Lines

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Background: Dysfunctional mutation in the p53 gene of neuroblastoma cells is very rare. However, abnormal p53 signal transduction pathway has been observed in many cases. We studied here the p53-dependent induction of p21 gene expression in several human neuroblastoma cell lines by focusing transcriptional regulation.

Methods: neuroblastoma cell lines used were CHP-134, NA, NB-1, and GOTO as p53 wild type cells and NGP, TGW, and SK-N-SH as p53 mutant cells. For p53 induction, 3.6 μM doxorubicin (DXR) treatment or transfection of a p53 expression plasmid, pXZ53^{wt}, was carried out. Induction of p53 and p21 proteins were determined by immunoblotting. For transcriptional activity, luciferase reporter gene assay was used. Protein-binding to DNA was studied by DMS-footprinting and EMSA.

Results: Expression of p21 protein in CHP-134, NA and NB-1 was detected by 12 hrs after DXR treatment accompanying with p53 induction. In spite of normal p53 induction of GOTO, p21 was apparently detected at 24 hrs. No significant changes were found in the p53 mutant cell lines. DMS-footprint analysis revealed that a novel element exist at approximately 40 bp upstream (-2281 to -2274: defined as site A) of p53 response element. One of the bands detected in CHP-134, NA, and NB-1 by EMSA with a site A probe was missing in GOTO. p53-dependency detected the EMSA was also confirmed by the luciferase activity. Transcriptional activity of site A was found in neuroblastoma cells, but not HeLa and colon cancer SW620 cells.

Conclusions: A novel element at the p21 promoter is found to be p53-dependent and seems to be specific to neuroblastoma cells. Identification of the factor binding to the element is under investigation.

B121 Epigenomic Profiles of Neuroblastomas with and without the CIMP

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Background: The CpG island methylator phenotype (CIMP) was closely associated with poor survival in Japanese and German neuroblastoma (NBL) cases with hazard ratios of 7-22. The prognostic power was independent from *TrkA* expression, DNA ploidy, and age. Almost all cases with N-*myc* amplification exhibited CIMP, and the presence of CIMP had strong prognostic power even in cases without N-*myc* amplification [Abe M, Cancer Res, 65, 828, 2005; Cancer Lett, 247, 253, 2007]. Here, detailed epigenomic profiles in the CIMP(+) and (-) NBLs were analyzed by DNA methylation mircroarray

Methods: Two NBL cell lines, IMR32 and SKNSH, two CIMP(+), two CIMP(-) primary NBLs, and the normal adrenal medulla were analyzed. Methylated DNA was collected by immunoprecipitation using anti-methylcytosine antibody (MeDIP). Microarray analysis was performed using Human CpG island (CGI) array (27,800 CGIs, 237k probes).

Results: Known maker CGIs for the CIMP were highly methylated in the CIMP(+) NBL cell lines and primary NBLs. In addtion, CIMP(+) IMR32 and SKNSH cell lines had 5,001 and 3,479, respectively, methylation positive probes, corresponding to 3,520 and 2,615 CGIs, respectively. The presence of methylation is now being validated.

Conclusions: CIMP(+) NBL cell lines and primary NBLs are likely to have extensive methylation around the genome.

B122 Identification of the Transcriptional Network Essential for the Tumorigenicity of Neuroblastoma Cells

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Background: Neuroblastoma cells undergo neuronal differentiation in response to retinoic acid (RA) treatment, leading to a loss of their tumorigenic capacity. We hypothesize that the genes that are downregulated upon induction of differentiation are required for maintaining the tumorigenicity.

Methods: The human neuroblastoma BE(2)-C cells were treated with 10 μM RA for 10 days. Total RNAs were isolated from untreated and RA-treated cells and analyzed by microarray using Human OneArray (Phalanx Biotechnology), a whole genome microarray containing more than 30,000 polynucleotide probes with each probe mapped to the latest draft of the human genome. Two entirely independent samples were used for microarray analyses.

Results: A total of 1039 genes were downregulated by ≥2-fold in RA-treated samples compared to untreated cells. The downregulated genes are involved in transcriptional regulation, cell division, DNA replication and repair, spindle organization and biogenesis, ribosome biogenesis, and cellular biosynthesis, reflecting the fact that differentiation is associated with inhibition of cell cycle progression and reduction in related biosynthetic activities. We are particularly interested in the downregulated genes coding for transcription factors, as they are ultimately responsible for cell fate decisions. There are a total of 181 downregulated genes encoding DNA-dependent transcription factors. We mapped the transcription factor gene list to the Gene Ontology biological process classification and identified 26 genes previously implicated in regulation of development and morphogenesis. This shorter gene list includes Sox10 and ASCL1, two genes known for their roles in the maintenance of neural crest stem cells and neural progenitor cells, respectively. Also on the shorter list is SIX3, which promotes the proliferation of neural progenitor cells by inhibiting BMP4-induced differentiation.

Conclusions: Our analysis suggests that the transcription factors essential for maintaining the stem cell state of neuroblastoma cells are also critical for their tumorigenic capacity.

B123 Alterations in SHC Signaling in Human Neuroblastoma

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Background: Shc proteins act as adaptors in several signaling pathways that regulate a number of basic cellular processes including proliferation, differentiation and programmed cell death.
Shc isoforms are differentially expressed in tissues: most cells express ShcA proteins while ShcC isoforms are specific for nervous system. ShcC isoforms were shown to be essential for neuronal function. Notably, embryonic brain expresses ShcA proteins which disappear around birth and are replaced by ShcC in adult brain. Involvement of ShcA or ShcC proteins in oncogenic transformation of cells of the nervous system has not been sufficiently studied.
Neuroblastomas are the most common solid malignancy of childhood.
Therefore, we studied the expression of Shc isoforms in human neuroblastoma.

Methods: Shc isoforms were studied by immunohistochemistry of archival paraffin-embedded biopsies of 15 neuroblastoma patients (All are MYCN negative). Tumor cell cultures were analyzed by immunoblotting and RT-PCR for the presence of ShcA and ShcC.

Results: We found that the type of Shc isoforms changes during tumorogenesis: ShcC expressed by normal neurons is gradually replaced by ShcA in neuroblastoma. Moreover, changes in Shc expression levels correlated with tumor grade - Biopsies of patients defined "infant" contained high level of ShcA protein but no ShcC protein and biopsies of patients defined "non-infant" contained both Shcs. More over, seven different neuroblastoma cell cultures expressed high level of ShcA protein and only five of the cell cultures expressed ShcC protein at low level.
PCR experiments showed the presence of ShcC-mRNA in all lines except one. Interestingly, the most malignant cell line was ShcC-negative and contained no ShcC-mRNA.

Conclusions: Collectively, these results support our hypothesis that the co-regulation of ShcA and ShcC proteins has a role in tumor growth and that Shc isoforms level could be indicative for tumor aggressiveness.

B124 GRP75 Promotes the Neuronal Differentiation of Neuroblastoma Cells

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Background: Neuroblastoma is a heterogeneous neoplasm. Our recent data have demonstrated that the tumor behavior and the differentiation of neuroblastoma are closely associated with the expression of glucose-regulated protein 75 (GRP75). We thus aimed to characterize the functional role of GRP75 in the molecular mechanism governing the neuronal differentiation of neuroblastoma.

Methods: A human neuroblastoma cell line SH-SY5Y was used as a model system to explore the function of GRP75 in the differentiation of neuroblastoma. The expression and cellular localization of GRP75 in SH-SY5Y cells corresponding to retinoic acid (RA)-induced neuronal differentiation were examined by confocal microscopy and Western blotting. The neuronal differentiation of SH-SY5Y cells induced by RA was also assessed by the neurite length and the expression of neuron-specific enolase (NSE) in response to the down-regulation of GRP75 by RNAi.

Results: We found that RA-treated SH-SY5Y cells exhibit a much higher level of GRP75 with a pan-cytoplasmic distribution and prominent nuclear translocation whereas those treated with DMSO show a lower level of GRP75 with a juxta-nuclear pattern. Using real-time PCR and Western blotting, we further confirmed that the levels of GRP75 transcript and protein in RA-treated differentiated cells were markedly increased, consistent with the notion that GRP75 expression correlates with the differentiation of NB. The neuritic localization of GRP75 can also be vividly observed in RA-treated SH-SY5Y cells, implicating a possible function of GRP75 in neurite formation. Furthermore, the RA-induced neuronal differentiation in SH-SY5Y cells transfected with a GRP75-targeting siRNA was significantly reduced, while RA induced an approximately 4-fold increase in neuronal differentiation of mock-transfected SH-SY5Y cells.

Conclusions: The present findings clearly recapitulate our clinical data and substantiating an essential role of GRP75 in the differentiation of neuroblastoma.

B125 A Novel Signaling Modulator in Neuroblastoma: Fibroblast Growth Factor Binding Protein 3

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Background: Our investigation focuses on a novel Fibroblast Growth Factor (FGF) binding protein, FGF-BP3. The FGFs contribute to cellular proliferation and angiogenesis in many tumor types. FGFs bind receptor tyrosine kinases (RTKs) at the cell surface, activating the Ras pathway. We propose that FGF-BP3 functions as an extracellular chaperone of the FGFs, essential for FGF-mediated activation of downstream signaling via the Ras pathway. In our investigation, we have characterized the role of FGF-BP3 in the context of cellular localization, protein function, and signal modulation. We hypothesize that FGF-BP3 is overexpressed in neuroblastoma and required for the FGF-induced cellular proliferation and neuroblastoma development.

Methods: Quantitative real-time PCR was performed to evaluate the expression level of FGF-BP3 in neuroblastoma cell lines and tissues. Colony formation assays were conducted using siFGF-BP3 transduced SH-SY5Y cells to determine the role of FGF-BP3 in anchorage-independent growth of tumor cells. Western immunoblot analysis was performed to elucidate the role of FGF-BP3 in basic FGF (FGF-2)-induced signal transduction pathway in neuroblastoma. Phosphorylation of extracellular signal-regulated kinase (ERK 1,2) served as a marker for activation of the FGF-mediated signal transduction pathway.

Results: FGF-BP3 is highly expressed in neuroblastoma cell lines and tissues, by RT-PCR. Soft agar growth revealed inhibition of colony formation in SH-SY5Y cells with FGF-BP3 knockdown. Western immunoblot identified a strong pattern of ERK 1,2 phosphorylation in control groups of SH-SY5Y cells, after incremental treatment time with FGF-2, compared to the FGF-BP3 knockdown cells. Rescue supernatant with recombinant FGF-BP3 restores the ERK signal pathway in FGF-BP3 knockdown cells, verifying the critical role of the FGF-BP3 protein in the FGF-mediated signal transduction pathway in neuroblastoma.

Conclusions: FGF-BP3 is a secretory protein, specifically overexpressed in neuroblastoma, important for the tumorigenic nature of cells, and essential for the FGF-mediated signal transduction in neuroblastoma. FGF-BP3 may represent a potential therapeutic target in neuroblastoma treatment.

B126 Centrosome Amplification is Correlated with Ploidy Instability in Infant Neuroblastoma (NB) Tumors, but not with *MYCN* Amplification in Infant and Childhood NB Tumors or Cell Lines

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Background: Ploidy is one of important biologic features classifying heterogeneous NB tumors. We tried to clarify whether centrosome amplification is correlated with the ploidy status or *MYCN* amplification.

Methods: Tumor samples were obtained from 16 infants (<18 months), 10 children (>18 months) and 6 cell lines. We performed immunofluorescence-staining of centrosomes using anti-gamma-tubulin antibody, and FISH using D1Z2/D1Z1 and *MYCN*/D2Z1 probes.

Results: Infant NBs were classified into 8 diploid and 8 triploid tumors, and all childhood NBs into diploid tumors. *MYCN* amplification was not found in infant tumors, but in 5/10 childhood tumors and 6/6 cell lines. Ploidy instability defined as a mixed population of cells with trisomy 1, cells with tetrasomy 1 and/or cells with pentasomy 1 in more than 5% of cells in addition to a predominant diploid or triploid clone of cells. The instability was found in 7/8 infant diploid, 0/8 infant triploid and 1/10 childhood diploid tumors ($P<0.01$). Centrosome abnormality was defined as 3 or more centrosomes in a cell, and centrosome amplification as centrosome abnormality in more than 5% of cells. Centrosome amplification was found in 5/8 infant diploid, 1/8 infant triploid and 5/5 childhood diploid tumors without *MYCN* amplification, and in 3/5 childhood diploid tumors with *MYCN* amplification ($P=0.02$). While all 3 diploid cell lines showed no centrosome abnormality, all 3 triploid cell lines showed the abnormality in 1 to 11% of cells.

Conclusions: Ploidy instability and stability found in infant diploid and triploid tumors, respectively, were correlated with the high and low incidences of centrosome amplification. Triploid cells may arise through tripolar division of tetraploid cells from the mixed population of infant diploid tumors, and the resulting triploid cells may proliferate by bipolar division and form a triploid tumor. *MYCN* amplification was not correlated with centrosome amplification in NB tumors or cell lines.

Poster

B127 MYCN Downregulates Integrin Alpha1 to Promote Invasion of Human Neuroblastoma Cells

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Background: Neuroblastoma is a childhood tumor thought to arise through improper differentiation of neural crest cells. MYCN amplification is a prognostic factor that indicates highly malignant disease and poor patient prognosis. Integrins are important regulators of neuroblastoma attachment and migration and participate in many aspects of metastasis. However, the role of integrins in neuroblastoma metastasis, the leading cause of death from this disease, remains less well understood. The aim of this study was to elucidate the mechanism of MYCN and integrins in tumor metastasis by investigating whether reduction of MYCN expression could control tumor attachment and migration in neuroblastoma by regulating integrins.

Methods: The siRNAs for human MYCN and the control were transfected into the cell lines NB1 or NB19 that have MYCN amplification. The cells were analyzed RT-PCR and real-time PCR analysis for gene expression. Attachment assays were carried out using 24-well plates coated with laminin, collagen type IV, or fibronectin. *In vitro* transwell migration assays were performed using Costar Transwell inserts.

Results: Screening of neuroblastoma cell lines for integrin mRNA expression showed that integrin alpha1 expression was higher in lines such as SK-N-SH and NB69 that do not have MYCN amplification than in cell lines such as IMR32, NB1, NB9, and NB19 that have MYCN amplification. Knockdown of MYCN in NB1 and NB19 cells resulted in increased expression of integrin alpha1, which correlated with enhanced attachment to the extracellular matrix and reduced migratory activity.

Conclusions: These results show that MYCN may limit cell adhesion to the extracellular matrix and promote cell migration by downregulating integrin alpha1.

B128 *MYCN* Amplification Contributes to Dysfunction of the p53 Pathway in Neuroblastoma

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Background: Mechanisms of p53 dysfunction in neuroblastoma remain to be studied, since p53 mutations are rare in neuroblastoma. Here we investigated whether *MYCN* amplification is involved in p53 dysfunction.

Methods: Expressions of *p53* and *p21*, a critical transcriptional target gene of *p53*, were examined by quantitative reverse transcription-PCR in 65 primary neuroblastomas (10 *MYCN* amplified (MNA) and 55 *MYCN* non-amplified (non-MNA)), and in two MNA and a non-MNA neuroblastoma cell lines. To investigate whether *MYCN* amplification has an effect on the transduction from *p53* to *p21*, we knocked down *MYCN* expression using RNAi (siMYCN) in the MNA cell lines of intact p53.

Results: The expression level of *p21* was significantly lower in the MNA tumors than that in the non-MNA tumors ($P < 0.01$), whereas the expression level of *p53* was higher in the MNA tumors than in the non-MNA tumors ($P < 0.05$). Similar results were obtained in the MNA and non-MNA cell lines at both the transcript and protein levels, suggesting that p53 was unable to induce p21 in the MNA neuroblastomas. Knockdown of *MYCN* expression in the MNA cell lines significantly elevated the expression level of p21 even though it reduced p53 at both the transcript and protein levels. Furthermore, the *MYCN* knockdown induced G1 cell cycle arrest, growth inhibition and neuronal differentiation in the MNA cell lines.

Conclusions: *MYCN* overexpression in *MYCN*-amplified neuroblastoma may directly or indirectly suppress *p21* expression, and may be a substitute mechanism of p53 dysfunction in neuroblastoma.

B129 The Role of Retinoic Acid Receptors in the Differentiation and Apoptosis of Neuroblastoma

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Background: 9-cis retinoic acid (9cisRA) and all-trans retinoic acid (ATRA) induce neuroblastoma cell differentiation whereas the synthetic retinoid, fenretinide, induces apoptosis. Retinoids signal via retinoic acid receptors (RARα, β, γ) and retinoid X receptors (RXRα, β, γ). Previous work shows that RARγ over-expression had no effect on apoptosis induced by fenretinide, an RARβ/γ-selective retinoid. However, RARγ over-expression altered the response to ATRA by inducing apoptosis rather than differentiation. Published work in other cell types suggests RAR-specific activation may determine whether neuroblastoma cells undergo differentiation or apoptosis in response to ATRA, if true, RAR-specific analogues may improve neuroblastoma therapy by targeting appropriate cellular responses and thereby reduce side effects. The aim of this study was to test the hypothesis that RARα over-expression would increase differentiation and decrease apoptotic responses to retinoids.

Methods: RARα cDNA was cloned into the pcDNA4/TO vector under control of a tetracycline-inducible promoter. This construct was stably transfected into SH-SY5Y neuroblastoma cells expressing the tetracycline repressor. Western blotting verified RARα over-expression in response to tetracycline treatment. Responses to ATRA, 9cisRA and fenretinide were studied using assays for cell viability, apoptosis and cell morphology.

Results: RARα was expressed in a tightly-regulated manner by varying the dose and time of tetracycline treatment. Cells over-expressing RARα showed different morphological and cell attachment properties compared to cells over-expressing RARγ. RARα over-expression had no effect on cell proliferation induced by ATRA and 9cisRA, but altered the morphological response to ATRA and 9cisRA producing less cell-neurite elongation and a greater proportion of flat, rounded cells.

Conclusion: These results suggest over-expression of RARα and over-expression of RARγ produce different retinoid-dependent responses and indicates differences between RARα and RARγ functions. Although the mechanism of these effects needs to be further clarified, these results suggest that receptor specific analogues may be used to target specific retinoid responses.

B130 Stem Cells are Targets of Micro-Foci Inducing Virus (MFV) in Neuroblastoma Carcinogenesis

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We have documented the presence of a novel virus (Micro-Foci inducing Virus–MFV) in cultures from tumours obtained in a cancer-cluster of paediatric neuroblastoma –NB- (Rovigatti 1992). Its presence was later confirmed in all aggressive NB tumors, studied so far (U. Rovigatti 2004). Involvement of Neural Crest Stem Cells in NB carcinogenesis has been documented, particularly by the group of Robert A. Ross (Ross and Spengler 2007).

Three strategies were employed: 1) Infection of young parents of the Fisher-344, Sprague-Dawley e Winstar strains; 2) A very sensitive telomerase-activity assay (Kim and Wu 1997) ; and 3) Infection/transformation assays at different MOI (multiplicity of infection) spanning approx. 12 log.s of MFV dilutions (Forrest and Dermody 2003) (U. Rovigatti 2004).

1) Adult animals are resistant to pathogenesis induced by MFV. To the contrary, progenies of injected parents display different frequencies of NRS induction (approx. 50% in Sprague-Dawley, up to > 95% in Fisher-344).
2) Telomerase assays indicate that this enzymatic activity is increased approx 30 folds over mock-infected controls in cells after acute infection and up to 150 folds in transformed cells.
3) Serial dilution experiments show that the number of affected targets (cell transformation) is proportional to cell inoculums rather than virus titer. Similar values of foci (10-30/ $5x10^6$ cells) were obtained for MFV dilutions of $10^{-1}/10^{-9}$.
These results add further evidence for the presence of stem cell targets (SCT) in NB, particularly in association with MFV, a novel ds-RNA virus.

An underlying infection by MFV with persistence and vertical transmission offers a promising model for explaining Stem Cells involvement and derangement in Neuroblastoma carcinogenesis.

B131 Detection of CpG Island Hypermethylation of Caspase-8 in Neuroblastoma Using Oligonucleotide Array

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Background: Neuroblastoma is a tumor that presents a high rate of spontaneous regression but other subtype shows aggressive phenotype. Caspase-8 gene (*CASP8*), which encodes a key enzyme at the top of the apoptotic cascade, potentiates apoptosis in neuroblastoma. Recently, *CASP8* is frequently inactivated in unfavorable neuroblastoma through DNA methylation. To detect methylation of CpG island in 5' flanking region of *CASP8*, we produced a self-made oligoarray.

Methods: Genomic DNA was isolated from a total of 41neuroblastoma cases obtained before any treatments. After bisulfate modification, modified DNA was amplified by PCR using a set of biotinylated primer in the *CASP8* gene and then hybridized on self-made oligoarray using a polycarbodiimide-coated slide (Carbo Station™, Nisshinbo Industries Inc.) Hybridization signal was visualized using the conventional ABC method for coloring platform to eliminate to use of special machine to detect these signals. To determine the methylation status of this region, sequence analysis was performed in PCR products as well as 10 clones from each sample after TA cloning. We also examined *CASP8* expression in real time RT-PCR.

Results: In 6 regions of *CASP8* CpG island, 31 of 41 cases showed at least 1 methylated site and 6 cases were hypermethylated (> 3 sites). Sequence of clone samples revealed that oligoarray was more sensitive to detect heterogeneity of methylated and unmetylated tumor cells than direct sequence. The cases with hypermethylated *CASP8* CpG island showed significantly low expression levels of *CASP8* and significantly poorer outcome of the patients.

Conclusions: In this study, methylation status of *CASP8* CpG island was significant difference between favorable and unfavorable subtype. To detect the CASP8 methylation status, our oligoarray is easy to use for risk stratification in neuroblastoma.

B132 Combination of IFNγ with Trail and Cisplatin or Etoposide to Induce Apoptosis in Neuroblastoma Cells

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Background: We used IFNγ and cisplatin or etoposide combined with TRAIL to treat the SY5Y cells and study the molecular mechanisms of TRAIL-resistance of NB cells, and whether combination of these drugs could overcome the resistance in order to provide experimental basis for the clinical application of TRAIL.

Methods: The expressions of Caspase8 mRNA and protein were detected by RT-PCR and Western-blot. The effects of IFNγ, TRAIL, IFNγ+TRAIL, IFNγ+Caspase8 inhibitor+ TRAIL and IFNγ+ cisplatin / etoposide + TRAIL on the growth and apoptosis of SY5Y cells were detected by MTT and FCM. The relative Caspase 8 activity was measured by colorimetric assay.

Results: Caspase 8 was undetectable in SY5Y cells but an increased expression of Caspase 8 were found after treatment with IFNγ. SY5Y cells were not sensitive to TRAIL, but SY5Y cells expressing Caspase 8 after treatment with IFNγ were sensitive to TRAIL. The effect of TRAIL on SY5Y cells expressing Caspase 8 was diminished by Caspase 8 inhibitor. Cisplatin and etoposide could enhance the sensitivity of TRAIL on SY5Y cells. The relative Caspase 8 activity of SY5Y cells in IFNγ+TRAIL group was much higher than those of control group, IFNγ group, TRAIL group and inhibitor group. There was no marked difference among IFNγ+TRAIL group, IFNγ+ cisplatin / etoposide +TRAIL group.

Conclusions: IFNγ could sensitize SY5Y cells to TRAIL-induced apoptosis and this may be realized by the upregulation of Caspase 8 . Cisplatin and etoposide could enhance the effect of TRAIL on SY5Y cells.

B133 Promyelocytic Leukemia-Nuclear Body Formation is an Early Event Leading to Retinoic Acid-Induced Differentiation of Neuroblastoma Cells

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Background: Neuroblastoma is one of the most common cancers in children. Neuroblastoma differentiation is linked to the presence of the promyelocytic leukemia (PML) protein. Retinoic acid, a powerful differentiation-inducer in vitro, is a potent agent for the treatment of neuroblastoma.

Methods: Using two different human neuroblastoma cell lines, SH-SY5Y and LA-N-5, PML nuclear bodies (PML-NB) formation was assayed during retinoic acid treatment. The mechanisms underlying these phenomena were analyzed.

Results: We show here that PML protein leads to the formation of nuclear bodies (PML-NB) after only 1 h of retinoic acid treatment and that this formation is mediated by the extracellular signal-regulated kinase (ERK) pathway. Inhibition of protein kinase C also leads to formation of PML-NB via the ERK pathway. Both sumoylation and phosphorylation of PML in an ERK-dependent pathway are also required for formation of PML-NB. Finally, we show that PML-NB formation in neuroblastoma cells is associated with neurite outgrowth.

Conclusions: These results support the proposal that the formation of PML-NB is correlated with the differentiation of neuroblastoma cells.

B134 Antagomir-17-5p Abolishes the Growth of Therapy-Resistant Neuroblastoma through p21 and BIM

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We identified a key oncogenic pathway underlying neuroblastoma progression: specifically, MYCN, expressed at elevated level, transactivates the miRNA 17-92 cluster, which downmodulates p21 and BIM translation by interaction with their mRNA 3? UTR. Overexpression of miRNA 17-92 cluster in MYCN-not-amplified neuroblastoma cells strongly augments their in vitro and in vivo tumorigenesis. In vitro or in vivo treatment with antagomir-17-5p abolishes the growth of MYCN-amplified and therapy-resistant neuroblastoma through p21 and BIM upmodulation, leading respectively to cell cycling blockade and activation of apoptosis. In primary neuroblastoma, tumor progression is linked to elevated MYCN expression, miR-17-5p upmodulation and p21 downmodulation. Altogether, our studies demonstrate for the first time that antagomir treatment can abolish tumor growth in vivo, specifically in therapy-resistant neuroblastoma.

B135 Human Neuroblastoma Cells are Characterized by Glycolipids Expression

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Background: Glycolipid molecules consist of a carbohydrate portion and a hydrophobic lipid backbone (ceramide), and are embedded in the outer leaflet of the cell membrane. These molecules are implicated in various cellular processes and glycolipids aberrantly expressed in tumors are linked to the malignant behaviour of many types of tumors. In case of neuroblastoma, it is known that glycolipid metabolism is correlated to the clinical and biological behavior of the tumor and thus it is expected that carbohydrate chain is a suitable molecule to characterize these cells. In this study we focused on the glycolipid expression in neuroblastoma cells.

Methods: Glycolipids of 10 human neuroblastoma cell lines were analyzed by high performance thin-layer chromatography (HPTLC) and liquid chromatography mass-spectrometry (LC-MS). We performed RT-PCR for genes related to neural differentiation and assessed the correlation between the expression of these genes and glycolipids.

Results: LC-MS analysis revealed that 15 ganglio-series gangliosides including GM3, GM2, GM1, GD1a, GT1a, GD3, GD2, GD1b, GT1b, acetylated gangliosides and 3 neolacto-series gangliosides were expressed in human neuroblastoma cells. Ten cell lines were classified into two groups, by expression level of ganglioside GD2. RT-PCR analysis revealed that expression of neural differentiation related genes, such as Phox2a, TrkC, neurofilament, Chromogranin A and N-Cadherin, were positively correlated with GD2 expression in neuroblastoma cell lines.

Conclusions: We have comprehensively analyzed glycolipid expression in neuroblastoma cell lines. These cells can be separated into two types by the expression level of ganglioside GD2 and it is suggested that the expression of GD2 is related to neural differentiation.

B136 Human Neuroblastoma Cells Stimulate the Expression of Interleukin-6 in Bone Marrow Stromal Cells: Identification of Galectin-3 BP as a Soluble Stimulatory Factor Produced by Neuroblastoma Cells

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Background: Bone metastasis is observed in approximately 56% of patients with metastatic neuroblastomas. We have previously demonstrated that neuroblastoma cells co-cultured with bone marrow stromal cells (BMSC) increase the expression of interleukin-6 (IL-6) by BMSC and that stimulation does not require cell-cell contact. In this study, we proceeded to the partial purification of the protein(s) secreted by neuroblastoma cells that stimulate IL-6 production by BMSC.

Methods: Serum-free culture media (up to 5 liters) from CHLA-255 neuroblastoma cells was concentrated 50x and applied to a series of chromatography columns. Fractions that eluted from the columns were examined for protein content by SDS-PAGE, for IL-6 stimulatory activity on cultured BMSC and for protein identification by Mass Spectrometry.

Results: Consistent with the soluble factor(s) being (a) protein(s), the IL-6 stimulatory activity in the supernatant of the neuroblastoma cells was lost upon treatment with trypsin (100 µg/ml) or heat (55°C x 30 min). Upon separation on heparin sepharose, 2 peaks of activity (heparin bound and unbound) were identified with the heparin bound fraction having the highest specific activity (99,570 pg IL-6/ml/mg protein). When this material was loaded on a DEAE column, an active fraction eluted with 0.2 M NaCl. After further separation by molecular sieve chromatography, 4 secreted proteins, including a galectin-3 binding protein (Gal-3BP), were identified in the active fraction. The presence of Gal-3BP protein was identified in all chromatographic fractions with activity by Western blot. Consistent with Gal-3BP being an IL-6 stimulatory factor produced by neuroblastoma cells, we observed that IL-6 stimulation by neuroblastoma conditioned medium was inhibited by an anti galectin-3 antibody and by lactose. Recombinant Gal-3BP also stimulated IL-6 expression in BMSC.

Conclusions: The data identifies Gal-3BP as one of the proteins secreted by neuroblastoma cells that stimulates the expression of IL-6 in BMSC.

B137 MYCN Sensitizes Neuroblastoma Cells to Apoptosis by Allowing a Specific p53 Post-Translational Modification

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Background: The role of MYCN and p53 in governing cell responses to DNA damage is an important field that is re-gaining momentum in neuroblastoma (NB) research. Although MYCN amplification (MNA) has not been clearly linked to drug resistance, MYCN amplified neuroblastomas most often progress very rapidly despite intense chemotherapy. In sharp contrast, MYCN overexpression sensitizes MYCN single copy (MNS) NB cell lines to apoptosis. The involvement of p53 in the DNA damage NB cell responses has been largely overlooked, possibly due to its infrequent mutation in this neoplasia.

Methods and Results: Here we report that MYCN sensitize MNS NB cells to the effect of the clastogenic drug bleocin. We show that the typical G2/M arrest and micronuclei formation induced by bleocin are converted into an apoptotic response upon MYCN expression. We observed a larger increase in the expression of proapoptotic p53 target genes, such as Noxa, DR5 and AIP, and a reduced expression of p21 specifically in apoptotic versus non-apoptotic cells. Supporting the significant role of p53, its knock-down via RNAi prevented bleocin induced apoptosis in MYCN overexpressing cells. Surprisingly, we only observed minor differences in p53 accumulation and phosphorylation at ser15 between MYCN induced and uninduced cells. In contrast, bleocin treatment caused the appearance p53 ser46 phosphorylation in MYCN overexpressing, but not in MYCN repressed cells, suggesting that this different p53 post-translational status might be responsible for the apoptotic response. Consistent with these results MNA NB cell lines, some of which with inactive p53, appeared more resistant to bleocin treatment. The pathways responsible for a different p53 post-translational processing are being investigated.

Conclusions: Our data indicate that a fully functional p53 could be recruited into an apoptotic pathway in MNS NB cells depending on MYCN expression while the same pathway might be blunted in MNA cells.

B138 Polyamine Depletion Strategies to Enhance Neuroblastoma Therapeutics

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Background: *MYC* deregulation portends poor outcome in neuroblastoma (NB) yet decisive downstream biopathways remain obscure. Polyamines are essential cations supporting *MYC* functions and are elevated in cancer. *MYCN*-amplified NBs expand polyamines through alterations in myriad polyamine regulators. Further, Odc1 inactivation by DFMO inhibits tumor progression in *TH-MYCN* mice (ANR 2006). We therefore sought to optimize polyamine depletion.

Methods: Cox inhibition (enhance polyamine export) and Amd1 inhibition (SAM486; Novartis) was assessed for cytotoxicity and polyamine depletion in combination with DFMO and cytotoxics in human and murine NBs. Potentiating combinations were tested in *TH-MYCN* mice.

Results: High-risk NBs require increased polyamine biosynthesis: *MYCN*-amplified NBs deregulate nearly all polyamine enzymes while non-amplified tumors deregulate *ODC1* ($p<0.001$). Mechanistically, *ODC1* copy number gain is frequent (48%; including co-amplification with *MYCN* in 4%) leading to overexpression. Odc inactivation with DFMO inhibited NB cell lines in vitro (median 40%) while SAM486 was more potent (median 70%). Spermidine and spermine were more depleted with SAM486, and only SAM486 inhibited *TH-MYCN* neuroblasts. In vitro synergy was apparent with SAM486 and cisplatin (augments *SAT* activity) but not doxorubicin. Diclofenac and sulindac markedly inhibited NB while celecoxib effects were variable. No Cox inhibitor demonstrated synergy with polyamine depletion. In vivo, DFMO led to reduced putrescine but maintained spermidine and spermine through compensatory *AMD1* upregulation. We therefore assessed dual Odc/Amd1 inhibition using DFMO (1% in water) and SAM486 (5 mg/kg IP 3x/wk). DFMO/SAM486 protected against NB in *TH-MYCN* hemizygous mice (1/26 developed tumor) compared with monotherapy or controls (14/35; $p<0.01$). *TH-MYCN* homozygous mice also had extended TFS (51 versus 34 days; $p<0.01$) with DFMO/SAM486. Importantly, polyamine depletion synergized with both cisplatin and cyclophosphamide in vivo to prolong TFS ($p<0.01$).

Conclusions: High-risk NBs manifest polyamine dependence that can be exploited therapeutically. Clinical investigation of this approach is warranted.

B139 Identification of Neuroblastoma Cancer Stem Cells

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Background: The cancer stem cell (CSC) hypothesis suggests that rare multipotent cells with indefinite potential for self-renewal drive the onset and growth of tumors. Although the existence of CSCs in leukemia and some solid tumors is established, CSCs have not yet been clearly identified in neuroblastoma (NB). NB cells should qualify as NB-CSCs if they are characterized by long-term self-renewal, multipotency, generation of many progeny and NB-initiating ability upon transplantation into mice.

Methods and Results: To evaluate if NBs contain cells capable of long-term self-renewal *in vitro* under conditions that promote expansion of adult neural stem cells, four fresh post-surgery primary NBs and two NB metastases from bone marrow of consenting patients were dissociated and plated at low density. The frequency of neurosphere-forming cells ranged between 0.01% and 0.5% in a primary sphere formation assay. Next, we performed limited dilution assays. In contrast to primary neurospheres derived from stage 4S-NBs without amplified *MYCN*, neurospheres of stage 4 NBs with amplified *MYCN* were expandable for many passages resulting in the generation of clonal cell lines. To elucidate the phenotypes of these clonally expandable cells, both mRNA and protein expression of neural stem cell markers were assessed using real-time RT-PCR and flow cytometry. The number of cells positive for these markers was increased compared to established NB cell lines. To elucidate the NB-initiating ability *in vivo*, cells were implanted into nude mice. Compared with established NB cell line-derived tumors, NBs developed at both lower seeding density and much slower rate.

Conclusions: Both primary NBs and NB metastases contain a minor fraction of cells capable of long-term self-renewal *in vitro*, which express neural stem cell markers and give rise to tumors *in vivo*.

B140 Unlike Other Cancers, Neuroblastoma Contains a High Frequency of Tumor-initiating Cells with Stem Cell-Like Behavior

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Background: Stem cells or tumor-initiating cells (TICs) have not been identified for neuroblastoma (NB), and evidence indicates that like normal neural stem cells, neuronal cancer stem cells irreversibly differentiate and lose their stem cell behavior in serum-containing media in tissue culture. This study functionally identifies TICs in NB cell lines.

Methods: Established NB cell lines were cultured in serum-containing medium (FCS) or adapted to medium that supports the growth of normal neural crest-derived stem cells (SCM). Growth rates, colony-forming capability and limiting dilution assays were performed *in vitro*. *In vivo* tumorigenicity of the five cell lines was compared in an orthotopic, xenograft model. Self-renewal ability was assessed in serial xenograft transplantations.

Results: Three of five NB cell lines detached and grew as neurospheres in the SCM. Growth rates differed between SCM and FCS but appeared to have no correlation to morphology or *in vivo* tumorigenicity. Methylcellulose assays showed no significant differences between FCS and SCM-cultured cells. In limiting dilution assays, KCNR cells have an estimated precursor frequency of 1/1.81, and initial *in vivo* studies have shown that unsorted, 10-cell injections have resulted in tumors. There were no significant differences in tumor-forming ability in the five cell lines cultured in RPMI versus SCM at any cell dose. Unsorted KCNR cells were serially transferred in mice at 100-cell and 10-cell doses, with 5/5 quarternary mice forming large tumors at both cell doses.

Conclusions: NB cell lines cultured in serum-containing medium contain cells with stem-cell like properties and are highly tumorigenic at low, unsorted cell doses, which is similar to primary neuroblastoma tumor cells (Hansford LM et al). These results indicate that neuroblastoma, an embryonal tumor of childhood, may retain its undifferentiated stem cell population despite tissue culture conditions that may differentiate the stem cells of adult neuronal tumors.

B141 Midkine is Involved in the Initiation and Progression of Neuroblastoma

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Background: MYCN transgenic (Tg) mice were reported as a model for neuroblastoma. These mice spontaneously develop neuroblastoma whose origin is the celiac ganglion. We have been studying to elucidate the molecular mechanisms for the initiation and progression of neuroblastoma utilizing this animal model. We are focusing on the growth factor Midkine. Midkine is overexpressed in human neuroblastoma specimens. Furthermore, the plasma level of Midkine is correlated with other prognostic factors.

Methods: First, we examined Midkine expression in MYCN Tg mice. We dissected following tissues: i) celiac ganglion at precancerous state, ii) neuroblastoma at initial stage, iii) neuroblastoma at terminal stage, and carried out RT-PCR and western blotting. We monitored the tumor progression by MRI. Second, we generated Midkine-deficient MYCN Tg mice to directly verify the role of Midkine in carcinogenesis and development of neuroblastoma. We crossed MYCN Tg mice with Midkine knockout mice, and examined the phenotype in terms of its tumor incidence until the age of 20-week old.

Results: Midkine was strongly expressed in the celiac ganglion at precancerous stage, suggesting that Midkine is involved in the initiation of neuroblastoma. Furthermore, Midkine was also intensively expressed in initial and terminal tumor tissues. Because the receptors for Midkine (e.g. LRP1) were simultaneously expressed in tumor tissues, neuroblastoma cells were supposed to utilize Midkine in an autocrine manner. Moreover, Midkine$^{-/-}$ MYCN Tg mice showed delayed tumor progression and suppressed tumor incidence with significant difference. These results strongly indicate that Midkine is involved in both initiation and progression of neuroblastoma.

Conclusions: Our results indicate that Midkine has important roles in the pathogenesis of neuroblastoma. It can be a potent target for the molecular therapy. We are currently examinig the Midkine targeting by antibody or small peptide derived from Midkine receptor.

B142 N-myc Gene Expression: Impact on Leukocyte Infiltration in 3D Neuroblastoma Spheroids

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Background: High N-myc expression is associated with advanced neuroblastoma stage and poor prognosis, but the relationship between N-myc and immunity has remained obscure. Multicellular neuroblastoma spheroids are a 3D in vitro model system that can reflect the pathophysiological in vivo situation of avascular neuroblastoma microregions and micrometastatic sites; in particular the core region of spheroids well mimics hypoxic conditions of neuroblastoma.

Methods: To investigate whether N-myc gene expression together with hypoxia affects leukocyte infiltration we used 72 hours cocoltures of peripheral blood mononuclear cells (PBMCs) and preformed 4 days old SHEP21N spheroids. Both the starting spheroids colture and the following spheroids-PBMCs cocolture was grown with (N-myc- , bare SHEP21N N-myc expression) or without (N-myc+ , high SHEP21N N-myc expression) tetracycline. The distribution of hypoxia and leukocyte infiltration was determined from 5-µm-thick paraffin-embedded spheroids sections using respectively monoclonal antibodies to pimonidazole (Hypoxiprobe kit) together with antibodies to hypoxic markers (e.g. HIF1α, HIF2α, VEGF, CaIX) and appropriate leukocyte-specific antibody (CD3, CD20 or CD68). Moreover using Affymetrix GeneChip we studied the differences in gene expression profile of N-myc+ and N-myc-SHEP21N spheroids. The gene expression data were analyzed using GeneSpring GX 7.3 software.

Results: We found that distribution of hypoxic regions and hypoxic markers was similar both in N-myc+ and N-myc- SHEP21N spheroids while infiltration of leukocytes, especially macrophages, was detectable only into N-myc- SHEP21N spheroids. Regarding gene expression experiments we found that some chemokines (e.g. CXCL12 and CXCL14) were upregulated in SHEP21N spheroids that barely express N-myc gene relative to SHEP21N spheroids with high N-myc expression.

Conclusions: Our data suggest a negative correlation between overexpression of a prognostically relevant oncogene, N-myc, and leukocyte infiltrate into neuroblastoma tumors. Therefore N-myc mediated tumorigenesis may be coupled with mechanisms of immune escape that are dependent on N-myc involvement in the regulation of immunologically relevant genes.

B143 MYCN Destabilizers for the Treatment of *MYCN* Amplified Neuroblastomas

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Background: It has been reported that targeted silencing of MYCN in *MYCN* amplified neuroblastoma by "antigene" or siRNA results in growth suppression and apoptosis, suggesting that MYCN destabilizing agents may prove beneficial to patients with *MYCN* amplified neuroblastoma. Because MYCN stability is under the control of RAS pathways (RAF/MEK/ERK and PDK1/PI3K/AKT), inhibition at any point of these pathways may lead to destabilization of MYCN. In this study, we examine the effect of three types of drugs on MYCN expression and growth of *MYCN* amplified neuroblastoma cell lines: OSU-03012 (a 3-phosphoinositide-dependent kinase-1 inhibitor), 17-AAG/17-DMAG (Hsp90 inhibitors), and S(+) Ibuprofen (a NSAID).

Methods: Western blot assay was used to detect MYCN and Caspase-3. MTS assay was used to determine growth effect of the drugs.

Results: Treatment of *MYCN* amplified neuroblastoma cells with OSU-03012 at the dose of 10 μM resulted in destabilization of MYCN within 3 hours. Accordingly, OSU-03012 exhibited significant growth suppressive effect on these cell lines. Nonetheless, OSU-03012 induced little Caspase-3 activation. 17-AAG also destabilized MYCN, but it required a longer incubation period (>18hours) to show its effect. 17-AAG was effective even at 320 nM, but its effect was only steadily increased thereafter. This response was reflected in their growth suppressive effect on *MYCN* amplified neuroblastoma cells. Ibuprofen, though it is not known for inhibiting RAS signaling pathways, also caused a rapid MYCN destabilization at 0.5 mM dose and showed a significant growth suppressive effect in *MYCN* amplified neuroblastoma cell lines. Ibuprofen induced a weak Caspase-3 activation in these cell lines, which occurred after 18 hours of its treatment.

Conclusions: MYCN destabilizing agents may be considered an effective therapeutic strategy for MYCN amplified neuroblastomas. Combination of the above compounds may have synergistic effect on MYCN destabilization, growth suppression and apoptosis induction in *MYCN* amplified neuroblastomas.

B144 Growth-Promoting Effect of Monocyte-Derived Interleukin 6 (IL-6) in *MYCN* Non-Amplified Metastatic Neuroblastoma

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Background: Two-thirds of metastatic, high-risk neuroblastomas lack *MYCN* amplification, and the sources of growth promotion in these tumors are unknown. From studies of model tumors and other human tumors, inflammation is known to contribute to tumor growth. We determined if inflammation-related interactions between neuroblastoma cells and leukocytes can be a source of tumor promotion.

Methods: *In vitro* and *in vivo* models of inflammatory-tumor cell interactions and primary tumors and bone marrows (BMs) from patients were studied.

Results: Four of five neuroblastoma cell lines expressed IL-6Rα, and treatment with rhIL-6 increased cycling neuroblastoma cells from 28.4 ± 4% to 39.2 ± 1.1% (P=0.03) as measured by BrdU incorporation. Conditioned medium from three neuroblastoma cell lines induced IL-6 production by normal PBMC but not by PMN cells. Negative magnetic sorting of PBMC revealed that monocytes were the only source of IL-6. In a NOD/SCID subcutaneous neuroblastoma model, co-injection of IL-6-transduced CHLA-255 cells and luciferase-transduced CHLA-255 cells (reporter cells) significantly enhanced growth of the reporter cells. Flow cytometric analyses of BM specimens obtained from five patients with known metastases revealed IL-6 accumulation in up to 50% of CD33+CD14+ myelo-monocytic cells. None of 16 neuroblastoma-free BMs had detectable IL-6-positive cells. Overnight incubation of IL-6-negative BM cells with CHLA-255 supernatant induced IL-6 expression in the CD33+CD14+ population in 7 of 16 cases. Finally, gene expression analysis of 127 MYCN non-amplified primary tumors from patients with metastatic disease using a TaqMan® Low Density Array revealed expression of IL-6 and CD14 above the median level was associated with a poor 5-year event-free survival: 33% vs. 55% and 35% vs. 53% (P<0.05).

Conclusions: Although neuroblastoma cells do not express IL-6, they can induce expression of this cytokine by monocytes in primary and metastatic (BM) sites. IL-6 can then have a paracrine growth-promoting effect on tumor cells.

B145 Inhibitors of Histone Deacetylase and Proteosome in Combination Results in Super-Induction of MYCN Expression and Massive Cell Death in *MYCN* Amplified Neuroblastomas

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Background: The inexorable progression of *MYCN* amplified neuroblastoma has been a challenge for treatment of this disease. Studies from our group have identified several therapeutic agents that destabilize MYCN and inhibit growth of *MYCN* amplified neuroblastoma cells. We have also shown that inhibitors of histone deacetylase (HDAC) and proteosome enhance favorable neuroblastoma gene expression in neuroblastoma cell lines and inhibit their growth *in vitro* and *in vivo*. In this study, we investigate the effect of Trichostatin A or TSA (an HDAC inhibitor) and Epoxomycin (a proteosome inhibitor) alone and in combination on MYCN expression and growth of *MYCN* amplified neuroblastoma.

Methods: MTS assay was used to assess growth effect of the drugs. Western blot assay was used to detect MYCN and Caspase-3.

Results: TSA alone down-regulated the expression of MYCN in *MYCN* amplified neuroblastoma cell lines (IMR5, CHP134 and NLF). In contrast, Epoxomycin enhanced the expression of MYCN in these cells. Intriguingly, treatment of these *MYCN*-amplified neuroblastoma cells with TSA in combination with Epoxomycin resulted in a super-induction of MYCN. The TSA+Epoxomycin combination had an additive growth inhibitory effect on the neuroblastoma cell lines examined in comparison to the single-drug treatments. Caspase-3 was also activated in response to the drug treatments. In consistent with these observations, forced over-expression of MYCN resulted in growth suppression of IMR5 cells.

Conclusions: MYCN expression levels are at equilibrium to sustain growth of *MYCN* amplified neuroblastoma cells. Either down regulation or super-induction of MYCN results in growth inhibition and/or apoptosis of *MYCN* amplified neuroblastoma cells. Inhibitors of HDAC and proteasome in combination may serve as an effective therapeutic approach against the most aggressive *MYCN* amplified neuroblastoma.

B146 Prokineticin Signaling and Cancer Stem Cells in Neuroblastoma

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Background: Neuroblastoma (NB), derived from improperly differentiated neural crest cells (NCCs), possesses a unique propensity to exhibit either a spontaneously regression or an unrestrained growth. Emerging evidence indicates that the cancer stem cells (CSC) are the critical determinants for these enigmatic clinical outcomes. We have previously demonstrated that Prokineticin signaling is crucial in the growth and neuronal differentiation of NCCs and is implicated in the NB progression. In this project, we further delineated its implication in NB CSCs.

Methods: Expression profiles of Prokineticin receptors (PK-R1 and PK-R2) in different NB subclones were analyzed using real-time PCR. Implication of Prokineticin in the NB CSCs was directly demonstrated using flow cytometric analysis. Clonegenicity, migration capability and proliferation of the Prokineticin responsive subpopulations were also examined in this study.

Results: PK-R1 and PK-R2 were differentially expressed in various malignant NB stem cell and neuronal subclones. In particular, these receptors were expressed at higher level in a malignant neuronal subclone (SH-SY-5Y) than its parental clone (SK-N-SH), further suggesting the implication of Prokineticins in the growth of the malignant neuroblastic subpopulation. Subsequent flow cytometric analysis directly showed that Prokineticin-1 profoundly increases the CSC population of SH-SY-5Y cells (c-kit$^+$/GD2$^+$/p75^{NTR+}/CD133$^+$). Isolated CSCs consistently expressed both PK-R1 and PK-R2 and these CSCs were still highly responsive to Prokineticin-1 treatment. In addition, Prokineticin-1 also increased the CD133$^+$/p75^{NTR+}/c-kit$^+$ and CD133$^+$/p75^{NTR-} subpopulations in two other NB stem cell lines. These p75^{NTR+} cells were highly proliferative, clonegenic and migratory, may contribute to the malignant phenotype of the NB. The tumorigenic potential of these subpopulations is currently under investigation.

Conclusions: Our current data suggests that Prokineticin signalling favours the tumor growth by promoting the growth of the CSCs and/or malignant neural crest precursors. Understanding the novel role of Prokineticin signaling will have profound implications for the diagnosis and therapeutic interventions for NB.

B147 N-Cadherin: Potential Mediator of Metastatic Neuroblastoma?

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Background: Identification of biological factors responsible for the metastasis of neuroblastoma cells is imperative as it will trigger the development of targeted therapeutics. N-cadherin (CDH2), a cell-cell adhesion molecule, may play a pivotal role in the dissemination process. In this study, the expression, function and clinical relevance of N-cadherin in neuroblastoma is studied.

Methods: N-cadherin gene and protein expression levels were quantified in 10 neuroblastoma cell lines. The influence of N-cadherin inhibitors (GC-4 antibody, thalidomide and ADH-1 peptide (gift Adherex)) on the morphology, aggregation, proliferation and metabolic activity of neuroblastoma-derived CLB-GA and SK-N-FI cells was studied. Furthermore, the association between the N-cadherin/catenin complex and the cytoskeleton was assessed using capping and immunoprecipitation assays. Finally, the association between N-cadherin mRNA expression in primary tumor samples from 41 neuroblastoma patients and the presence or absence of metastasis was evaluated.

Results: N-cadherin was expressed in all cell lines. The inhibitors had no influence on the morphology or aggregation of CLB-GA and SK-N-FI cells. However, GC-4 impaired the proliferation and metabolic activity of CLB-GA, while no effect on SK-N-FI was seen. These findings are suggestive of a non-functional N-cadherin complex in SK-N-FI. This hypothesis was confirmed by capping assays. The association between the N-cadherin/catenin complex and the cytoskeleton, essential for functional N-cadherin, was defective in SK-N-FI. Immunoprecipitation assays showed a reduced interaction between the N-cadherin/β-catenin complex and α-catenin. In neuroblastoma patients, a significant association between reduced N-cadherin mRNA expression and metastatic disease was found.

Conclusions: Functional assays revealed a defect in the N-cadherin mediated cell adhesion in SK-N-FI cells. The loss of function was associated with an impaired interaction between the N-cadherin/β-catenin complex and α-catenin. Reduced N-cadherin mRNA expression was associated with metastatic disease. Both the presence of a non-functional adhesion molecule or low N-cadherin expression may result in a higher propensity for metastasis.

B148 Biological Functions of Caspase-8 in Neuroblastoma Progression

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Background: The role of caspase 8 in neuroblastoma has been controversial. Caspase 8 expression is clearly suppressed in a large percentage of human neuroblastoma patients. In our hands, caspase 8 expression clearly decreased tumor cell metastasis via the induction of apoptosis downstream of integrins. Nonetheless, studies among different patient groups have generated conflicting results as to its prognostic value, and it is clear that at least some aggressive neuroblastoma do express caspase 8.

Methods: To stimulate resistance to apoptosis, we knocked down caspase 3 expression in neuroblastoma expressing or lacking caspase 8, and measured metastasis in both avian and murine models. In vitro assessments of cell migration and signaling responses following integrin-mediated substrate attachment were performed.

Results: Surprisingly, we found that caspase 8+3- tumor cells exhibited significantly enhanced metastasis relative to 8-/3- tumors, in contrast to 8+3+ cells in which metastasis was profoundly suppressed. In the case of apoptosis resistant cells, we find that caspase 8-promoted cell adhesion and migration. Increased migration does not require proteolysis of caspase 8, but rather requires nonenzymatic activities of the caspase catalytic domain, and its interactions with the focal adhesion complex.

Conclusions: Caspase-8 plays multiple roles in NB tumorigenesis, dependent upon the cellular context. With certainty, we see that caspase 8 promotes adhesion and migration, even as it leads to apoptotis among cells entering an inappropriate microenvironment. Among apoptosis resistant cells, this increased migration promotes metastasis and tumor aggression.

B149 Interleukin-6 in the Bone Marrow Microenvironment Promotes the Growth and Survival of Neuroblastoma Cells

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Background: The tumor microenvironment plays an important contributory role to cancer progression. In neuroblastoma, the second most common solid tumor in children that frequently metastasizes to the bone marrow, tumor cells stimulate the expression of interleukin-6 (IL-6) by bone marrow mesenchymal cells (BMMC) which activates osteoclasts.

Methods: Here we have examined whether IL-6 has a paracrine effect on neuroblastoma cells co-cultured with bone marrow mesenchymal cells.

Results: We first demonstrated that neuroblastoma cells do not express IL-6 but express a functional IL-6 receptor complex (IL-6R) that signals through STAT-3 and Erk 1/2. The signaling is enhanced in the presence of soluble IL-6R. We then observed that the presence of BMMC increases neuroblastoma cell proliferation and protects tumor cells from drug-induced apoptosis. Supporting an involvement of IL-6, these effects were suppressed by an anti-IL-6R blocking antibody. Activation of IL-6R in neuroblastoma cells stimulated their proliferation and protected them from etoposide-induced apoptosis. IL-6 also induced the expression of Cox-2 in neuroblastoma cells with concomitant release of PGE2, which increased the expression of IL-6 by BMMC, providing an amplification loop. Supporting a role for IL-6 in patients with neuroblastoma, we detected IL-6 and sIL-6R in the serum of 12 patients with neuroblastoma bone metastasis, and their levels decreased upon treatment with zoledronic acid and cyclophosphamide. BMMC isolated from 2 patients with neuroblastoma bone marrow metastasis also produced IL-6, and production was increased in the presence of serum-free conditioned medium from neuroblastoma cells.

Conclusions: Altogether the data indicate that IL-6 contributes to the formation of a bone marrow microenvironment that is particularly favorable to the progression of metastatic neuroblastoma cells.

B150 Galectin-1 Expression is Necessary but not Sufficient for BDNF-Mediated Migration and Invasiveness of TrkB-Expressing Neuroblastoma Cells

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Background: Galectin-1 has emerged as an interesting cancer target since it is involved in modulating cell proliferation, cell death and cell migration, all of which are linked to cancer inititation and progession. We previously identified galectin-1 mRNA to be up-regulated in patients with aggressive, relapsing neuroblastoma (NB) and found that galectin-1 protein was up-regulated in an *in vitro* model of TrkB-expressing NB.

Methods: The effects of galectin-1 on cell migration and invasiveness were monitored in the presence or absence of neurotrophins in SY5Y cells stably transfected with TrkA/NTRK1 (SY5Y-TrkA), TrkB/NTRK2 (SY5Y-TrkB) or empty vector. For this purpose, galectin-1 was either down-regulated by sequence-specific siRNAs or recombinant galectin-1 was added exogenously. Levels of galectin-1 were monitored by real-time PCR and Western blotting.

Results: In line with our proteomic analyses, Galectin-1 mRNA is induced by BDNF treatment of SY5Y-TrkB. BDNF treatment of SY5Y-TrkB enhances invasiveness and migration, which can be impaired by transient transfection using Galectin-1 specific siRNA. Addition of exogenous Galectin-1 in the absence of BDNF could not fully restore migration and invasive capacity. SY5Y-TrkA and vector controls were non-invasive and displayed little migration in all conditions analysed.

Conclusions: Endogenous Galectin-1 is necessary but not sufficient for the migration and invasiveness of neuroblastoma cells expressing TrkB. Targeting Galectin-1 might be a promising approach for therapy of high-risk, TrkB-expressing neuroblastoma.

Poster

B151 Accumulation of Segmental Chromosome Alterations in Neuroblastoma Samples Studied by Array-CGH at Relapse

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Background: It has been suggested that in neuroblastoma (NB) segmental chromosomal changes detected at diagnosis may be associated with an increased risk of relapse, but little is known about the evolution of the genomic profile at the time of relapse.

Methods: We have performed genomic profiling of paired NB samples obtained at the time of diagnosis and relapse from 15 patients by array-CGH using an in-house BAC/PAC array with a resolution of 1 clone/Mbase.

Results: At diagnosis, in 5 tumors, only whole chromosome copy number variations were detected. At relapse, one of these cases showed an identical genomic profile, whereas in 4 others 1-3 new, additional segmental chromosomal alterations were found. In 10 other tumors, at diagnosis, segmental chromosome changes were detected in addition to whole chromosome copy number variations. At relapse, in one case an identical genomic profile was observed, and in one case the segmental alterations were no longer observed. The remaining 8 cases showed 1 - 6 new, additional segmental alterations. Overall, additional breakpoints at relapse occurred both on those chromosome arms frequently altered in NB (1q, 2p, 3p, 4p, 11q, 14q, 17q) and those not frequently altered in NB (5p, 6p, 8pq, 9pq, 10p, 13q, 18q, 19q). They were observed both in patients having received chemotherapy (n=6) and surgery only (n=6) in their primary treatment.

Conclusion: At the time of relapse, 12/15 cases showed additional segmental chromosomal alterations which might have been present in a subclone at diagnosis, or might have occurred secondarily either induced by treatment or spontaneously, conferring additional selective advantage to the tumor cells.

B152 XRCC4 Expression is Dependent on the Neurotrophin Receptor Status and Influences Genomic Stability and Differentiation of Human SY5Y Neuroblastoma Cells

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Background: Expression of the TrkA neurotrophin receptor is associated with good prognosis in neuroblastoma, whereas TrkB expression is a marker for aggressive tumor progression and high genomic instability. DNA double-strand repair (DSB) capacity is a core factor for maintaining chromosomal stability. We used the human neuroblastoma cell line, SY5Y, to analyze the influence of different Trk receptors on nonhomologous end joining (NHEJ), the major pathway of DSB repair in vertebrates.

Methods: A cell-free assay was used to analyze the efficiency of NHEJ in the SY5Y cell lines. Gene expression was stably silenced using an EBV-based siRNA-vector system. XRCC4 expression was measured by Real time PCR and Western blotting.

Results: SY5Y-TrkA cells displayed significantly higher repair efficiency than SY5Y-TrkB cells. Reanalysis of gene expression data from the SY5Y model system for DNA repair genes revealed a significant up-regulation of XRCC4, which is essential for NHEJ, in SY5Y-TrkA cells compared to SY5Y-TrkB cells. Silencing of XRCC4 expression in SY5Y-TrkA cells using siRNA decreased the repair capacity to that of SY5Y-TrkB cells, showing that different NHEJ activities is caused by varying XRCC4 expression. XRCC4-silenced SY5Y-TrkA cells were much less differentiated, similar to the phenotype of SY5Y-TrkB cells. This indicates that XRCC4 influences neuroblastoma differentiation, since TrkA-expression was similar in both cell types. XRCC4 expression was strongly reduced in cells bearing a KFG-mutation in the TrkA gene, which also affects signal transduction. KFG is a binding site for the SNT/FRS-2 protein, and the deletion of this motif has an impact on the activation of the Ras/MAPK pathway.

Conclusions: Our data confirm a functional relationship between Trk receptor status, DSB repair capacity and genomic stability in SY5Y neuroblastoma cells. We demonstrate for the first time that the XRCC4 protein has an impact on neuroblastoma cell differentiation and is at least partly regulated by the Ras/MAPK pathway. Further analysis of the regulation of genomic stability and differentiation might help to explain the molecular pathogenesis in neuroblastoma in more detail.

B153 An *in vivo* Method for Detecting Extracellular ATP Release in Neuroblastoma Microenvironment

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Background: ATP accumulates at sites of trauma or inflammation, but information about its role in the tumor microenvironment is lacking. Techniques for measuring ATP in the extracellular environment are still rudimentary. Recently, a novel method for measuring extracellular ATP was developed by engineering a chimeric luciferase-folate receptor construct in which the luciferase cDNA was appended to targeting sequences derived from the folate receptor. The chimeric protein, called pmeLUC (plasma membrane luciferase), is targeted to the plasma membrane and detects ATP in extracellular space close to the plasma membrane. Stable pmeLUC transfectants (HEK293-pmeLUC cells) may function as "reporters" of the interstitial tissue ATP concentration in vivo, and thus help to clarify whether neuroblastoma (NB) microenvironment is characterized by an ATP concentration higher than healthy tissue. This is relevant since we have previously demonstrated that ATP sustains NB growth in vitro (Raffaghello L. *et al.*, Cancer Res. 2006).

Methods: HEK293 were transfected with pmeLUC using Lipofectamine 2000 and stable clones were selected. Expression of luciferase was evaluated by RT-PCR and FACS analysis. In vivo bioluminescent imaging was performed with highly sensitive cooled CCD camera, mounted in a light-tight imaging chamber (IVIS 100 System™). NB bearing mice were inoculated with HEK293-pmeLUC and then subjected to IVIS analysis at different times.

Results: HEK293-pmeLUC express luciferase and detect ATP in a range of 5-10 μM. HEK293-pmeLUC were completely silent when inoculated in healthy tissues, after i.v. or i.p administration. In contrast, analysis of NB bearing mice, after HEK293-pmeLUC injection, disclosed several light-emitting foci in the abdomen. Accordingly, direct luminometric analysis of the excised NB masses showed that light-emitting foci coincided with tumour metastasis.

Conclusions: In this study, we demonstrated that i) NB generate an environment characterized by high levels of extracellular ATP, and ii) HEK293-pmeLUC is a useful probe for measuring extracellular ATP levels in vivo.

B154 Neuro-Endocrine Secretory Protein 55 (NESP55): A Novel Marker for Tumor Hypoxia in Neuroblastoma

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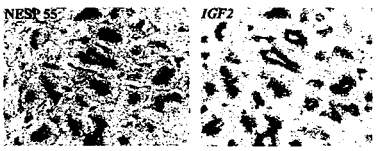
Background: Chronic diffusion-dependent hypoxia is a common phenomenon in tumors, with multiple therapeutic implications. Previous observations suggest that such extreme regional hypoxia causes neuroblastoma (NB) cells to undergo a neuronal-to-neuro-endocrine transition. Hallmarks for this hypoxia-dependent phenotype are *VEGFA* and *IGF2* co-expression and a chromaffin-like cell morphology. Frequently, portions of the hypoxic tumor compartment also feature cytoplasmic chromogranin A (CgA) immunoreactivity; a traditional neuro-endocrine marker. NESP55 is a novel member of the chromogranin family of neuro-secretory granule proteins, recently reported to be co-expressed with CgA in NB. In this study we have tested the hypoxia-dependence of NESP55 expression in clinical and experimental NB.

Methods: Hypoxic induction of *NESP55* was tested by qRT-PCR in five different neuroblastoma cell lines. *In situ* hybridization (ISH) and immunohistochemistry (IHC) were used for detection of NESP55 mRNA and protein, respectively, at the cellular level in cell line-derived tumors xenografted on mice and from a clinically representative set of NBs/ganglioneuromas. Controls for tumor hypoxia were *VEGFA* and *IGF2* expression, analyzed in consecutive sections. IHC and ISH was also applied to fetal tissues, representing weeks 6 through 20 of sympathetic nervous system development.

Results: In the NB cell lines *NESP55* induction by hypoxia was 3- to 53-fold (mean 19-fold). In sections of NB, NESP55 immunoreactivity was strong and strikingly co-localized with *IGF2/VEGFA*-expressing tumor regions (Fig). NESP55 ISH and IHC revealed strict co-localisation. In the fetal sympathetic nervous system NESP55 immunoreactivity was unambiguously chromaffin-specific.



Conclusions: Expression of NESP55 in NB/ganglioneuroma is: (*1*) an excellent marker for chronic hypoxia and (*2*) a strong evidence for a neuro-endocrine phenotype of this tumor compartment. NESP55 IHC and ISH are therefore useful tools for studying hypoxia as a determinant for regional differentiation in this group of tumors and for distinguishing a chromaffin tumor phenotype from ganglion cell-like differentiation.

Poster

B155 Tumor Derived Endothelial Microvessels: Formation Kinetics and Role in Tumor Growth in an Orthotopic Model of Human Neuroblastoma

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Background: Neuroblastoma (NB) associated endothelial microvessels can originate from primary tumors and human microvessels are found in tumors formed by the *MYCN* amplified human HTLA-230 NB cell line or the *MYCN* non-amplified ACN NB cell line in immunodeficient mice. Here we have investigated the kinetics of formation of tumor derived endothelial microvessels and their contribution to NB growth in immunodeficient mice.

Methods: HTLA-230 cells ($1x10^6$/mouse) were injected orthotopically in the left adrenal gland of athymic (nu/nu) mice. Tumor bearing mice were treated with the anti-human CD31 monoclonal antibody (mAb) Moon-1 (IgG1) or isotypic control (450 µg/mouse) every three days until sacrifice, starting on day 3 from tumor injection. Paraffin-embedded tumor tissue sections were stained by immunofluorescence with antibodies to human(h)CD31, mouse(m)CD34, hCD105, hVon Willebrand Factor (VWF), GD2, NB84, prostate-specific membrane antigen (hPSMA), smooth muscle actin (SMA). *MYCN* amplification and murine DNA were detected by FISH using specific probes. Apoptosis was evaluated by TUNEL and proliferation by Ki67 staining.

Results: Human microvessels developed in orthotopic NB tumors by day 7 and represented 50% of total endothelial vessels by day 15. They were functional since contained red blood cells. These vessels were $CD31^+$, $mCD34^-$, $CD105^+$, VWF^+, $GD2^+$, $NB84^+$, $PSMA^+$, and showed *MYCN* amplification. SMA^+ pericytes coating human microvessels were of mouse origin. Scanty chineric human/mouse microvessels were detected. Treatment with CD31mAb, but not isotypic control, reduced human microvessels by 50% ($p=0.022$) without any effect on tumor growth and was associated with 75% increase of murine endothelial microvessels ($p<0.0001$) and rapid regeneration (+40%) of human microvessels ($p=0,002$).

Conclusions: Tumor derived human endothelial microvessels in orthotopic NB expressed GD2 and NB84, as well as PSMA. Their contribution to tumor growth remains difficult to define since, following CD31mAb depletion, they were quickly replaced by new vessels of both human and murine origin.

B156 Novel TrkA Extracellular Isoforms in Human Neuroblastoma

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Background: TrkA, the high-affinity tyrosine kinase receptor for neurotrophins, 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 intra-cellular signaling pathways was carried out.

Methods: RNA extracted from 62 primary neuroblastoma tumors and 14 human neuroblastoma cell lines was reversely transcribed and cDNAs were subject to two independently designed PCRs specific for the portion of the gene coding the TrkA extracellular domain. PCR products were analyzed and semi-quantified by capillary electrophoresis. Identification of novel variants was confirmed by direct sequencing. Results were correlated with clinical and biological data.

Results: Two novel isoforms (namely TrkA-IV, TrkA-V) were identified in 48 of 62 neuroblastoma tumors and in 11 of 14 cell lines. TrkA-IV isoform lacks exons 5, 6 and 7, while TrkA-V misses exon 7, both resulting in frame-shift. Expression of the isoforms did not correlate with any of the generally acknowledged prognostic factors (i.e. age, histology, *MYCN* amplification, 1p deletion). The difference in 5-years event-free survival of patients having high level versus low level isoforms' expression (28% vs 41%) was not statistically significant.

Conclusions: Preliminary structural and functional in silico predictions suggest that the novel isoforms might encode TrkA proteins that have a modified extracellular domain followed by a truncated C-terminal tail, presumably being a soluble form of the receptor. This discovery challenges the 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 TrkA isoforms in neuroblastoma pathogenesis.

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B157 Expression of TWEAK and Fn-14 in Neuroblastoma; Implications in Tumorigenesis

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Background: Tumor necrosis factor-like weak inducer of apoptosis (TWEAK), a member of the tumor necrosis factor (TNF) family of cytokines, acts on responsive cells via binding to the cell surface receptor Fn-14. TWEAK binding to Fn-14, or constitutive Fn-14 overexpression, activates the NF-*k*b signalling pathway, which is known to play an important role in immune and inflammatory processes, oncogenesis, and cancer therapy resistence.

Methods: Expression of TWEAK and Fn-14 was assessed by RT-PCR, western blotting and immunohistochemistry on neuroblastoma primary tumors (n=33) and cell lines (n=7). Effect of TWEAK stimulation was analysed by proliferation assays, western blotting and ELISA. Matrix metalloprotease (MMP) production was assessed by performing zymography.

Results: High expression of TWEAK and Fn-14 was detected in all neuroblastoma cell lines and primary tumors of all clinical stages and biological subsets. Exogenous addition of TWEAK to neuroblastoma cell cultures increased cell survival, proliferation and chemoresistance. This effect was partly due to activation of NF-*k*b signaling and increased expression of anti-apoptotic proteins. TWEAK induced cyclooxygenase-2 (COX-2) expression and subsequent prostaglandin E2 (PGE2) release in neuroblastoma cells, thereby inhibiting apoptosis, promoting proliferation and angiogenesis. Moreover, we observed that TWEAK induced release of MMP-9 in neuroblastoma cells suggesting that TWEAK may play a role in the invasive phase of neuroblastoma tumorigenesis.

Conclusions: High expression of TWEAK and Fn-14 in neuroblastoma tumors and cell lines indicate that TWEAK is an important regulator of neuroblastoma cell proliferation, invasion, and survival, thereby providing a potential new option for therapy.

B158 Arsenic Trioxide Induced All Cycle Arrest in Neuroblastoma Cells with or without MYCN Over-Expression & the Response Had No Correlation with their Effect on Chromosomal Passenger Complex

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Background: Arsenic Trioixde (AS_2O_3) can induce cell cycle arrest and apoptosis in various cancer cells. However, the underlying mechanisms are not fully understood. Other mitotic arrest-induced drugs such as paclitaxel and vincristine act on chromosomal passenger complex (CPC) which consists of four proteins "survivin, aurora-B, INCENP & borealin". Their coordination is essential for regulation of kinetochore-microtubule attachment; thereby anaphase will be blocked until all chromosomes achieve a bipolar attachment to the mitotic spindle. It is unclear that whether AS_2O_3 also induces cell cycle arrest via disruption of CPC and whether such change will correlate with the *MYCN* expression.

Methods: Neuroblastoma cell line Tet-2N (gift of Prof. M. Schwab) with tetracycline control *MYCN* expression status was used. In addition, pEGFP transfected Tet-2N cells were used for detection of microspindle abnormality after AS_2O_3 therapy. Proteins of the cells treated with above cytotoxic drugs were extracted for determination of their survivin, aurora-B, INCENP & borealin expression by Western blot.

Results: AS_2O_3 induced higher percentage of abnormal mitotic spindle in *MYCN* overexpressed neuroblastoma cells (AS_2O_3 treated 28% vs untreated 12%). Among AS_2O_3 treated cells, it was also slightly higher among *MYCN* over-expression (28%) than non-expression (25%) cells. However, this abnormal mitotic spindle response pattern had no correlation with the change in CPC. No matter *MYCN* was over-expressed or non-expressed, both survivin & aurora-B were activated at 24hr after the treatments with palictaxel, vincristine or AS_2O_3, but were then downregulated at 48hr. This response pattern was in general more pronounce with palictaxel and vincristine than AS_2O_3. Similar pattern were noted in the response of INCENP & borealin to palictaxel or vincristine but not AS_2O_3.

Conclusions: AS_2O_3 exerted part of its cytotoxic effect on microtubule assembly. However, its effect on CPC is less prominent than that of classical anti-microtubule agents such as paclitaxel and vincristine. The *MYNC* expression level had no correlation with its effect on CPC.

Poster

B159 Role of Histone Deacetylase 8 in Neuroblastoma Cells

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Background: The control of histone acetylation and deacetylation by histone acetyl transferases and histone deacetylases (HDACs) plays an important role in regulating transcriptional programs in normal cells, and deregulation of this epigenetic key process has been linked to human cancer. We want to dissect the functional role of class I, II and IV HDAC family members for controlling biological properties of malignancy in neuroblastoma cells.

Methods: Functional role of HDAC8 in proliferation (growth curve), clonogenic growth (softagar assays) and neuronal differentiation (marker upregulation) in the neuroblastoma cell line BE(2)-C was investigated by the use of small-interfering RNAs (siRNAs).

Results: Silencing of the class I family member HDAC8 with RNA interference revealed that this enzyme plays a role in tumor cell proliferation, cell cycle progression and clonogenic growth. Additionally, knockdown of HDAC8 induced neuronal differentiation with outgrowth of neurite-like structures and upregulation of neuronal markers, like neurofilament (mRNA and protein), which is in contrast to the knockdown of other class I family members.

Conclusions: On the one hand our data show that HDAC8 contributes to the malignancy of neuroblastoma cells and on the other hand that distinct HDAC family members control specific cellular functions.

B160 Effects of TrkB and BDNF Level on Chemoresistance in Neuroblastoma

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Background: Neuroblastoma(NB) is a pediatric solid tumor derived from neural crest precursor cells. It is very resistant to current treatment protocols, including high dose chemotherapy. The mechanisms of chemoresistance are very complex. The recent studys have shown that the level of tyrosine kinase receptor (TrkB) and brain-derived neurotrophic factor (BDNF) is high in poor prognosis NB tumors.The aim of this research was to explore the effects of TrkB and BDNF level on the chemotherapy sensitivity in neuroblastoma by using the NB cell line SH-SY5Y in vitro.

Methods: We use SY5Y NB cell line to detect the expression of TrkB protein after the treatment all trans-retinoic acid(ATRA) by Western-blot; detecting the cell survival rate by MTT; We treat the cells with different concentration of ATRA in combo with BDNF and Cisplatin and detect apoptosis rate by FCM.We also detect the morphous of the apoptosis cell by TEM.

Results: (1) The expression of TrkB protein was positive after the treatment of ATRA(1,10,100nmol/L) for five days. It was ATRA dose dependent. (2) The difference of the survival and apoptosis rates between the low dose BDNF(10ng/ml)+ ATRA(10nmol/L)+ Cisplatin(5ug/ul) group and the CP alone group were not significant (P>0.05). While the survival rate the high dose BDNF(>50ng/ml) in combo with the same concentration of ATRA(10nmol/l) and Cisplatan(5ug/ul) were significantly lower than that of the CP alone group while the apoptosis rate in on the contrary(P<0.01). The effect is dose dependent of BDNF(>50ng/ml).If we lower the concentration of ATRA to 1nmol/L,the effect disappear.If we use higher concentration of ATRA(>10nmol/L) in combo with high concentration of BDNF and Cisplatin, The survival rates will be increased while the apoptosis rate will be decreased significantly when compared with CP alone. And it's ATRA dose dependent.(3)To survey the shap of the cell apoptosis by TEM : Some of the cells in the CP alone group were as followed : chromatin agglutination or side ,appearing apoptosis change, the shape of group ATRA(10nmol/L)+BDNF(50ng/ml)+CP were as followed : the intranuclear chromoplasma were well-distributed,the nuclear membrathe were clear, mitochondria, ribosome, solvent can be seen.

Conclusions: The sensitivity of SY5Y to CDDP was affected by the level of TrkB and BDNF, The higher the level of TrkB and BDNF, the lower sensitivity of SY5Y to CDDP.

B161 Crucial Role of ER Chaperone in Autocrine-Loop-Drived Carcinogenesis

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Background: Midkine (MK) is a heparin-binding growth factor. Although MK is hardly pressed in normal adult tissues, it is up-regulated tumor tissues. Plasma MK level is also significantly correlated with prognosis of neuroblastoma patients. Furthermore, MK knockdown leads to suppression of tumor growth in nude mice. Therefore, the important role of MK in carcinogenesis and/or tumor progression is strongly implicated. One of the receptors of MK is LRP, and they form an autocrine-loop in cancer cells and protect cells from apoptosis. However, the regulation for their production is not fully understood. To investigate this, we examine roles of receptor-associated protein (RAP), a specialized ER chaperone for LRP.

Methods: First, we examined expression of RAP in human colorectal cancer specimens. Secomdly, we examined the effects of insufficiency of RAP to cells. Thirdly, we constructed LRP fragment with ER-retension signal and evaluate its effects to MK-LRP autocrine loop.

Results: RAP was up-regulated in human cancer tissues, and its expression was correlated with MK expression. Insufficiency of RAP caused the premature binding of MK and LRP in the ER, and resulted in ligand-receptor aggregation and suppression of MK secretion and LRP maturation *in vitro*. Consistent with this, retinoic acid treatment of TGW cells led to increase of intracellular MK expression, but not MK secretion. An LRP fragment trapped MK in the ER, and consequently suppressed MK secretion. The LRP fragment also induced LRP aggregation probably through trapping RAP in the ER, and reduced LRP transported onto the cell surface.

Conclusions: Our results indicate that the premature ligand-receptor interaction plays a negative regulatory role for their production, and ER chaperones, which rescue cells from this negative effects and form autocrine-loop, are essential to carcinogenesis

B162 The Apoptosis-Inducing Effect of IFNγ and TRAIL on Neuroblastoma Cells

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Background: To study the effect of IFNγ on TRAIL-induced apoptosis in neuroblastoma cells and its possible molecular mechanisms.

Methods: The expression of Caspase 8 was detected by RT-PCR and immunohistochemistry method . The effects of IFNγ,TRAIL, IFNγ+TRAIL and IFNγ+Caspase 8 inhibitor+ TRAIL on the growth and apoptosis of KCNR cells were detected by the methods of MTT and FCM . The relative caspase8 activity was measured by colorimetric assay.

Results: Expression of Caspase 8 was not detected in KCNR cells but an increased expression of Caspase 8 was found after treatment with IFNγ.KCNR cells were not sensitive to TRAIL, but IFNγ-treated KCNR cells were sensitive to TRAIL and it was correlated with the concentration of TRAIL; The relative Caspase 8 activity in IFNγ+TRAIL group was much higher than those of TRAIL group and inhibitor group . zIETD-FMK could block the activation of Caspase 8 and reduce the apoptosis of KCNR cells induced by TRAIL.

Conclusions: IFNγ can sensitize KCNR cells to TRAIL-induced apoptosis and this may be realized by the up regulated expression of Caspase 8.

B163 Fenretinide-Induced Caspase-8 Activation and Apoptosis in Metastatic Neuroblasts

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Background: Resistance of high risk metastatic neuroblastoma (HR-MNB) to induction and high dose chemotherapy (CT) raises a major therapeutic challenge in paediatric oncology (overall survival, 5 years, <30%). Responding patients are treated by maintenance CT and an antiproliferative and apoptotic inducer, the synthetic retinoid fenretinide (4-HPR). Recent studies demonstrated that NB metastasis process is enhanced by the simultaneous loss of caspase-8 and integrin α3β1 preventing Integrin-Mediated Death (IMD) to occur (Stupack *et al.*, 2006). As the role of caspase-8 appears critical in preventing metastasis, we aimed at studying the effect of 4-HPR on caspase-8 expression in metastatic neuroblasts.

Methods & Results: The human *MYCN*-amplified NB experimental model, - the IGR-N-91 model derived from an involved bone marrow of HR-MNB patient, - is able to disseminate *in vivo* from the primary nude mouse tumor xenograft (PTX) into myocardium (Myoc) and bone marrow (BM) of the animal. Using such a model, we observed in metastatic BM and Myoc neuroblasts caspase-8 expression downregulation contrasting with a significant up-regulation in PTX cells. This caspase-8 loss paralleled a concomitant decrease of α3- and β1-integrins while caspase -3, –9, -10, Bcl-2 or Bax expressions were unchanged. Data show that in BM, compared to PTX cells, 4-HPR up-regulates caspase-8 expression that correlates a higher sensitivity to apoptotic cell death. No changes were noted in the β1-integrin or caspase-3, -9, -10 and Bcl-2 expressions. Stable caspase-8-silenced SH-EP cells appear more resistant to 4-HPR-induced cell death compared to control or caspase-10-silenced SH-EP cells. As apoptosis is restored in the VP16-resistant BM cells, thus 4-HPR synergizes with drugs.

Conclusions: These results demonstrate that 4-HPR i) up-regulates caspase-8 expression and ii) induces apoptotic cell death in HR-MNB through caspase-8 activation. This study provides basic clues for using fenretinide in clinical treatment of HR-MNB patients, and even challenges the concept of including 4-HPR in the induction CT of these patients.

B164 MYCN-Amplified Neuroblastoma with Favorable Histology

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Background: MYCN amplification is known to prevent cellular differentiation and to promote mitotic and karyorrhectic activities in neuroblastoma. Accordingly, MYCN-amplified tumor typically shows an appearance of either undifferentiated or poorly differentiated subtype with a high mitosis and karyorrhexis index (MKI). MYCN-amplified neuroblasts are also often characterized by their prominent nucleoli. Though vast majority of MYCN-amplified tumors is classified into Unfavorable Histology (UH) by the International Neuroblastoma Pathology Classification, there are rare cases presenting with Favorable Histology (FH).

Methods: Among 63 neuroblastoma cases (FH 32 cases, UH 31 cases; MYCN amplified 10 cases, MYCN non-amplified 53 cases) filed in our department during a period of 2001 and 2007, there were four cases of MYCN-amplified neuroblastoma having FH. Clinical and pathological characteristics of those 4 cases were analyzed.

Results: All 4 patients (M:F=3:1) were diagnosed between 6 and 13 months of age. All had adrenal primary. Three patients (stage 1, 3, 4) are alive 82 months, 5 weeks, and 5 weeks after diagnosis, respectively. One patient with stage 4 died 8 months after diagnosis. All tumors showed an appearance of neuroblastoma, poorly differentiated subtype with a low MKI. Prominent nucleoli were not found in the neuroblastic cells. Though MYCN amplification was clearly detected by FISH, immunohistochemical staining for MYCN protein was negative in all those tumor tissues. Furthermore, a cell line (PT005) established from bone recurrence of stage 1 tumor showed no MYCN RNA expression by RT-PCR.

Conclusions: Patients with MYCN-amplified and FH neuroblastoma in our series were all diagnosed under 18 months of age and had adrenal primary. Discrepancy between molecular property (MYCN amplification) and histologic manifestation (FH) in those rare tumors could be attributable to a lack of MYCN protein expression in spite of gene amplification.

B165 The Autotaxin Gene is Upregulated in Advanced Stage Neuroblastoma

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Background: Neuroblastoma is the most common extra-cranial solid tumour of childhood. The molecular etiology of this heterogeneous, frequently fatal, tumour is poorly understood, although MYCN amplification, observed in about 25% of tumours is strongly correlated with poor survival. We have recently identified an additional gene, autotaxin (ENPP2) which may also contribute to poor outcome. 6/7 MYCN amplified and 2/6 MYCN non-amplified neuroblastoma cell lines showed significant upregulation of autotaxin. Autotaxin is a phospholipase D which catalyses the production of lysophophosphatidic acid and sphingosine-1-phosphate, signaling lipids which evoke hormone and growth factor like responses, resulting for example, in the stimulation of cell proliferation, motility and migration. The purpose of our study was to assess the frequency of autotaxin expression in neuroblastoma tumours and its relationship to other genetic and clinical parameters of the disease.

Methods: Immunohistochemistry was used to detect autotaxin protein in a panel of 81 paraffin embedded neuroblastoma tumours of all stages, with and without MYCN amplification. Tumours were scored as positive if >25% of neuroblasts showed autotaxin staining.

Results: Autotaxin was detected in only 3 of 26 localized neuroblastomas (stages 1 and 2) and in 0/4 stage 4S tumours. However 5 of 14 stage 3 neuroblastomas and 25 of 37 stage 4 neuroblastomas were positive.
Among stage 3 and 4 neuroblastomas the frequency of autotaxin staining was similar in both MYCN amplified and non-amplified tumours (62% and 57% respectively).

Conclusions: Our results demonstrate that approximately 60% of stage 3 and 4 neuroblastomas are positive for autotaxin expression using immunohistochemistry. We conclude from the significantly more frequent ($p<0.001$) autotaxin staining in advanced stage tumours that autotaxin is likely to have a role in the pathogenesis of neuroblastomas, both with and without MYCN amplification.

B166 Determination of MYCN Gene Amplification in Archival Neuroblastic Tumors by Chromogenic in Situ Hybridization

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Background: Determination of the MYCN gene status is required for risk stratification in neuroblastoma. Fluorescence in situ hybridization (FISH) on touch preparations is a major method to determine the gene status. However, this assay is not always feasible in routine clinical practice. In this study, we evaluate the feasibility of chromogenic in situ hybridization (CISH) on formalin fixed paraffin-embedded tissues (FFPE).

Methods: MYCN gene amplification was determined by CISH and FISH on 20 neuroblastic tumor FFPE samples. The results of CISH and FISH were compared for each tumor. The MYCN expression was also analyzed by immunohistochemistry.

Results: By CISH assay, MYCN gene signals were easily identified under light microscope in 15 of 20 samples. The genes were detected as small discrete dots or large clusters in the nucleus and the tissue architecture was easily recognized. We found a very good concordance between CISH and FISH on FFPE samples. In 7 cases for which the MYCN status was previously determined by FISH on touch imprints, complete concordance was obtained between CISH on FFPE and FISH on touch preparations. We considered that CISH is a practical alternative to FISH to identify MYCN status due to its accuracy and relative low cost. In all MYCN amplified tumors, MYCN overexpression was observed by immunohistochemistry.

Conclusions: High concordance between CISH and FISH indicated that CISH is a valuable tool for assessment of MYCN status in pathology laboratory.

B167 Allogeneic Hematopoietic Stem Cell Transplantation for Relapsed Neuroblastoma

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Background: Relapse after high dose chemotherapy remains the main cause of treatment failure in high-risk neuroblastoma. To assess the effect of allogeneic immune reaction against neuroblastoma, we treated four patients with allogeneic hematopoietic stem cell transplantation (allogeneic HSCT), who relapsed after high-dose chemotherapy (HDC) with autologous HSCT.

Patients and Methods: Four patients with relapsed neuroblastoma underwent allogeneic HSCT. Their disease had relapsed after 9-18 months after HDC with autologous HSCT. Prior to allogeneic HSCT they received chemotherapy and radiotherapy with or without surgery. The conditioning regimen included busulfan (16mg/kg) and fludarabine (100-180mg/sqm). Two patients received bone marrow transplantation from mother (1 HLA-matched, 1 HLA-mismatched); two patients unrelated cord blood transplantation (HLA-1-locus-mismatched). GVHD prophylaxis was short-term MTX and tacrolimus.

Results: All showed successful hematopoietic engraftment. Three regimen-related toxicites were observed: thromobotic microangiopathy (n=1), venoocclusive disease (n=1) and hemophagocytic syndrome (n=1) in separate patients. Three patients developed grade II-III acute graft-verus-host disease (GVHD) and chronic GVHD. One of them developed bronchiolitis obliterans dependent on oxygen treatment. The disease recurred in two patients; one adjacent to the initial recurrent site and the other in bone marrow, 3 and 5 months after allogeneic HSCT, respectively. The other two patients are alive disease-free at 15 and 28 months after allogeneic HSCT. The patient with recurrence in bone marrow thereafter received ex vivo-expanded donor CD4+ lymphocyte infusion, which led to transient improvement of the disease.

Conclusion: These results suggest the existence of graft-versus-tumor effect in neuroblastoma and that allogeneic HSCT is an effective treatment for high-risk neuroblastoma.

B168 Mitochondrial Toxicity Induced by Fenretinide in Neuroblastoma Cells

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Background: Fenretinide is a vitamin A analogue which induces oxidative stress and cell death in neuroblastoma. The precise mechanism underlying the production of Reactive Oxygen Species (ROS) and cell death in neuroblastoma is not known yet. However, it has been suggested that mitochondrial respiration is essential for the prooxidant and apoptotic effects of fenretinide. In this study, we investigated the effect of fenretinide on the mitochondrial respiratory chain complexes and ROS production.

Methods: Fenretinide-induced ROS was analysed in control cells and cells depleted of mitochondrial DNA (Rho-zero cells) in the presence and absence of the antioxidants MitoQ and trolox. Complex I, II and ATP synthesis were analyzed *in vivo* in digitonin-permeabilized neuroblastoma using the substrates malate (complex I) and succinate (complex II). Mitochondrial membrane potential was measured using JC-1.

Results: A decrease of ATP-synthesis and inhibition of the electron flow through the respiratory chain was observed in all neuroblastoma cell lines after 4h incubation with fenretinide. The amount of ROS induced by fenretinide in Rho-zero cells was approximately 45% less than in control cells. Furthermore, the mitochondrial antioxidant MitoQ almost completely scavenged the total amount of ROS in 3 out of 4 neuroblastoma cell lines. In addition, trolox completely inhibited the accumulation of fenretinide-induced ROS and cytotoxicity. Surprisingly, a decreased mitochondrial membrane potential was also observed in the presence of antioxidants.

Conclusion: The decreased ROS production in Rho-zero cells and the scavenging effect of MitoQ suggest that fenretinide-induced ROS is at least partly generated in mitochondria. Our results demonstrated that fenretinide inhibits the ATP-synthesis in mitochondria via inhibition of various complexes of the mitochondrial respiratory chain. The impaired electron flow of the mitochondrial respiratory chain most likely underlies the loss of mitochondrial membrane potential and increased ROS production by fenretinide.

B169 Arsenic Trioxide Affected Microtubules Assembly in Neuroblastoma Cells with or without MYCN Over-Expression & the Response Had No Correlation with Their Effect on Chromosomal Passenger Complex

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Background: Arsenic Trioixde (AS_2O_3) can induce apoptosis in various cancer cells. However, the underlying mechanisms are not fully understood, but related to microtubule polymerization like other anti-microtubule drugs such as paclitaxel and vincristine. We hypothesized that the resulting loss of tension between kinetochore and microtubule increased the number of neuroblastoma cells expressing chromosomal passenger proteins (CPPs), including survivin, aurora-B, INCENP & borealin. It is unclear that whether AS_2O_3 also induces apoptosis via CPPs and whether it will correlate with the *MYCN* expression.

Methods: Neuroblastoma cell line Tet-2N (gift from Prof. M. Schwab) with tetracycline control *MYCN* expression status was used. In addition, pEGFP transfected Tet-2N cells were used for detection of microspindle abnormality after AS_2O_3 therapy. The expressions of survivin, aurora-B, INCENP & borealin after treatment of AS_2O_3 were determined by Western blot. Palictaxel and vincristine were used as a positive control.

Results: AS_2O_3 induced higher percentage of abnormal mitotic spindle in *MYCN* overexpressed neuroblastoma cells. Among AS_2O_3 treated cells, it was also slightly higher among *MYCN* over-expression than non-expression cells. However, this abnormal mitotic spindle response pattern had no correlation with the change in CPPs. No matter *MYCN* was over-expressed or non-expressed, both survivin & aurora-B were activated at 24hr after the treatments with palictaxel, vincristine or AS_2O_3, but were then downregulated at 48hr. This response pattern was in general more pronounce with palictaxel and vincristine than AS_2O_3. Similar pattern were noted in the response of INCENP & borealin to palictaxel or vincristine but not AS_2O_3.

Conclusions: AS_2O_3 exerted part of its cytotoxic effect on microtubule assembly. However, its effect on CPC is less prominent than that of classical anti-microtubule agents such as paclitaxel and vincristine. The *MYCN* expression level had no correlation with its effect on CPC.

B170 Effects of Combined Notch and VEGF Blockade in Experimental Neuroblastoma

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Background: While VEGF blockade has been validated as a therapy in human cancers, virtually all patients receiving this therapy ultimately progress, suggesting that alternative mechanisms contribute to tumor persistence. Recent data support critical roles for the Notch pathway in both tumor formation and angiogenesis. We have previously shown that experimental human neuroblastoma is relatively resistant to VEGF blockade. We hypothesized that targeting both VEGF and Notch signaling would more effectively disrupt angiogenesis in our model.

Methods: We blocked Notch signaling in human NGP Neuroblastoma cells by expressing a novel construct based on the Notch1 extracellular domain (N1ECDFc). 10^6 NGP-N1ECDFc or NGP-LacZ cells were implanted intrarenally in nude mice (N=60 each), treating half of each group with the anti-huVEGF antibody bevacizumab (BV) or vehicle biweekly for 5 weeks. Tumors were analyzed by immunohistochemistry for vascular markers and real-time PCR for angiogenesis-related gene expression. Tumor hypoxia was quantified by computer-assisted analysis of pimonidazole staining.

Results: BV treatment reduced tumor weight (0.94 versus 2.42 grs in controls, $p<0.05$), but adding N1ECDFc did not further restrict growth (0.96 versus 0.94, p=NS). Immunostaining for endothelial and pericyte markers demonstrated disrupted vasculature in N1ECDFc+LacZ tumors, whereas BV+LacZ tumors displayed defective pericyte coverage. BV + N1ECDFc tumors exhibited both discontinuity and pericyte loss, and were quantitatively more hypoxic than BV or N1ECDFc-exposed tumors. However, expression of the hypoxia-regulated chemokine CXCL12/SDF-1 was significantly reduced by N1ECDFc in vitro and in vivo, even during co-treatment with BV.

Conclusions: Our data suggests that Notch inhibition in NGP neuroblastoma increases hypoxia as compared to VEGF blockade alone. In addition to morphologic changes in vessels, N1ECDFc decreases tumor expression of SDF-1. Thus, Notch blockade may not only directly affect vascular cells but may decrease SDF-1-mediated recruitment of vascular progenitors. Thus, Notch inhibition may act via multiple mechanisms to limit tumor vascular remodeling during VEGF blockade.

B171 Activating Mutations of ALK in Neuroblastoma

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The gene encoding the receptor tyrosine kinase anaplastic lymphoma kinase (ALK), which is mainly expressed in the neural system, has been shown to be amplified in a subset of neuroblastoma (NB) cases. We have now identified somatic mutations of ALK in NB cell lines and primary tumors. In one case, mutation affects the amplified allele, therefore resulting in an exclusive expression of the mutant form. In the other cases, both the wild-type and mutant alleles are expressed. Two hot spots of missense mutations have been identified. Both types of mutations are single base substitutions within the kinase domain, one being observed in the activation loop. Western blot analysis using antibodies directed against specific phosphorylated forms of ALK could demonstrate the activation of the mutant proteins. Sequencing of additional samples is ongoing. Invalidation of the ALK gene was investigated in one of the NB cell lines exhibiting a mutant ALK protein using shRNA lentiviral plasmids. Infection with 2 different shRNA resulted in efficient silencing of the gene, as checked by Western blot analysis of ALK. The inhibition of ALK leads to a marked reduced proliferation of the cells and focus formation. These data clearly implicate ALK in the pathogenesis of NB. Its targeting by genetic or pharmacological tools will likely represent a key strategy for therapy of NB patients with mutated ALK.

Poster Discussion: Translational Research

TR29 *MYCN-non* Amplified Metastatic Neuroblastoma with Good Prognosis and Spontaneous Regression: A Genomic Portrait of Stage 4S

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Background: *MYCN*-non amplified infantile metastatic neuroblastoma (stage 4 NB) show 3 distinct patterns of presentation and associated prognosis. Babies under 12 months of age elicit two subtypes associated with good prognosis and frequent tumor regression, *i.e.*, NB stage "4S", and stage 4 NB without this presentation ([1yr$^-$]). In contrast, children older than 12 months ([1yr$^+$]) show a dismal outcome. To gain basic knowledge on stage 4S, a genomic survey of stage 4 NB tumors of the 3 clinical patterns was carried out.

Methods: Differential genome and transcriptome analyses of 29 *MYCN* - non amplified stage 4 NB tumors series (n=29) including 4S (n=12) were performed using 1Mb BAC microarrays and Agilent 22k probes oligo-microarrays. Firstly, mRNA chips data following filtering yielded informative genes before unsupervised hierarchical clustering to identify relationship among tumor samples. Gene clusters were confirmed by quantitative RT-PCR and then validated using a tumors test set (n=22). Secondly, the data were explored directly to sort out ontologies, functional pathways and interaction networks (SBIME software, Kauffmann A *et al. Oncogene* 2007)

Results & conclusion: Chromosomal landscape of infants tumors (whole chromosomes gains or loss) differ radically from that of older children (segmental chromosomes gains or loss) in agreement with the National French Neuroblastoma CGHa study (Janoueix *et al*, 2008 *ANR* meeting Abstract, Chiba). Our transcriptome analysis confirms the marked genomic difference within stage 4 NB between infants and children in accordance with recent findings that tumors transcriptome profiling can identify subgroups with different outcomes (Azgharzadeh *et al, JNCI* 2006).
The combination of genes clusterings with the *S.B.I.M.E.* data provides a molecular stage 4S NB portrait in facets regarding neuro-ectodermal embryogenesis in agreement with recent findings (Fischer *et al, Clin. Cancer Res*.2006), mitochondrial and fatty acids metabolism activities, transcription factors expression and inflammatory/immune response.

TR30 Meta-Mining of Neuroblastoma and Normal Fetal Neuroblast Gene Expression Profiles Reveals Candidate Therapeutic Small Molecules

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Background: The Connectivity Map is a new tool that reveals potential therapeutics based on comparison of gene expression profiles of diseased cells and a database of gene expression profiles in response to known therapeutic compounds. We have applied this tool to neuroblastoma (NB) based on data of an integrative meta-analysis of genomic and gene expression data from primary NB tumors and the unique profile of normal fetal neuroblasts.

Methods: Data from four NB gene expression studies for a total of 210 tumors were re-analyzed. Probe-identifiers from different platforms were matched and analysis was performed on genes common to all platforms. After assignment of 146 tumors to one of the three major genomic subgroups (1: numerical aberrations only, 2A: 11q-/17q+, 2B: *MYCN* amp/17q+), differential gene expression analysis was performed to identify subgroup discriminating signatures which were used for Connectivity Map database analysis.

Results: Our meta-mining approach revealed a 132-gene classifier for genomic subgroup discrimination with 91% accuracy, thus reflects the presence of genomic subgroup specific gene expression alterations. Next, lists of genes differentially expressed between fetal adrenal neuroblasts and NBs belonging to the different genomic subgroups were analyzed using the Connectivity Map database. Based on similarities in expression profiles a putative therapeutic effect for NB was predicted for seven compounds. Cell viability analysis effectively demonstrated a significant effect for six of these molecules: HSP90 inhibitors 17-AAG and monorden, dopamine blocking agent fluphenazine, histon deacetylase inhibitor trichostatin, inhibitor of the AKT/mTOR pathway rapamycin as well as a novel agent with an unknown mechanism of action.

Conclusions: This proof-of-principle study indicates that an integrative genomic meta-analysis approach with inclusion of neuroblast data enabled the identification of promising compounds for treatment of children with NB. Further studies are warranted to explore in detail the therapeutic potential of these compounds.

TR31 Specific Gene Expression Profiles and Unique Chromosomal Abnormalities are Associated with Regressing Tumors Among Infants with Disseminated Neuroblastoma

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Background: Neuroblastoma tumors (NBTs) have the highest incidence of spontaneous remission, especially among the subgroup of stage 4s NB affecting infants. Clinical distinction of stage 4s from lethal stage 4 can be difficult, but critical for therapeutic decisions. The identification of distinct biological markers for these NB subgroups will aid accurate classification.

Methods: Twenty-nine infants < 12 months with NBTs (10 stage 4s and 19 stage 4) treated at MSKCC, were evaluated by allelic and gene expression analyses. An additional set of stage 4 NBTs diagnosed at < 18 months was available for differential gene expression analyses.

Results: All stage 4s patients underwent spontaneous remission while only 47% of newly diagnosed stage 4 patients survived despite combined modality therapy. Stage 4 tumors were 19/19 near-diploid/tetraploid, 8/19 MYCN amplified, 50% had 1p36 LOH, 43% had 1p34-p31 and/or 14q LOH and 37% had 11q and/or 1p22 LOH. Stage 4s tumors were 7/8 near-triploid, none MYCN-amplified and LOH was restricted to 11q. Pair-wise comparison analyses between stage 4 < 12 months and 4s tumors revealed distinct gene expression profiles associated with each subgroup. A statistically significant portion of genes mapped to chromosome 1 (p<0.0001), 90% with higher expression in stage 4s, and chromosome 11 (p=0.0054), 91% with increased expression in stage 4. When the age cut-off was extended to 18 months, expression profiles between 4s and infant 4 were less definite and association with specific chromosomal regions was not observed

Conclusions: Distinct chromosomal aberrations are responsible for unique gene expression profiles associated with spontaneously regressing or aggressive NB, providing the biological basis for the distinct clinical behavior. These expression profiles may provide new insights in the genetic control of spontaneous remission in neuroblastoma.

TR32 Myc-Pathway Activation and Stage of Neuronal Differentiation Identify More Malignant Neuroblastomas Independently of Clinical Stage or Risk Classification

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Background: The childhood cancer neuroblastoma arises in the developing sympathetic nervous system and is a genotypically and phenotypically heterogeneous disease. Prognostic markers of poor survival probability include amplification of the *MYCN* oncogene and an undifferentiated morphology. While these features discriminate high- from low-risk patients with precision, identification of poor outcome low- and intermediate-risk patients is more challenging.

Methods: We defined *a priori*-based gene expression signatures representing Myc transcriptional targets and transcripts associated with stage of neuronal differentiation. By using a rank-based score we correlated the activities of the Myc and differentiation pathways to patient outcome in two large neuroblastoma gene-expression microarray data sets.

Results: We show that differential overexpression of Myc transcriptional targets and low expression of genes involved in sympathetic neuronal differentiation predict relapse and death from disease independently of current clinical stage or risk classifications. This was evident not only for high-risk patients, but was also robust in identifying groups of poor prognosis patients otherwise judged to be at low- or intermediate-risk for adverse outcome.

Conclusions: Our results suggest that pathway-specific gene expression profiling might be useful in the clinic to adjust treatment strategies for children with neuroblastoma.

TR33 High-Resolution aCGH Identifies Different Risk Groups in Neuroblastoma, but does not Exclude Metastatic Recurrences

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Background: Array-based analysis of tumours outperforms conventional cytogenetic methods and enables a more precise classification of genomic alterations.

Methods: Primary tumour material of 171 neuroblastoma patients at initial diagnosis was analyzed using high-resolution oligonucleotide array-based comparative genomic hybridization (aCGH) to investigate patterns of gains and losses (ADM-2 algorithm). Hybridization was done with whole genome oligonucleotide arrays of 43.000/99.000 (109/62 tumours) human sequences yielding an average resolution of 35kb/15kb. Chromosomal aberrations were categorized into risk categories and correlated with outcome.

Results: Of 171 patients analyzed, 36 patients (21%) showed gains and losses of whole chromosomes, 31 patients (18%) segmental changes only and 100 patients (59%) demonstrated a mixture of numerical and segmental aberrations. Four patients (2%) exhibited no aberrations. Amplification of *MYCN* (MNA) occurred in patients with segmental (n=17) and numerical/segmental (n=13) aberrations. Five risk groups were defined: a) numerical changes of whole chromosomes (n=36), b) segmental chromosome gains/losses (n=14), c) segmental and numerical aberrations (n=87), d) MNA with numerical ± segmental aberrations (n=13) and e) MNA without numerical ± segmental aberrations (n=17). Survival data showed different outcome (3-y-OS: 100% vs. 86±9% vs. 85±4% vs. 54±14% vs. 52±13%; p<0.001). This classification was restricted to three groups: a) only numerical aberrations, b/d) segmental aberrations (± numerical aberrations) without MNA and c/e) segmental aberrations (± numerical aberrations) with MNA. Prognosis results showed significantly better outcome in the group with numerical aberrations compared to segmental groups (3-y-OS: 100% vs. 85±3% vs. 52±9%; p<0.001; 3-y-EFS: 63±8% vs. 46±5% vs. 39±9%; p<0.001). Recurrences of the low risk group were mainly local (11/15), but also metastatic (4/15; 2 from stage 1, 2 from stage 4S).

Conclusion: Although aCGH and *MYCN* status are powerful predictors of prognosis in neuroblastoma, metastatic recurrences cannot be excluded by these tools.

TR34 High-Resolution aCGH Reveals No Significant Breakpoint Position on Chromosome 17 with Clinical Impact in Neuroblastoma

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Background: In several studies, chromosome 17q gain and particular breakpoints (e.g. ERBB2) were reported to be prognostically relevant in neuroblastoma. We addressed this issue using a novel chromosome analysis method.

Methods: Primary tumour material of 182 patients was analyzed using high-resolution oligonucleotide array-based comparative genomic hybridization (aCGH). Results were correlated with clinical and survival data. Breakpoint regions of chromosome 17q were studied in detail dynamically by maximally selected rank statistic distribution.

Results: Chromosome 17 aCGH analysis showed partial gain in 105/182 patients (58%), numerical gain in 62/182 patients (34%) and no aberration in 15/182 patients (8%). The percentage of partial gain was correlated to stage (stage 1 48%; stage 2 46%; stage 3 56%; stage 4 81%, stage 4S 32%; p<0.001). Overall survival was significantly better in patients with numerical gain compared to partial gains or without any chromosome 17 aberrations (3-y-OS: 94±4% vs. 76± 4% vs. 71±12%; p=0.001). 101/105 patients (96%) with partial gain included the telomere region of chromosome 17, while the proximal breakpoint position was heterogeneous. Without plateaus the most proximal and distal breakpoints mapped at position 22 Mb (17cen) and 59 Mb (17q23.2). The distribution of breakpoint was not correlated to stage, *MYCN* amplification or 1p-deletion. Subgroup analysis of differently chosen breakpoints within 17q revealed no correlation to outcome (stage 4, all ages; stage 4 >1 yr; *MYCN* amplification). Additionally, bioinformatic analysis of our cohort using dynamic rank statistics examined a complete lack of correlation between 17q gain breakpoints and outcome.

Conclusion: Patients with numerical gain had a better outcome, whereas patients with partial gain and even without aCGH chromosome 17 aberrations had a worse outcome. In contrast to other studies, we could not identify a particular breakpoint with impact on prognosis in patients with partial chromosome 17q gain.

TR35 Molecular Diagnostics of Neuroblastoma: Report from the International Neuroblastoma Biology Risk Grouping (INRG) Committee

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Background: Neuroblastoma serves as the paradigm for utilizing tumor genomic data for patient prognosis and treatment allocation. However, there is no worldwide consensus on markers, methodology or data interpretation, inhibiting translational research efforts. The International Neuroblastoma Risk Group (INRG) Biology Subcommittee was charged with identifying highly prognostic genetic aberrations to be included in this risk stratification schema. The committee was also asked to formulate precise definitions for *MYCN* amplification and standardized methods for analyzing genetic aberrations in NB tumors.

Methods: The Biology subcommittee of the INRG working group was entrusted with developing an international consensus on all aspects of neuroblastoma molecular genomic diagnostics, including future directions. Consensus was achieved at the September 2005 conference in Whistler, Canada.

Results: Consensus was reached to include *MYCN* status, 11q23 allelic status, and ploidy in the INRG Classification System based on the recommendations of the INRG Biology Subcommittee, the results of statistical analyses, and the clinical relevance of the individual genetic factors. Standardized operating procedures (SOPs) for analyzing these genetic factors were formulated, and the criteria for *MYCN* amplification were defined. The INRG Biology working group also recommended prospective evaluation of chromosomes 1p36 and 17q23-25 because of the prognostic significance of these factors. Thus, genetic characterization of neuroblastomas according to INRG guidelines will require information regarding the tumor cell content and certified reference laboratories with expertise in the genetic assays described. In addition, recommendations for future approaches for analyzing NB biology using pan- or multi-genome profiling assays were addressed.

Conclusions: Neuroblastoma treatment planning is closely related to tumor cell genomic features, and it is likely that a panel of DNA-based biomarkers will be used in future risk assignment algorithms. Consensus on methodology and interpretation of these increasingly complex assays is essential for uniform INRG classification thus facilitating international cooperative clinical and translational research studies.

TR36 The E.E.T.-Pipeline: An Interdisciplinary Concept for Translating Neuroblastoma Genome Data into Significant Medical Progress

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Background: Treatment of neuroblastoma and other embryonal tumors (ET) is a challenge for the pediatric oncologist. Innovative translational research is required to exploit available genomic data and implement state-of-the-art technologies to overcome the deficits of current diagnostic and treatment strategies.

Methods: We set up a consortium of researchers, clinicians and industrial partners with extensive clinical and technological expertise to establish a pipeline for the comprehensive development and validation of novel diagnostic tools in addition to efficient preclinical drug development for neuroblastoma and other ET.

Results: Our approach includes the validation of established chip-based diagnostic platforms including analysis of genes previously shown to be effected in neuroblastoma, as well as generation of neuroblastoma-specific data on novel array-based platforms for the development of diagnostics at the microRNA and serum proteomics levels. Novel bioinformatic solutions are developed for the meta-analysis of generated high-throughput data. An existing database designed to warehouse complete clinical and experimental data for neuroblastoma is further extended to include other ET entities as well as a virtual ET-biobank to improve sharing of patient samples. Functional characterization of the most promising molecular targets previously identified by the partners is a foundation for entry into a drug development pipeline. This pipeline consists of a representative cell line panel for *in vitro* drug testing and orthotopic mouse models to evaluate new treatment modalities *in vivo*.

Conclusions: Here we present our concept for a coordinated effort to facilitate the necessary integration of research capacities for translating genome data into significant medical progress and provide representative interdisciplinary data obtained in the first 12 months of the consortium's work.

TR37 A DNA Methylation Profile Predicts Outcome In Advanced Stage, High-Risk, Neuroblastoma Patients

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Background: Neuroblastoma patients are stratified into three risk groups (high, intermediate and low) for their assignment to the optimal therapeutic regimen. However, the clinical and biological criteria for this stratification are still imperfect predictors of outcome.
The aim of our study was to identify thresholds of DNA methylation that could discriminate, within a clinically homogeneous group of stage 4 neuroblastoma patients, those likely to have a favorable outcome from those presenting fatal disease.

Methods: We have utilized a "class discrimination" strategy to determine the predictive value on the outcome at 60 months of different levels of methylation measured by quantitative Pyrosequencing analysis in a cohort of 53 patients at high risk.

Results: Our results demonstrated that the thresholds of methylation defined by ROC analysis for two genes: *14.3.3σ* and *RASSF1A* can distinguish with high significativity and independently from all known predictors, the patients presenting favorable outcome from those with progressing disease (Hazard Ratio (HR) = 8.44; 95% Confidence Intervals (95% CI): 2.86-24.88 for the two genes combined. The level of methylation in the tumors of the high risk "long survivors" was comparable to that of the tumors derived from the patients at lower risk and to that of benign neuroblastic tumors.

Conclusions: The quantitative analysis of methylation is an accurate predictor of survival in neuroblastoma patients and leads us to hypothesize that a subset of patients considered at high risk, but displaying low levels of methylation, could be assigned at a lower risk group and, in perspective, could be eligible for a less aggressive therapeutic regimen.

TR38 Differential Expression of Genes Mapping to Recurrently Abnormal Chromosomal Regions Provide the Biological Basis of Ploidy in Neuroblastic Tumors

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Background: Neuroblastic tumors (NBTs) represent a heterogeneous spectrum of neoplastic diseases associated with multiple genetic alterations. Structural and numerical chromosomal changes are frequent and are predictive parameters of NBTs outcome. We performed a comprehensive analysis of the biological entities constituted by NBTs with different ploidy status.

Methods: Gene expression profiling of 49 primary NBTs with ploidy data was performed using oligonucleotide microarray. Forty-six primary NBTs were further analyzed by Quantitative Real-Time Polymerase Chain Reaction (Q-PCR); array-Comparative Genomic Hybridization (aCGH); and Fluorescent *in situ* Hybridization (FISH) to investigate the correlation between aneuploidy, chromosomal changes and gene expression profiles.

Results: Gene expression profiling revealed distinct gene expression profiles associated with near-triploid and near-diploid/tetraploid NBTs. A statistically significant portion of genes mapped to 1p36 (P=0.01) and 17p13-q21 (P<0.0001), described as recurrently altered in NBTs. Over 90% of these genes showed higher expression in near-triploid NBTs and the majority are involved in cell differentiation pathways. Specific chromosomal abnormalities observed in NBTs, 1p loss, 17q and whole chromosome 17 gains, were reflected in the gene expression profiles. Comparison between gene copy number and expression levels suggests that differential expression might be only partly dependent on gene copy number. Clonal heterogeneity was observed in all NBTs, with marked complexity in near-diploid/tetraploid tumors.

Conclusions: Gene expression profiles of NBTs with different ploidy status revealed differential gene expression at chromosomal regions associated with NBT outcome. Our results show that NBTs genetic abnormalities are complex, heterogeneous, and transcribe into gene expression profiles that define their biological behavior.

Poster

TR39 Molecular Profiling of Genetic Changes by a Multi-Genomic Approach for Neuroblastoma Subtyping and Therapy Stratification

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Background: International efforts to refine risk assessment (INRG) in neuroblastoma patients are essentially based on tumor genetics. Therefore, an overview of structural aberrations and amplifications of the neuroblastoma tumor genome, which includes all well known structural changes, is urgently needed and requires a multi-genomic, high throughput, robust and reliable approach enabling the simultaneous detection of a multitude of genetic aberrations. Neuroblastoma specific Multiplex Ligation-dependent Probe Amplification (MLPA) technique is able to identify gains or losses of up to 115 different genomic loci (covering the ten chromosomal regions of highest interest plus reference loci) within a given sample. MLPA can be considered as a 'semi-high throughput' technique as 13 or more different tumor samples can be analyzed in one assay.

Methods: All together, 169 tumor and 5 bone marrow samples from 124 patients were analyzed by MLPA and in parallel with I-FISH (*MYCN*, 1p, 17q, 11q) and FCM. 14 samples were tested in parallel with array-CGH and co-operative testing was done in 6 European countries.

Results: No discordances in the detection of structural aberrations between MLPA and I-FISH and/or array-CGH have been found with the exception of cases with tumor heterogeneity. Mixing experiments revealed detection of structural aberrations even in cases with 50% tumor cell content and detection of *MYCN* amplification in cases with even lower tumor cell content. As expected, no structural/segmental aberrations were found in low stage favorable tumors, whereas structural aberrations were associated with higher stages and/or unfavorable outcome.

Conclusions: Thus, this new routine technique offers a unique chance to greatly extend the picture of genomic changes in neuroblastomas on a large series of tumors because a genomic data set of the most frequently altered loci on a statistically adequate number of clinically well documented patients with similar treatment can be generated in reasonable time.

TR40 Practical Application and Validation of a Customized Expression Microarray-Based Diagnostic System toward the New Risk Classification of Neuroblastoma

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Background: To provide novel prognostic tools for Neuroblastoma (NBL) and contribute to select the appropriate therapy for the patients with NBL, we previously developed a practically useful mini-chip carrying top-ranked 200 genes that can predict the prognosis of NBL at the accuracy of approximately 90%. An original computational algorithm for outcome prediction, which provides survival probabilities (a '*posterior*' value which ranges between 0 and 1, '0' corresponds to the worst) at 5-years after diagnosis, is accompanied to our system. Here we present the preliminary results from the ongoing vaidation study.

Results & Discussion: By using the mini-chip, we have analyzed gene expression profiles of 76 NBLs including 16 *MYCN*-amplified tumors obtained by surgery since 2005. The median follow-up duration is approximately 21 months (from 1 to 55 months). Overall, the distribution of expression profiles of 76 tumors was almost similar to those of the 136 samples previously used for the construction of prediction algorithm as well as those of 50 samples used at the beginning for the independent test of the mini-chip: two major gene clusters were displayed by F and UF (highly expressed in favorable and unfavorable NBLs, respectively) genes. The *MYCN* expression was extremely high in all *MYCN*-amplified tumors except one, while it was very low in the *MYCN*-nonamplified tumors whose prognosis was unfavorable. Other UF genes were highly expressed commonly to the *MYCN*-amplified as well as -nonamplified tumors with unfavorable phenotype, suggesting that similar molecular pathways might be involved in these subsets. No significant correlation was observed between *MYCN* and other UF genes' expression (Pearson test). 37 tumors with *posterior* <0.5 included 6 dead samples, and 39 tumors with *posterior* ≥0.5 included 3 dead cases (one with metatasis to central nervous system and one with prenatal NBL). Thus, our customized mini-chip has so far worked well for establishing personalized medicine of NBL, though it needs much longer follow up for validation.

TR41 Evaluation of Twenty-Six Housekeeping Genes in Neuroblastoma Using Expression Profiling

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Background: Quantitative reverse transcription–polymerase chain reaction (qRT-PCR) is widely used to study gene expression. The suitability of reference or housekeeping genes as endogenous controls has not been sufficiently investigated in neuroblastoma (NB). We first identified 26 commonly used reference genes in the studies of human solid tumors including NB. The relative stability of these 26 genes was then investigated using a NB tumor array.

Methods: Gene expression profiles were done on 86 NB tumor samples using the Affymetrix U95A-E arrays. These 86 patients belonged to five risk groups at diagnosis: loca-regional (stages 1, 2 and 3) >18 months of age, stage 4 >18 months, local-regional ≤18 months, stage 4 ≤18 months, and stage 4S). Microarray data were normalized using the RMA method. Expression difference between risk groups was examined using an ANOVA model and an F test. The expression level stability was further evaluated using software NormFinder and geNorm.

Results: The 26 genes were represented by 63 probesets on the U95 arrays. Among them, three genes ALAS1, LMNB1, POLR2A showed significant differences in expression among the five risk groups and therefore deemed unsuitable. According to NormFinder, the 10 most stable genes (in descending order of stability) were YWHAZ, SDHALP1, UBC, POLR2F, M-RIP, SDHA, ACTB, PPIA, SDHALP2, and FAM48A. geNorm identified YWHAZ and M-RIP as the best gene pair.

Conclusions: Using the U95A-E expression profiles of 86 NB tumors, of the 26 most commonly used references genes, YWHAZ, SDHALPI, UBC, POLAR2F and M-RIP appeared most stable within and among different risk groups in NB. Confirmation by qRT-PCR using an independent set of tumors seems warranted.

TR42 Chromosomal Expression Profiles of Genetically Favorable and Senescent Neuroblastoma Cells

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Background: Genetically favorable neuroblastomas (NB) frequently undergo complete spontaneous regression or maturation without any cytotoxic therapy. The regression phenomenon found in patients with localized or 4s tumors is linked to a specific genomic pattern whereby the absence of *MYCN* amplification is only one of the genetic hallmarks of these tumors. Despite the fact that the genotype of these tumors is well characterized, the molecular mechanisms underlying the spontaneous regression process (of whole or tumor residuals after surgery) are still unknown. Another mechanism leading to non aggressive NB phenotype is the phenomenon of cellular senescence found in *MYCN* amplified neuroblastoma cell lines which have expulsed the amplified gene during the senescence process.

Methods: In order to learn whether the global chromosomal expression profile is similar in both cell types we applied the comparative expressed sequence hybridization (CESH) technique. The expression profile of genetically favorable NBs (n=3) and those of senescent NB cell lines (n=4) was compared. In both instances non senescent, *MYCN* amplified, NB cell lines were used as reference. The genomic status was analyzed by I-FISH, CGH and MLPA.

Results: Genetically favorable tumors display a highly differential expression pattern. High expression was observed predominantly in AT rich chromatin, while the aggressive NBs displayed high expression at different loci (for example 2p23-24, i.e. *MYCN* locus) and in predominantly GC rich chromatin fractions. Surprisingly, when comparing the chromosomal expression profile of genetically favorable NBs with those of senescent NB cells, a similar expression pattern was found in both cell types.

Conclusion: Despite the fact that the two cell types studied are very different concerning their genesis, the global chromosomal expression profiles of genetically favorable and senescent NB cells are similar.

TR43 Expression of HER2/neu is Uncommon in Childhood Neuroblastomas but not in Human Neuroblastoma Cell Lines

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Background: Alterative therapy is needed for advanced neuroblastoma patients with relapse or progress after chemotherapy. We investigated childhood neuroblastomas and human neuroblastoma cell lines for Expression of HER2/neu (c-erbB-2), epidermal growth factor family, which is a target against trastuzumab.

Methods: We treated 20 patients with advanced neuroblastomas (age >1 year, stage III or IV) between April 1992 and November 2007. Serial paraffin embeded biopsy samples obtained from all but one case. We investigated HER2/neu expression in 19 childhood advanced neuroblastomas semiquantitively by means of a certified immunohistochemical system. We also evaluated HER2/neu expression in 6 human neuroblastoma cell lines (NB1, KUYS, NB69, IMR-32, SKNSH, and GOTO) by flowcytometric analysis.

Results: Flow cytometric analysis revealed that HER2/neu had been highly expressed in 3 neuroblastoma cell lines (NB1, KUYS, and GOTO). However, immunohistochemical studies showed that none of 19 childhood neuroblastoma biopsy samples expressed HER2/neu, regardless of the presence or the absence of MYCN amplification.

Conclusions: These data suggest that HER2/neu should not be considered as a convincing candidate for molecular target therapy, and that HER2/neu might not be relevant prognostic factor in neuroblastoma.

TR44 Serum Profiling of Neuroblastoma Patients Using SELDI Mass Spectrometry Reveals Neuroblastoma-Specific Patterns and Novel Potential Marker Proteins

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Background: In neuroblastoma (NB), serum markers such as ferritin, LDH, NSE and catecholamines are well established as useful diagnostic and prognostic tools. Innovative proteomic technologies might identify additional tumor-specific proteins in patient body fluids.

Methods: We used surfaced-enhanced laser desorption ionization mass spectrometry (SELDI-MS) to analyze the serum proteomes of 48 NB patients in comparison to 44 patients with Wilms' tumors and 42 healthy children. Serum fractions were analyzed on three different chip types, and the spectra obtained were analyzed for individual peak and pattern differences using bioinformatic methods and the CiphergenExpress software. Peptide mass fingerprinting and MS/MS technology allowed the identification of selected differentially expressed proteins, which were validated using immunodepletion from serum samples followed by SELDI re-analysis as well as ELISA of individual patient serum samples.

Results: Peak resolution was improved by fractionating serum samples prior to SELDI-MS analysis. A pilot study identified three serum fractions and two chip surfaces which visualized the most differences between sera from NB patients and healthy controls. Both analysis methods identified single peaks as possible NB marker proteins. We could confirm the presence of elevated serum amyloid A (SAA) in patients with stage 4 tumors as compared with stage 4S tumors, as previously reported. However, we could not confirm SAA as a NB-specific marker compared to control serum due to the large variation of SAA levels in the entire cohort analyzed. Analysis of the mean group spectra using Support Vector Machines (SVM) allowed the classification of NB sera (91.7% accuracy), control patient sera (92.9% accuracy) and Wilms' tumor sera (76.5% accuracy) in the independent test set using the SVM 3-group classifier.

Conclusions: We present NB-specific SELDI-MS patterns, the validation of a previously reported serum marker protein for high-risk NB and novel potential marker proteins identified in the serum of neuroblastoma patients.

TR45 Identification of GRP75 as an Independent Favorable Prognostic Marker of Neuroblastoma by a Proteomics Analysis

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Background: Neuroblastoma is a heterogeneous neoplasm. Detailed biologic discrimination is critical for the effective treatment of this disease. Since the tumor behavior of neuroblastoma is closely associated with the histological state of differentiation, we thus aimed to identify novel differentiation-associated markers of neuroblastoma with prognostic implication.

Methods: A human neuroblastoma cell line SH-SY5Y was used as a model system to explore potential biomarkers for the differentiation of neuroblastoma by proteomic analyses. Seventy-two neuroblastoma tumor tissues were subsequently investigated by immunohistochemistry to validate the correlations between the expression of a novel prognostic marker, various clinicopathologic and biologic factors, and patient survival.

Results: Using two-dimensional differential gel electrophoresis (2-D DIGE), we found a total of 24 spots of proteins in SH-SY5Y cells whose expression was enhanced following differentiation. Glucose-regulated protein 75 (GRP75) was unambiguously identified as one of the five proteins that were dramatically up-regulated following differentiation. Immunohistochemical analyses of 72 neuroblastoma tumor tissues further revealed that positive GRP75 immunostaining is strongly correlated with differentiated histologies ($P < 0.001$), mass-screened tumors and early clinical stages ($P = 0.016$ and $P < 0.001$ respectively), but inversely correlated with MYCN amplification ($P = 0.010$). Univariate and multivariate survival analyses demonstrated that GRP75 expression is an independent favorable prognostic factor.

Conclusions: The present findings clearly demonstrated that our proteomics-based novel experimental paradigm could be a powerful tool to uncover novel biomarkers associated with the differentiation of neuroblastoma. Our data also substantiate an essential role of GRP75 in the differentiation of neuroblastoma.

TR46 Identification of Peripheral Blood Markers of Neuroblastoma Using Proteomic Profiling

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Background: There are currently no accurate or reproducible methods to detect early recurrence in children with high risk neuroblastoma (HRNB). We propose that examination of plasma samples from these patients as well as samples from patients with low risk neuroblastoma (LRNB) and healthy pediatric controls (HPC) will identify proteins that are specifically associated with active HRNB.

Methods: We screened plasma from 35 HRNB patients, 20 LRNB patients, and 20 HPC. The samples underwent immunodepletion and then isoelectric point fractionation. Control samples showed an intraexperimental CV of 20%. We then performed surface-enhanced laser desorption ionization time of flight mass spectrometry (SELDI-TOF MS) on duplicate samples from each of the six fractions with CM10 Protein Chip Arrays (Ciphergen, CA) at low and high laser settings.

Results: *Comparison of HRNB and LRNB*: Using the support vector machine (SVM) classifier and a significance level of 0.005, we classified HRNB from LRNB with a sensitivity of greater than 90% and positive predictive value of 80%. Of the 4 peaks identified, two appeared to be isoforms of one protein and two isoforms of another. One of the pairs had m/z values of 11,544 and 11,711, which correspond to the previously described protein serum amyloid A. The second pair had m/z values of 4964 and 4966, a peak that has not been previously described to our knowledge. *Comparison of HRNB and HPC*: Using SVM classifier, we classified HRNB from HPC with a sensitivity of greater than 94% and positive predictive value of 94%. Of the 6 peaks identified, three appeared to be isoforms of one protein with m/z values of 17,212, 17,263, and 17,328.

Conclusion: With the use of immunodepletion and fractionization, the comparative SELDI-TOF MS method identified 3 proteins whose expression may serve as markers of active disease in the peripheral blood of patients with HRNB.

Poster

TR47 CDK2 Targeting is Synthetic Lethal to MYCN Expressing Cancer Cells

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Background: Targeting a gene that is synthetic lethal to a cancer relevant mutated gene provides a mechanism to specifically kill tumour cells and spare normal cells. Recent papers have shown that C-Myc over-expression in several cell types results in increased sensitivity for CDK1/2 inhibitors. We now show that neuroblastoma cells with N-Myc over-expression are sensitivity for CDK2 inhibition.

Results: Targeted silencing of CDK2 resulted in massive apoptosis only in N-Myc expressing neuroblastoma cells. Silencing of N-Myc could rescue cells from apoptosis after CDK2 inhibition and over-expression of N-Myc sensitizes non N-Myc amplified neuroblastoma cells for apoptosis after CDK2 silencing. Profiling of N-Myc amplified neuroblastoma cells after CDK2 silencing shows a strong response of p53 target genes and protein analysis shows p53 stabilization and nuclear translocation. We could confirm this N-Myc dependent P53 induced apoptosis using a CDK2 inhibiting small molecule (Roscovitin).

Conclusion: These results validate CDK2 inhibitors as a potential MYCN selective cancer therapeutics.

TR48 The Role of UBE4B and EGFR Trafficking in Neuroblastoma

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Background: Signaling through growth factor receptors is important in neuroblastoma pathogenesis. Chromosome 1p36 is commonly deleted in neuroblastoma tumors and is associated with a poor prognosis. *UBE4B*, a gene in 1p36, has been reported mutated in high-risk neuroblastoma. We have found a direct interaction between UBE4B and hrs, a protein required for epidermal growth factor receptor (EGFR) trafficking, suggesting a link between EGFR trafficking and neuroblastoma pathogenesis. We have analyzed the role of UBE4B in the EGFR pathway in neuroblastoma cell lines.

Methods: The expression of UBE4B, hrs and EGFR were analyzed by quantitative Western blot in a panel of 7 human neuroblastoma cell lines (SH-EP, SK-N-AS, SK-N-SH, KCNR, SH-SY5Y, LA155N, NGP). EGFR degradation rates were determined by examining the kinetics of cellular EGFR depletion following a pulse of ligand.

Results: UBE4B levels were lowest in SKNAS and highest in NGP cells. Hrs levels were lowest in SKNSH cells and higher in other cell lines. EGFR levels were lowest in NGP and KCNR and highest in SKNAS cells. UBE4B levels were correlated with known 1p deletions. EGFR degradation rates were slowest in SKNAS cells and therefore correlated with cellular UBE4B levels. The low degradation rates were correlated with high cellular levels of EGFR.

Conclusions: Expression levels of UBE4B are correlated in neuroblastoma cell lines with chromosome 1p deletions. Cell lines with lower levels of UBE4B degrade EGFR at a markedly slower rate, correlated with higher cellular EGFR levels. We hypothesize that UBE4B affects cell growth by interacting with hrs, directing EGFR for degradation. In its absence the ability of a cell to sort growth factor receptors for degradation is inhibited, resulting in growth factor receptor overabundance and uncontrolled cell growth. These results support the testing of EGFR inhibitors in future trials for children with neuroblastoma.

TR49 Use of Corticosterone for an Improved Selective Uptake of mIBG / 6–FDA in Neuroblastoma Cells in Competition with Cells Expressing Extraneuronal Monoamine- / Organic Cation-Transporter

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Background: Radiolabeled mIBG (SPECT) and [^{18}F]6-Fluorodopamine (PET) can be used for imaging of neuroblastoma and pheochromocytoma cells expressing noradrenaline- and/or dopamine transporters (NAT/DAT). However, catecholamines and some of their derivatives are taken up also by cells which express extraneuronal monoamino- / organic cation transporters (EMT/OCT). The aim of this study was to clarify whether mIBG is also incorporated by these cells and, if so, whether EMT/OCT can be selectively inhibited in order to enrich mIBG and 6-Fluordopamine (6-FDA) in neuroblastoma cells.

Methods: SK-N-SH (neuroblastoma), PC-12 (pheochromocytoma), CAKI-1 (kidney carcinoma) and HEK-293 cells transfected with EMT, OCT-1 and OCT-2, respectively, were used for uptake studies. Uptake of [^{3}H]dopamine, [^{3}H]noradrenaline, and [^{131}I]mIBG was measured in suspension cultures and in transwell systems in the absence and presence of desipramine and corticosterone.

Results: The uptake of [^{3}H]dopamine (which is incorporated like 6-FDA) was in the order SK-N-SH > PC-12 >> CAKI > HEK-293:OCT-2 ~ HEK-293:EMT > HEK-29: OCT-1. In contrast, [^{131}I]mIBG was best incorporated into EMT /OCT-expressing cells (CAKI >> HEK-293:EMT ~ HEK-293:OCT-2 > HEK-293:OCT-1 > SK-N-SH ~ PC-12). Inhibition studies showed that unlike desipramine, an highly effective inhibitor of catecholamine/mIBG uptake in SK-N-SH and PC-12 cells, corticosterone (10^{-4}M) effectively inhibited mIBG/catecholamine uptake in EMT- and OCT-2 expressing cells, but only slightly in SK-N-SH and PC-12 cells.

Conclusions: mIBG was even better enriched in cells which express EMT and OCT-2 than in cells expressing NAT (neuroblastoma/pheochromocytoma). However, corticosterone strongly inhibited mIBG- and catecholamine uptake in EMT/OCT-, but only slightly in NAT/DAT- expressing cells. Therefore, corticosterone may be able to shift the uptake of [$^{131/123}$I]mIBG or [^{18}F]6-FDA to neuroblastoma- and pheochromocytoma-cells and could therefore be used in combination with [^{123}I]mIBG or [^{18}F]6-FDA for an improved imaging.

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TR50 Neuroblastoma Cell Death by Casiopeinas

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Background: Neuroblastoma is a very frequent solid tumor in childhood. They present markedly divergent clinical and biological factors with prognostic relevance. Casiopeinas® are a family of molecules with an active Cu^{2+} core and an aminoacid gives them tumoral specificity. Evidence shows that Casiopeinas® generate reactive oxygen species that could be responsible for the apoptotic phenomenon in tumoral cells. Our hypothesis is that Casiopeinas IIgly and IIIia have an apoptotic effect in neuroblastoma.

Methods: Using SK-MSN cells we determined viability by sulphorhodamine-B and some indicators of apoptosis; such as condensed nuclei recognized by Hoechst, and disruption of the mitochondrial transmembrane potential, identified with a cationic dye that exhibits a distinct fluorescence in healthy and apoptotic cells. Finally, the levels of glutathione-S-transferase were measured.

Results: All cases had some condensed nuclei. Treatment with Casiopeinas showed apoptosis as a green fluorescence sign, can not aggregate in the mitochondria due the altered membrane potential. Finally, treatment with Casiopeinas showed that glutathione did not play a role in the cellular defence against oxidative stress in SK-MSN cells and caspases could be concentration-dependent.

Conclusion: In conclusion, Casiopeinas IIgly and IIIia could be an important compound for treatment of neuroblastoma by apoptosis.

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Poster

TR51 Repression of BIRC5/Survivin by the Transcription Factor FOXO3/FKHRL1 Sensitizes Neuroblastoma Cells to Doxorubicin- and Etoposide-Induced Cell Death

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Background: The protein kinase B (PKB) and its target, the forkhead transcription factor FOXO3/FKHRL1 have been suggested as regulators of neurotrophin-mediated cell survival in neuronal cells. We therefore analyzed human neuroblastoma cells and found that FKHRL1 was phosphorylated, suggesting its inactivation. Since the inhibitor of apoptosis protein BIRC5/Survivin is regulated downstream of PKB and overexpressed in malignant neuroblastoma cells we analyzed whether FKHRL1 affects Survivin expression.

Methods: A 4OH-tamoxifen (4OHT)-regulated, phosphorylation-independent FKHRL1(A3)ER^{tm} allele was retrovirally introduced into SH-EP cells. Activation of transgenic FKHRL1 by 4OHT induced apoptotic cell death in a dose-dependent manner. To determine the impact of Survivin on FKHRL1,- doxorubicin,- and etoposide-induced apoptosis transgenic Survivin and short hairpin-expressing Survivin plasmids were retrovirally infected into SH-EP/FKHRL1 cells. Apoptosis induction by FKHRL1 and by the chemotherapeutics doxorubicin and etoposide was analyzed by flow cytometry. FKHRL1-mediated Survivin repression was determined by immunoblot and quantitative RT-PCR.

Results: The onset of FKHRL1-induced cell death was preceded by the repression of Survivin. Ectopic Survivin inhibited apoptosis but not cell cycle arrest by FKHRL1, whereas Survivin knock-down by shRNA technology sensitized for FKHRL1-induced apoptosis. In neuroblastoma cells, Survivin purified with the mitochondrial compartment, prevented the production of reactive oxygen species and reduced the loss of mitochondrial membrane potential and the release of Cytochrome *c* during FKHRL1-induced cell death. Low-dose activation of FKHRL1 sensitized for apoptosis by doxorubicin and etoposide, whereas the overexpression of Survivin diminished FKHRL1-sensitization to these chemotherapeutic agents.

Conclusions: We conclude from these data that the repression of Survivin modulates FKHRL1-induced apoptosis and drug-sensitization in neuroblastoma cells.

TR52 Intratumoural Heterogeneity of the *MYCN* Oncogene in Neuroblastoma

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Background: *MYCN* amplification is an important prognostic and therapy-stratifying molecular marker in neuroblastoma. The FISH method with detection of *MYCN* signals on a single cell level allows a more critical judgement of intratumoural *MYCN* heterogeneity.

Methods: The *MYCN*-Status was investigated by FISH in 1559 samples of 1278 patients at initial diagnosis and at relapse (touch preparations 45%, histological sections 41% and bone marrow 14%). *MYCN* heterogeneity was defined within one tumour as cluster or single cells (≥5 cells per slide) with amplification surrounded by non-amplified tumour cells, as *MYCN* heterogeneity between primary tumour versus metastasis, or as changing of *MYCN* status during the course of disease.

Results: Out of 1278 patients, 1014 showed no amplification (79,4%) and 244 had homogeneous amplification (19%), but 20 patients showed heterogeneity according to the *MYCN* status (1,6%). Of these patients with genetic heterogeneity, 12 had clusters of *MYCN* amplifications, four amplified single cells and two *MYCN* amplifications in the bone marrow but not in primary tumour. Two patients acquired *MYCN* amplification during the course of the disease. Eight of the 20 patients with *MYCN* heterogeneity were stage 4 and treated on high risk protocols. Among 12 patients with localized/4S disease, therapy was intensified in four patients because of *MYCN* amplified clusters, but not in eight patients (one intermediate risk, seven no chemotherapy). Eight of 12 patients on high risk regimes showed progression (7/8 stage 4; 1/4 localized/4S disease). Tumour progression was also observed in one patient with intermediate therapy and in 3/7 without chemotherapy (two local, one metastastic). 4/7 patients without chemotherapy are disease-free 213 to 3480 days after diagnosis (stage 1/2 patient with amplified single cells; two stage 4S patients with clusters).

Conclusion: *MYCN* heterogeneity is rare. Few children with low percentages of amplified cells and low risk neuroblastoma remained disease-free without chemotherapy.

TR53 FOXO3/FKHRL1 Promotes Epigenetic Activation of Caspase 8 and Sensitizes Neuroblastoma Cells for TRAIL-Induced Apoptosis

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Background: Deregulated neurotrophin signalling and lack Caspase 8 as a consequence of epigenetic gene silencing are frequently observed in aggressive neuroblastoma cells. Since the forkhead transcription factor FOXO3/FKHRL1 might be regulated downstream of neurotrophin receptors and the FKHRL1-target GADD45A has been recently shown to cause demethylation of silenced genes we investigated whether forced activation of FKHRL1 changes Caspase 8 expression and triggers apoptosis via the extrinsic death pathway in low passage neuroblastoma cells.

Methods: A 4OH-tamoxifen (4OHT)-regulated, phosphorylation-independent FKHRL1(A3)ER^{tm} allele was retrovirally introduced into three low passage, caspase 8-deficient neuroblastoma cell lines. Expression of transgenic FKHRL1, Caspase 8, GADD45A and TRAIL was analyzed by immunoblot and quantitative RT-PCR. The effect of FKHRL1 and transgenic Caspase 8 on cell survival was assessed by flow cytometry.

Results: Transgenic FKHRL1 transiently induced the death ligand TRAIL and spontaneous cell death in one out of three neuroblastoma cell lines. Retroviral expression of caspase 8 restored or accelerated apoptosis induction by FKHRL1 in all three cell lines, indicating that the extrinsic apoptosis pathway is critical for cell death decision in these cells. Interestingly, similar to the treatment with the demethylating agent 5-Aza-Cytidine, transgenic FKHRL1 *per se* caused a delayed re-expression of caspase 8 in all three cell lines. The re-expression of caspase 8 was preceded by strong induction of the demethylating enzyme GADD45A.

Conclusions: The combined data suggest that FKHRL1-induced cell death in neuroblastoma cells is critically modulated by the presence of caspase 8 and that FKHRL1 may relieve epigenetic gene silencing via repair-mediated DNA-demethylation.

TR54 A Differential Expression of Pleiotrophin and Midkine could Participate in Neuroblastoma Oncogenesis

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Background: PTN (pleiotrophin) and MDK (midkine) are highly homologous cytokines, expressed in neuroblastoma together with their receptors, in particular the anaplastic lymphoma kinase (ALK). PTN expression is associated with a good prognosis, while data are still unclear about MDK expression and prognostic value in NB. We hypothesized that MDK and PTN play differential roles in neuroblastoma oncogenesis and we therefore compared their expressions in tumors and normal tissues.

Methods: 101 patients (median age 2.5 years, range: 0–13) with a resected neuroblastoma (16 stage I; 7, II; 22, III; 48, IV and 8, IVS) were included. Tissue microarrays were constructed containing 101 primary tumors, 39 paired metastases and 56 paired normal tissues. Immunohistochemical staining was performed on sections with antibodies directed against ALK, PTN or MDK and the intensity was evaluated by a semi-quantitative score based on the percentage of positive cells. The Wilcoxon signed rank test was applied for the comparison of paired data.

Results: 99% of the tumors expressed ALK, 66% MDK and 50% PTN, while the expressions of these proteins in normal tissues were found in 97% cases for ALK, 34% for MDK and 47% for PTN. Interestingly, ALK and MDK had higher expression scores in tumors as compared with paired normal tissues ($p<0.0001$) while PTN showed an inverse tendency, being more expressed in normal tissues ($p=0.07$). It is noteworthy that we did not find any relation between the protein expressions and survival or prognosis factors in this series of tumors. We are currently evaluating the role of the pathway using stably transfected NB cell lines.

Conclusions: Our data suggest that the ALK-PTN-MDK pathway could participate in neuroblastoma oncogenesis. The "switch" between the expressions of PTN and MDK after transformation is of particular interest, making MDK a potential target for novel therapies.

Poster

TR55 Topotecan Inhibits Activation of Vascular Endothelial Growth Factor Production and Angiogenic Activity by Hypoxia-Inducible Factor (Hif)-1α and Hif-2α in Human Neuroblastoma

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Background: Neuroblastoma, the most common pediatric extracranial solid tumor, elaborate angiogenic peptides, and the extent of angiogenesis correlates with tumor progression and poor clinical outcome. Hence, inhibition of angiogenic factor production represents an important modality of therapeutic intervention. One of the major drives to tumor angiogenesis an a challenge of therapy is hypoxia, a decrease in oxygen tension that characterizes the tumor microenvironmen.

Methods: We investigated the effects of the topoisomerase-1 inhibitor, topotecan, on hypoxia-induced production of the proangiogenic mediator, VEGF, in advanced-stage MYCN-amplified human neuroblastoma cell lines.

Results: We demonstrate that pharmacological concentrations of topotecan counteracted hypoxic induction of VEGF. This effect was paralleled by decreased angiogenic activity of conditioned medium from hypoxic NB cells *in vivo* in the chorioallantoic membrane (CAM) assay. Functional studies of the VEGF promoter utilizing luciferase reporter constructs demonstrated the requirement for both hypoxia-inducible factor (HIF)-1α and -2α in the activation of VEGF gene transcription by hypoxia, because: (i) targeted knockdown of either HIF-1α or HIF-2α accumulation in response to hypoxia by RNA interference counteracted hypoxia ability to activate VEGF transcription and resulted in a parallel inhibition of VEGF mRNA and protein induction; (ii) overexpression of either protein under normoxia by transfection with expression vectors resulted in VEGF promoter transactivation, which was abrogated by mutation in the HIF-1-binding site.

Conclusion: Topotecan treatment significantly decreased VEGF promoter transactivation by hypoxia by targeting the expression of both subunits. A similar pattern of results was obtained in cells treated with the hypoxia-mimetick agent, desferrioxamine. These findings have important implications for neuroblastoma treatment.

TR56 Implications of *EPHA2* Expression in Neuroblastoma

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Background: Neuroblastoma is unique because of its striking clinical bipolarity. Survival rate of children with unfavorable neuroblastoma has been among the lowest despite extensive efforts to treat this disease. We have been interested in studying functions of EPH receptors and ephrin ligands in neuroblastoma. Subsequently, we have identified members of the EPH/ephrin receptor/ligand families as favorable neuroblastoma genes (*EPHB6*, *EFNB2*, and *EFNB3*). It has been suggested that EPHA2, also known as a member of the EPH family receptor kinases, can either promote or suppress cancer cell growth depending on cellular contexts. This study investigates the biological significance of EPHA2 in neuroblastoma.

Methods: *EPHA2* expression in neuroblastoma cell lines and primary tumors was examined by quantitative RT-PCR. MTS assay was employed to investigate the growth effect of EPHA2 on neuroblastoma cells *in vitro*. Western blot analysis was used to detect protein expression. Kaplan-Meier and Cox regression analyses were used to determine the effect of *EPHA2* expression on survival of neuroblastoma patients.

Results: Tumorigenic N-type neuroblastoma cell lines expressed low levels of *EPHA2,* but inhibitors of DNA methylation and histone deacetylase enhanced its expression. In contrast, hypo-tumorigenic S-type neuroblastoma cell lines expressed high levels of *EPHA2*. Forced expression of *EPHA2* in N-type neuroblastoma cell lines resulted in growth suppression. Nonetheless, survival analysis indicated that high *EPHA2* expression was not associated with a good disease outcome of neuroblastoma. *EPHA2* expression was correlated with the expression of *EPHB4* and *EFNB1,* which are also p53 target genes, in primary neuroblastomas. In addition, EPHA2 expression was augmented concomitantly with p53 up-regulation by doxorubicin in neuroblastoma cells *in vitro*.

Conclusions: *EPHA2* is a growth suppressive gene of neuroblastoma, and targeted pharmaceutical enhancement of EPHA2 by non-cytotoxic agents may be an attractive therapeutic approach for treatment of children with unfavorable neuroblastoma.

TR57 The Development of a New Murine MYCN-Amplified Neuroblastoma Cell Line

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Background: Neuroblastoma is the most common solid extra cranial tumor in childhood accounting for 8–10% of all childhood cancers. High-level expression of MYCN plays an important role in maintaining the malignant phenotype of neuroblastoma and is the most significant predictor of rapid tumor progression and poor prognosis overall. The *MYCN* oncogene is amplified in more than 40% of patients with advanced disease. Especially for these patients the development of new adjuvant therapeutic strategies is desperately needed.
Recent studies suggested that MYCN is a suitable target for tumor immunotherapy, but up to now, a syngeneic *MYCN*-amplified neuroblastoma mouse model is not available to examine new therapeutic strategies *in vivo*.

Methods: Here, we report for the first time the establishment of a *MYCN*-amplified murine neuroblastoma cell line syngeneic to A/J mice. For this purpose we used NXS2 cells. NXS2 is a murine neuroblastoma cell line derived from A/J mice with one copy of the MYCN oncogene characterized by low MYCN protein expression. We stably integrated the MYCN cDNA into NXS2 cells by using a lentiviral system based on the vectors pMD2G, pMDLg/pRRE, pRSV Rev and pPRIE7.

Results: Stable transduction was verified by real-time PCR and Western-Blot, revealing significantly higher expression levels of MYCN compared the human MYCN-amplified cell line Kelly, which contains about 120 copies of the *MYCN* gene. Additionally, the expression of the new NXS2-MYCN cells is about 25 fold higher than in the wildtype NXS2 cells. Characterization of the murine *MYCN*-amplified cells *in vitro* and *in vivo* is currently under investigation. Results will be presented at the meeting.

Conclusions: In summary, we report the development of a new murine *MYCN*-amplified neuroblastoma cell line providing for an important new syngeneic model for the *in vivo* evaluation of new therapeutic strategies for this highly aggressive type of neuroblastoma.

TR58 Tumor Cell Senescence in *MYCN* Amplified Neuroblastoma: Senescence Initiation and Possible Effects of Senescent Tumour Cells

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Background: Neuroblastoma (NB) cells with *MYCN* amplification can be induced to expulse the amplified gene by micronucleus formation using low hydroxyurea (HU) doses *in vitro* and to enter the senescence pathway. Previous work has shown loss of *MYCN* copies, reduction/cease of proliferation, decrease of telomeric lengths and presence of senescence-associated-β-galactosidase in the so-called F-cells. So far, the cause leading to *MYCN* expulsion, the expression pattern of the senescent cells and thus, their possible effects on non-senescent cells, were unknown.

Methods: Two NB cell lines (STA-NB-9/-10) with *MYCN* amplification were treated with 75/100μM HU and tested at different time points using: dihydrorhodamine 123 (DHR), MitoSox, 10-N-nonyl acridine orange (NAO) and JC-1 (relative mitochondrial mass) staining (evaluation by FACS) for oxidative stress and mitochondrial impairment; replication behaviour by FACS analyses; γH2AX and Western blot (p53, phospho p53, p21) for indication of DNA damage. Expression pattern was analyzed by: immunofluorescence (IF), RT-Q-PCR (36 genes) and microarray.

Results: DHR, MitoSox, NAO and JC-1 staining revealed a significant increase of superoxide/peroxide/peroxynitrite concentrations and mitochondrial impairment during the course of HU treatment. Cell cycle analyses showed an increase of S-phases while cell number decreased in the first days of treatment. Nuclear/micronuclear γH2AX staining increased and showed co-localizations with *MYCN*. Further, an increase of (phospho) p53 and p21 was encountered. Expression pattern analysis revealed about 800 differentially expressed genes in the senescent cell population (amongst them: *CDKNs, CLU, TGL, FN1, CD44, IGFBPs, INHBA, SERPINB5, THBS1, COL18A1, SPARC, INHBA, TIMP1, LIF, IL6, 7, 8, TSLP, LAMP2, PA28, MHCI, B2M, TAPBP, CALR, NKG2D ligands*).

Conclusions: We suspect a combination of replication delay and oxidative stress leading to DNA damage as the main causes for *MYCN* expulsion and senescence induction. The change of expression could point to antiproliferative, angiogenesis inhibiting and possibly immunogenic effects of the senescent F-cells.

TR59 Allogeneic Bone Marrow Transplants are Significantly more Efficient than Autologous Transplants in Treatment of Murine Neuroblastoma

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Autologous transplants of bone marrow cells (BMC) using high dose multiagent chemotherapy and/or total body irradiation (TBI) are effective in children with high risk neuroblastoma (NB), but most patients relapse. Few clinical studies have compared autologous and allogeneic bone marrow transplantation (BMT) in NB patients using identical preparatory regiments. Allogeneic transplant in these patients may induce graft versus tumor (GVT) effect against NB after HLA-matched/mismatched allogeneic BMT, renewing interest in allogeneic BMT for treatment of high risk neuroblastoma. We studied allogeneic responses to NB in a murine model.
An important role of T-cell mediated immunity is attributed on the basis of the faster growth rates of Neuro2-a cells in immunocompromized NOD SCID mice (n=8) as compared to growth in immunocompetent tumor-congenic A mice (n=15, $p<0.01$). Subsequently, A/J mice ($H2K^a$) received 10^6 Neuro2-a cells (subcutaneous) followed by TBI at 700 rad and transplantation of $5\text{-}10x10^6$ BMC from A/J ($H2K^a$) or C57BL mice ($H2K^b$). Irradiation and transplantation of syngeneic BMC (n=11) slowed tumor growth (at day 30) to 2077± 651 mm^3 vs. tumor in nonirradiated mice 3980±772mm^3 ($p<0.001$). At day 30 tumor sizes were 758±356 mm^3 and 908±420 mm^3 respectively after transplantation of allogeneic whole BMC and T-cell-depleted BMC ($p<0.001$ versus syngeneic transplants), indicating induction of an effective, yet insufficient, graft versus tumor reaction.
Neuroblastoma is a malignancy with low immunogenicity and few known specific antigens. As such the mechanism of GVT is cryptic. It is possible that the lack of MHC antigens expressed by NB leaves the tumor unprotected by the tolerogenic donor-host state induced by BMT, rending it accessible to immune alloreactivity. The mechanism of GVT in allogeneic transplant models deserves further exploration with regards to failure of innate and adaptive immunity to fight this tumor efficiently and with regards to the specific immune effectors of the allogeneic GVT reaction.

TR60 Significant Decreased Expression of PhosphoAKT in Mass Screening than in Standard Neuroblastoma

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Background: Neuroblastoma (NB) is a pediatric solid tumor with a poor outcome except in children younger than one year. Based on catecholamine urinary excretion, large mass screening (MS) programs have been organized in infants but felt to decrease the mortality secondary to this tumor. The hypothesis is that the detected tumors would present a spontaneous maturation or regression. In these two options, activation of AKT is involved. The objective was to analyze the level of activation AKT between MS NB and standard NB.

Methods: A first tissue microarray (TMA) with MS NB coming from Quebec and Japan contained 55 primitive tumors and 21 metastases (median age of 7 months, 16 stage 1, 15 stage 2, 9 stage 3, 6 stage 4 and 9 stage 4S). A second TMA contained 101 primary standard NB and 39 paired metastases (median age of 30 months, 16 stage 1, 7 stage 2, 22 stage 3, 48 stage 4 and 8 stage 4S). Immunohistochemical staining was performed using antibodies against, AKT, phosphoAKT, TRKB which is known as a poor prognosis factor of NB. Immunostaining intensity was evaluated by a semi-quantitative score based on the percentage of positive cells. The t-student test was applied for the comparison of protein expression between standard and MS NB.

Results: The expression of phosphoAKT was significantly higher in primitive tumors, in metastases, in stage 1 and in patient under one year of standard NB than in MS NB. AKT was only significantly more present in primitive and metastases of standard NB than in MS NB, as for TRKB.

Conclusions: The activation of AKT pathway is significantly higher in standard than in MS NB independently of age, stage and primitive of metastatic status of the tumor. This confirmed that NB diagnosed through MS differ biologically from standard NB.

TR61 Potential Use of Mesenchymal Stem Cells in Experimental Neuroblastoma Treatment

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Background: Mesenchymal stem cells (MSC) are non-hematopoietic stem cells that can act as immuno suppressors. Although poorly immunogenic, MSC may function as antigen-presenting cells (APC). MSC injected systemically in tumor bearing mice are attracted to the tumor site where they can inhibit or stimulate malignant cell growth. Aim of this study was to investigate the role of MSC as APC for NB associated antigens and their impact on NB growth.

Methods: MSC were expanded in vitro from the bone marrow (BM) of healthy donors or femurs of A/J mice. MSC immunophenotype was assessed by flow cytometry. MSC were transfected with pooled mRNA from NB cell lines using Transmessenger Transfection Reagent. IFN-γ and Granzyme-B release by human NB specific CTL was assessed by ELISPOT assays using Multiscreen-IP Millipore plates. CTL-mediated cytotoxicity was evaluated by 4 h ^{51}Cr release assay. Human and murine MSC were injected in pseudometastatic and orthotopic NB bearing immunocompetent or immunodeficient mice. In vivo tumor localization of MSC labelled with the fluorescent dye SP-DiI was evaluated by immunofluorescence.

Results: Human MSC transfected with NB mRNA but not unmanipulated were able to stimulate IFN-γ and Granzyme B release by NB-specific CTL, in an HLA-class I restricted manner. However, when tested in cytotoxic assays, CTL did not lyse either transfected or unmanipulated MSC at any E/T ratio.
Next, we showed that MSC i.v. injected in NB bearing mice specifically localized to NB metastases, but did not affect tumor growth, irrespective of the model used. We are currently evaluating the immunomodulating and antitumor activities of murine MSC transfected with NB mRNA in a pseudometastatic immunocompetent NB model.

Conclusions: This study shows that MSC transfected with NB mRNA challenged with specific CTL release cytokines but are not lysed. These MSC may modulate the anti-NB immune response in vivo.

TR62 A Review of Our Twenty-Year Experience with Established Neuroblastoma Cell Lines

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Background: Cell lines from neuroblastoma (NB) provide a reproducible resource. During the last 20 years we have established 16 NB cell lines. Here we report our major findings about the biological and molecular characters of NB by the use of these cell lines.

Methods and Materials: Fresh tumors or bone marrow cells were cultured in RPMI1640 with FCS. To date we have attempted to establish cell lines from 186 tumor samples.

Results and Discussion: A total of 16 cell lines were established (9% of all specimens) from 14 patients (13 stage 4 and 1 stage 3). Two additional cell lines were obtained from 2 of patients, one of which was obtained during a relapse and the other of which came from a different metastatic site on autopsy. Our major findings are as follows: 1) NB can be diagnosed by the analysis of cell surface membrane antigens that are stably expressed for long-term-culture (Cancer Res 1986), 2) One cell line was found to have CD10 antigen (Cancer Res 1985). 3) Retinoid or gamma-interferon differentiated into neuronal cells (Cancer Res 1987, 1989) and BrdU differentiated into Schwannian cells (Cancer Res 1988). 4) Four lines of substrate-adhesive (S)-type cells were found to have smooth-muscle-cell phenotypes, which strongly suggests that neural crest cells have the ability to differentiate into smooth-muscle cells in human systems (Int J Cancer 1991, Diagn Mol Pathol 2000). 5) Although establishment of an cell line from a tumor indicates that the tumor was growing aggressively *in vivo*, and is nearly always fatal, one cell line was established from a stage 4 patient with *MYCN* amplification who has experienced long-term-event-free survival. 6) The *MYCN* DNA level in culture supernatants from cell lines with *MYCN* was not affected by storage temperatures or by freezing and thawing, indicating that quantitative real-time PCR is a promising method for measuring serum *MYCN* DNA level (J Clin Oncol 2005).

Poster

TR63 Clinical Implications of the Slight Increase for the Gene Dosage of MYCN Determined Using Quantitative PCR in Neuroblastoma

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Background: In recent years, it is recommended to determine MYCN status in neuroblastoma(NB) using the quantitative PCR method(Q-PCR) instead of the Southern blotting(SB). However, the significance of gene dosage of MYCN determined using Q-PCR in NB is still unclear. In order to assess the implications of the gene dosase determined by Q-PCR, we evaluated the MYCN status using Q-PCR method for DNA extracted from small areas of NB sample using laser captur microdissection (LCM).

Methods: We quantified MYCN gene dosages (MYCN/NAGK) of 63 primary NB frozen samples using Q-PCR. In all 63 samples, LCM from at least two small areas of each paraffin-embedded tissue was performed, then, we quantifed MYCN gene dosages of 243 microdissected tissues using Q-PCR. In 23 of 63 cases, the MYCN gene status was evaluated using FISH.

Results: Of 54 frozen samples with a single copy of MYCN based on SB, 12 samples showed the slight increase for the gene dosage of MYCN (4.00 ≧ MYCN/NAGK ≧ 1.84), and 9 of these 12 cases showed advanced stage. Of 12 cases, 1 case had several LCM areas which showed high copy numeber of MYCN (MYCN/NAGK ≧ 16) and several LCM areas which showed a single copy number of MYCN (1.84 ≧ MYCN/NAGK). Another case of 12 cases showed the slight increase for MYCN gene dosage (3.65 ≦ MYCN/NAGK ≦ 4.82) in all LCM areas, furthermore, the finding of MYCN gain was found using FISH. In other 2 of 12 cases, although any LCM areas did not show the slight incesae for the dene dosage of MYCN, the cells with MYCN amplification were found using FISH.

Conclusions: The slight increase for the gene dosage of MYCN by Q-PCR may means that NB tissue contains a small number of cells with MYCN amplification or a large number of cells with MYCN gain, which are associated with the progression of tumor.

TR64 Id2 Protein Expression in Neuroblastoma Patients: Initial Results

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Background: Although many prognostic factors are known in neuroblastoma, there is still no possibility to precisely predict the prognosis and disease outcome. The Id protein family is known to take part in the control of cell cycle and was proposed to be a risk factor of poor prognosis in neuroblastoma patients, independent of NMYC amplification.

Methods: 60 patients treated for neuroblastoma in our center during the last 15 years were retrospectively evaluated. Id2 protein expression was examined by immunohistochemistry on paraffin embedded tumor tissue. NMYC was evaluated with FISH – either the results obtained at diagnosis were analyzed or additional examinations were performed on paraffin embedded tumor section if NMYC had not been previously analyzed. All tumors were re-evaluated by pathologist according to INPC classification.

Results: All analyzed tumors showed the Id2 protein expression. The variety of strength and pattern of expression of this protein was observed in tumors from different patients. 11 patients had amplification of MYCN. No correlation was found between the presence and the pattern of Id2 expression, NMYC amplification and tumor histology according to INPC as well as with age and stage of the disease.

Conclusions: Although Id2 may take part in the pathogenesis of neuroblastoma, it probably does not correlate with well established risk factors and it is not an independent risk factor. The pattern and strength of expression does not depend on characteristic and presentation of neuroblastoma.

TR65 Chromosomal Alterations (unb11pLOH and 1pLOH) and Histologic Changes in Peripheral Neuroblastic Tumors

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Background: Peripheral neuroblastic tumors (pNTs) offer a model for investigating biologically and clinically relevant relationship between molecular/genetic alterations and their morphologic manifestations. Recent study demonstrated that Unb11qLOH and 1pLOH were independent prognostic factors for patients with MYCN non-amplified pNTs (N Engl J Med 353:2243, 2005).

Purpose of the Study: To investigate histologic features characteristic for unb11qLOH and 1pLOH pNTs.

Materials and Methods: Histologic sections of 383 MYCN non-amplified pNTs with known chromosomal status (using the same data from cases published in N Engl J Med) filed at the COG Neuroblastoma Pathology Reference Laboratory were reviewed. No tumors in Ganglioneuroblastoma, Intermixed and Ganglioneuroma category (34 cases) had those chromosomal alterations. Tumors in Ganglioneuroblastoma, Nodular (44 cases) were excluded from the study due to possible sampling error for LOH determination. Histologic features according to the International Neuroblastoma Pathology Classification (grade of neuroblastic differentiation-Grade and Mitosis-Karyorrhexis Index-MKI) and presence or absence of pleomorphic neuroblastic cells were analyzed on 305 "Neuroblastoma" tumors (Unb11qLOH 49 cases; 1pLOH 24 cases; Unb11qLOH & 1pLOH 11 cases; no chromosomal alteration 221 cases).

Results: Tumors with those chromosomal alterations had significantly higher incidences of Unfavorable Histology than tumors without alterations ($p<0.006$). No significant differences in distribution of Grade and MKI were observed between tumors with or without the chromosomal abnormalities. The most striking feature was frequent presence of large pleomorphic cells in unb11qLOH tumors. (71.7%, 43/60 in 11qLOH alone tumors or 11qLOH & 1pLOH tumors vs. 8.2%, 20/245 in non-11qLOH tumors, $p<0.0001$). Those cells were more frequently found in unb11qLOH alone tumors (69.4%, 34/49) than in 1pLOH alone tumors (12.5%, 3/24), $p<0.0001$.

Conclusions: Unb11qLOH, MYCN non-amplified "Neuroblastoma" tumors were characterized by frequent presence of pleomorphic neuroblastic cells. The results prompt us to further investigation on mechanism linking the chromosomal alteration and the unique morphologic change in pNTs.

TR66 HIF-2α in Perivascular Neural Crest-Like Neuroblastoma Cells

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Background: Neuroblastoma is a childhood tumor of the developing SNS, which in turn is derived from the neural crest. Hypoxia-Inducible Factor 2α (HIF-2α) is one of the main mediators of the cellular response to oxygen shortage, and is also specifically expressed in sympathetic ganglia and paraganglia during development. In neuroblastoma cells, HIF-2α is stabilized and active at physiological oxygen tensions and in neuroblastoma tumors, HIF-2α is expressed adjacent to blood vessels. Furthermore, HIF-2α protein expression in neuroblastoma is correlated to poor clinical outcome. We have further characterized HIF-2α expressing cells in neuroblastomas.

Methods: Tissue sections from neuroblastoma tumors were studied immunohistochemically. Tumor origin of the studied cells was confirmed by combined immunofluorescence and fluorescence in situ hybridization (FISH) for MYCN.

Results: In addition to hypoxic stabilization of HIF-2α in perinecrotic tumor areas, we found subsets of intensely HIF-2α positive tumor cells located in apparently non-hypoxic perivascular areas. Such cells were found to lack expression of the sympathetic differentiation markers TH and NSE while expressing neural crest and early sympathetic progenitor markers such as Notch-1, HES-1, c-Kit, dHAND and vimentin. HIF-2α expressing tumor-associated macrophages were frequently found close to these immature cells and both cell types expressed high levels of VEGF.

Conclusions: High levels of HIF-2α highlight neural crest-like tumor cells and tumor-associated macrophages that may both contribute to the aggressive phenotype of neuroblastomas by driving angiogenesis through high expressions of VEGF. Due to their localization in the same perivascular niche, our data suggest a potential therapeutic value in clinical targeting of neuroblastoma vasculature, as HIF-2α expression is correlated to unfavorable clinical outcome.

TR67 Extensive Validation of the Standardized Immunocytochemical Technique for Detection of Disseminated Neuroblastoma Cells: a SIOPEN Bone Marrow Subcommittee Study

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Background: Standardized analysis procedures are indispensable tools for multicenter studies evaluating the clinical significance of minimal residual disease in neuroblastoma. European reference laboratories therefore developed SOP's for the immunocytochemical (IC) detection and quantification of disseminated neuroblastoma cells. In order to guarantee the unambiguous identification of rare tumour cells, the SIOPEN Bone Marrow subcommittee decided to validate the standardized detection technique by comparing results of the IC assay to those of automated immunofluorescence plus FISH (AIPF), a technique which verifies the genetic make-up of detected cells.

Methods: In this multicenter study, 137 bone marrow samples or PBSC preparations (Israel: n=94; Czech Republic: n=43) from 34 SIOPEN high-risk neuroblastoma patients were analysed by immunocytochemistry (according to SOP's) and AIPF in a blinded way.

Results: Using immunocytochemistry, in 25/137 samples "criteria positive cells" (CPC's) were detected. In 23/25 samples also "FISH positive cells" (FPC's) were found. Nine samples scored positive for AIPF although no CPC's were found. The number of detected neuroblastoma cells in these discordant samples was low. Only in 3 samples more than 10 FPC's per 10^5 mononuclear cells were detected.
In 12/137 samples, solely IC NCIC's (not convincingly interpretable cells) were found. The malignant nature of the NCIC's was confirmed by AIPF in 3/12 samples. Again, the number of NCIC's detected in the AIPF$^-$ samples was generally low (< 10 NCIC's), indicating that the discordance may be caused by sampling error.
Finally, the correlation between the results of both techniques was calculated taking only IC CPC's and AIPF FPC's into account. A statistical significant correlation was found (p-value<0.001).

Conclusions: The high concordance between the results of both techniques suggests that most cells detected by immunocytochemistry are neuroblastoma cells. The standardized technique is reliable, relatively simple and cost-effective and enables the unequivocal identification of disseminated neuroblastoma cells in a multicenter setting.

TR68 Multi-target Model Using DCX and TH by QRT-PCR for the Detection of Residual Cells in High-Risk Neuroblastoma Patients

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Background: Detection of minimal residual disease (MRD) in bone marrow (BM),peripheral blood (PB) and peripheral blood stem cells (PBSC) is very important for high-risk neuroblastoma patient's follow up. We propose to develop a multi-target model by DCX and TH quantitative reverse transcriptase polymerase chain reaction (QRT-PCR) in order to detect MRD in neuroblastoma patients.

Methods: Relative quantification of DCX and TH was studied by QRT-PCR using Assays on Demand from Applied Biosystems (Oltra et al., 2005, Diagn Mol Pathol. 14: 53-57). We studied DCX and TH expression in 368 BM samples, 267 PB and 25 PBSC belonging to 104 high-risk patients (95 stage 4 and 9 stage 3) treated according cooperative national and international protocols.

Results:. We observed coincidence in the expresion of both markers in 85% of BM, 87% of PB and 92% of PBSC samples. The results obtained at different time points are shown in Table 1.

Table 1: Frequencies of patients expressing DCX or TH in two treatment periods, in PB and in BM aspirates. n refers to the number of patients.

	BM		PB	
	Diagnosis	**End Induction**	**Diagnosis**	**End Induction**
DCX+	75,3 %	37,8 %	58,3 %	12,5 %
TH+	76,7 %	28,9 %	50 %	15,6 %
Combined	78 %	40 %	65 %	22 %
n	73	45	60	32

Conclusions: Both DCX and TH mRNA are detected at high frequency in BM and PB from children with neuroblastoma. This multi-target model is more efective to detect residual cells in high risk neuroblastoma patients than a model based in one marker only. Moreover the combination of both markers could save the tumoral heterogeneity.

TR69 A Novel Celecoxib Derivate, OSU03012, Suppress Neuroblastoma Growth by Inhibiting β-Catenin Function and MYCN Expression

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Background: Neuroblastomas express cyclooxygenase 2(COX-2) and inhibition of COX-2 activity by celecoxib has profound effects on neuroblastoma growth *in vitro* and *in vivo* with promising clinical results. In addition to being a COX-2 inhibitor, celecoxib has also been shown to directly target the 3-phosphoinositide-dependent protein kinase-1 (PDK1), a protein involved in phosphatidylinositol 3-kinase (PI3K)/AKT signaling. We therefore investigated the effect and mechanism of OSU03012, a novel celecoxib derivate lacking COX-2 inhibitory activity, but retaining PDK1 inhibiting activity, on neuroblastoma growth.

Methods: Immunohistochemisty staining of a wide range of primary neuroblastoma tumors was done using phospho-specific antibodies for the detection of activated proteins in the PI3K/AKT signalling pathway. Measurements of OSU 03012 effects on neuroblastoma growth and mechanisms of growth inhibition was done using proliferation assays, flow cytometry, real-time PCR and immunoblotting.

Results: Significant expression of activated AKT and mTOR was detected in all primary neuroblastoma tissue samples, but not in non-malignant adrenal medullas. OSU03012 inhibited PI3K/AKT signal transduction and displayed antiproliferative effects on neuroblastoma cells by induction of mitochondrial transmembrane depolarization, activation of caspases, cleavage of PARP and induction of apoptosis. The concentration of OSU03012 that was associated with 50% inhibition of neuroblastoma cell growth (biologic IC_{50}) ranged from 0.3-1.5 μM compared to 26-32 μM for celecoxib. Neuroblastoma cells expressing high levels of MYCN were more sensitive to OSU 03012 compared to cell lines expressing low MYCN levels. Importantly, OSU 03012 inhibited nuclear translocation of β-catenin and downregulated expression of cyclin D1 and MYCN through a mechanism involving activation of GSK-3β.

Conclusion: Neuroblastoma tumors have activated PI3K/AKT signaling pathway. Our results suggest that OSU 03012 have therapeutic efficacy at low concentrations on aggressive neuroblastoma expressing high MYCN levels.

TR70 Tailored Neuroblastoma Therapy - Identification of Small Molecules that Induce Apoptosis in a MYCN-Dependent Manner

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Background: One of the key predictors of poor outcome in neuroblastoma is amplification of the *MYCN* oncogene which occurs in 40-50% of high-risk cases. Such amplification is associated with advanced-stage disease, rapid tumor progression and a survival rate of less than 15% despite novel advances in treatment strategies. Our approach is to identify low molecular compounds that selectively induce cell death in neuroblastoma cells with high MYCN expression

Methods: We have screened a chemical library of low molecular weight compounds in neuroblastoma cells with inducible MYCN expression to identify compounds that selectively can inhibit the proliferative function of MYCN and/or enhance its pro-apoptotic effect.

Results: We have identified 30 substances with specificity for MYCN overexpressing cells compared to non-expressing neuroblastoma cells. Based on structural analysis ten of these compounds have been further characterized. We have found that all of the substances interfere with the MYCN pathway by inducing apoptosis but that the mechanism of action is different. Some compounds decrease MYCN protein expression, others interfere with MYCN/Max binding to DNA and all inhibit cellular transformation. We are currently analyzing the compounds in a xenograft model in SCID mice order to test candidate compounds *in vivo*.

Conclusions: The *MYC* oncogene is deregulated in a wide variety of human tumors and is therefore an attractive target for novel cancer therapies. Using a cellular screening approach we have identified several low molecular weight compounds that predominantly suppress proliferation in MYCN-overexpressing cells compared to cells without MYCN expression.

In summary, our data suggest that cellular screening assays can be a powerful strategy for the identification of candidate substances that modulate the MYC pathway. These compounds can be useful tools for studying MYC function and may also be of therapeutic potential as leads for medical chemistry and drug development.

Poster

TR71 Chk1 Inhibitor AZD7762 Synergizes with Chemotherapeutic Agents in p53 Defective Neuroblastoma (NB)

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Background: Although rare at diagnosis, p53 pathway aberrations are frequently found at relapse in patients with advanced NB, which probably account for chemoresistance after an initial treatment response. AZD7762 (AstraZeneca) is a novel selective inhibitor of checkpoint kinase Chk1 with the potential to enhance the action of DNA-damaging agents in carcinomas. By expression array, Chk1 expression is high in NB cell lines and a subset of NB tumors. Here we test if NB cell lines are sensitive to AZD7762 and whether this drug can synergize with cytotoxic agents in NB tumor cell kill.

Methods: Cytotoxicity of AZD7762 alone or in combination with chemotherapeutic agents was measured by the WST-8 chromogenic proliferation assay on 5 NB cell lines. Calculation of IC_{50} and Combination Index (CI) values were based on the median-effect principle. CI<1 indicates synergism, CI=1 additive effects, and CI>1 antagonism.

Results: AZD7762 was cytotoxic to all NB cell lines tested (average IC_{50}=73.4 nM, range 38.2-94.9 nM). Two NB cell lines derived from the same patient before [BE(1)N, p53 wild type] and after [BE(2)N, p53 mutant] chemotherapy were equally sensitive to AZD7762 alone (IC_{50} was 93.7 and 94.9 nM, respectively). When AZD7762 was tested for synergy with DNA-damaging chemotherapeutic agents, CI was 1.24±0.18 for BE(1)N, and 0.60±0.21 for BE(2)N, consistent with strong synergism in the latter. Similarly for LAN1, which had p53 deletion, AZD7762 (IC_{50} 85.9 nM) synergized with gemcitabine (CI=0.19), topotecan, SN38, melphalan, etoposide, doxorubicin, and cisplatin (CI=0.63±0.13). In contrast, no synergy was found when AZD7762 was combined with vincristine (CI=1.14), a non-DNA damaging agent.

Conclusions: NB cell lines were highly sensitive to Chk1 inhibitor which synergized with conventional DNA-damaging agents, particularly in NB cells with nonfunctional p53 status. These findings provide a strong rationale for further *in vivo* preclinical studies.

TR72 The Multidrug Transporter Genes ABCC1/MRP1, ABCC3/MRP3 and ABCC4/MRP4 are Powerful Predictors of Clinical Outcome in Childhood Neuroblastoma

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Background: We have previously shown, both retrospectively and prospectively, that high-level expression of the multidrug transporter gene ABCC1/MRP1, is strongly predictive of poor outcome in the childhood cancer neuroblastoma (NEJM, 334:231-8, 1996; JCO, 24:1546-53, 2006), and that ABCC1/MRP1 can be regulated by the MYCN oncogene. The contribution of other ABCC family genes to clinical outcome in this disease has now been examined.

Methods: Real-time quantitative PCR was used to determine ABCC gene expression in a large prospectively accrued cohort (n=209) of primary untreated neuroblastomas from patients enrolled on POG biology protocol 9047.

Results: Established prognostic indicators including older age, advanced stage, and MYCN amplification were all predictive of poor outcome in the cohort. Amongst the ABCC family, high levels of ABCC1 and ABCC4, but low levels of ABCC3, were strongly associated with reduced survival and event-free survival (P<0.005) in the overall study population, and also in subgroups of patients lacking MYCN amplification. Following adjustment for the effect of MYCN gene amplification and other prognostic indicators by multivariate analysis, expression of ABCC1 (HR=2.3; p=0.03), ABCC3 (HR=2.7; p=0.0141), ABCC4 (HR=3.4; p=0.002) retained significant prognostic value for outcome, whereas age and MYCN amplification lost all prognostic significance. By combining the expression of these three transporter genes, patients could be stratified into groups having excellent, intermediate or poor outcome (EFS = 84%, 59%, 17%, respectively).

Conclusions: These data, suggest that ABCC1, 3 and 4 are amongst the most powerful prognostic markers yet identified for childhood neuroblastoma and as such represent important targets for potential therapeutic intervention.

TR73 Decitabine in Combination with Doxorubicin and Cyclophosphamide in Neuroblastoma and Other Solid Tumors - A Children's Oncology Group Phase 1 Consortium Study

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Background: Loss of caspase-8 expression contributes to apoptotic resistance and metastasis in neuroblastoma. Since decitabine increases caspase-8 expression and enhanced apoptosis in vitro, we conducted a phase I trial to determine the toxicity, pharmacokinetics and molecular effects of decitabine followed by doxorubicin and cyclophosphamide in children with refractory solid tumors.

Methods: Stratum A included children with any relapsed or refractory solid tumor; Stratum B included neuroblastoma patients only. Patients received a 1-hour decitabine infusion for 7 days, followed by doxorubicin (45 mg/m^2) and cyclophosphamide (1 g/m^2), repeating every 28 days. The starting decitabine dose was 5 mg/m^2/d. Pharmacokinetic studies were performed with the first dose. Methylation and expression analyses of caspase-8 and two biomarkers of methylation [(*MAGE-1* and fetal hemoglobin, (HbF)], were performed. Gene expression patterns were compared between pre and post treatment samples.

Results: The maximum-tolerated decitabine dose when administered daily for 7 days followed by doxorubicin and cyclophosphamide was 5 mg/m^2/d. The dose-limiting toxicities were neutropenia and thrombocytopenia. Three of 9 patients in Stratum A and 4 of 12 patients in Stratum B had stable disease for a median of 5 (range, 4-9) cycles. Decitabine was rapidly cleared from plasma with a $t_{1/2}$ of 18.4 ± 4.8 minutes. *MAGE-1* promoter demethylation was observed in 12/20 patients. HbF mRNA was increased in 14/16 patients. Demethylation and subsequent increased bone marrow caspase-8 mRNA expression was seen after treatment. Genes with altered expression were identified by microarray analysis.

Conclusions: Low-dose decitabine with doxorubicin and cyclophosphamide is associated with tolerable toxicity in children. Evidence of demethylation is observed, including demethylation of potential pro-apoptotic genes and surrogate markers of tumor demethylation. This study serves as the basis for future studies of demethylating agents in childhood cancer.

TR74 Antitumor Activity of the Selective MDM2 Antagonist Nutlin-3 in a Chemoresistant Model of Neuroblastoma

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Background: Restoration of p53 function by antagonizing its negative regulator MDM2 offers an appealing nongenotoxic approach for treatment of tumors with wild-type p53. Mutational inactivation of p53 is uncommon in neuroblastoma tumors at diagnosis and accounts for only a subset of multidrug-resistant neuroblastoma cases. In this study we investigated the antitumor activity of the small-molecule MDM2 antagonist nutlin-3 in a model of multidrug-resistant neuroblastoma that allows for study of treatment effects in relationship to p53 status.

Methods: The antiproliferative and cytotoxic effect of nutlin-3 was examined in chemosensitive (UKF-NB-3) and matched chemoresistant neuroblastoma cells characterized by high expression of P-glycoprotein and wild-type p53 (UKF-NB-3^{r}DOX^{20}) or high expression of P-glycoprotein and mutant p53 (UKF-NB-3^{r}VCR^{10}). Activation of the p53 pathway was assessed by real-time quantitative RT-PCR analysis of p53 target genes, flowcytometric cell cycle analysis, DNA fragmentation assay and measurement of caspase activity. Antitumor activity of nutlin-3 against multidrug-resistant neuroblastoma *in vivo* was explored using murine subcutaneous xenografts of UKF-NB-3^{r}DOX^{20} and UKF-NB-3^{r}VCR^{10} cells.

Results: Targeted inhibition of the p53-MDM2 interaction by nutlin-3 resulted in cell viability reduction, increased expression of p53 target genes, G1 cell cycle arrest and apoptosis in chemosensitive UKF-NB-3 and chemoresistant UKF-NB-3^{r}DOX^{20} cells to a similar extent, but not in UKF-NB-3^{r}VCR^{10} cells with mutant p53 (as expected). Treatment of mice carrying multidrug-resistant neuroblastoma xenografts with nutlin-3 selectively inhibited growth of p53 wild-type tumors (54% growth inhibition of UKF-NB-3^{r}DOX^{20} xenografts after three weeks of treatment), with induction of p53 target gene expression and caspase-3 activation evident after 36 h of treatment.

Conclusions: Antagonizing MDM2 function by nutlin-3 accomplishes the goal of p53 reactivation in neuroblastoma in a context of chemoresistance, provided that wild-type p53 is retained. These findings encourage clinical trials of selective MDM2 antagonists for treatment of neuroblastoma.

TR75 Schedule-Dependent Synergistic Cytotoxicity of Vorinostat and Flavopiridol against Multi-drug Resistant Neuroblastoma Cell Lines with Loss of p53 Function

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Background: As p53 loss of function confers high-level drug resistance in neuroblastoma, p53-independent therapies might have improved activity in recurrent neuroblastoma. We evaluated the cytotoxic activity of vorinostat, a novel histone deacetylase (HDAC) inhibitor, and flavopiridol, a novel pan-Cdk inhibitor on recurrent neuroblastoma cell lines.

Methods: The combination of vorinostat and flavopiridol was tested in 8 multidrug-resistant neuroblastoma cell lines: four with non-functional and mutated *TP53* (CHLA-90, CHLA-119, CHLA-172, and SK-N-BE2), one with p14 deletion (LA-N-6), and three with wild-type and functional *TP53* (CHLA-136, CHLA-79, SK-N-RA). Cytotoxicity was measured by digital image microscopy assay (DIMSCAN) and synergy was determined by combination indices (CI) calculated by CalcuSyn. We assessed the p53 expression level by Quantitative RT-PCR and immunoblot.

Results: LC90 values of vorinostat with single agent ranged from 0.81 to > 2 μM, and of flavopiridol ranged from 0.22 to > 0.4 μM in 8 multidrug-resistant cell lines. Sequential exposure to vorinostat followed by flavopiridol showed strong synergistic cytotoxicity (CI<0.8) in 4 multidrug-resistant cell lines with mutant *TP53* and 1 with p14 deletion. The LC90 of each in combination decreased to 0.24 to 1.02 μM for vorinostat and 0.048 to 0.20 μM for flavopiridol. By contrast, the combination showed modest synergy in 3 multidrug-resistant lines with functional p53. Expression of p53 was upregulated in CHLA-136 (wt *TP53*) treated with the combination but reduced in CHLA-172 and CHLA-90 (mt *TP53*).

Conclusions: Combining vorinostat and flavopiridol warrants further investigation as a novel combination therapy for recurrent neuroblastoma with p53-LOF. Decreasing mutant p53 expression may be a mechanism of action for this drug combination.

TR76 The Hsp90 Inhibitor, 17-DMAG, Inhibits Growth of Neuroblastoma Cell Lines in vitro and in vivo

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Background: The identification of novel agents as options for alternative multimodal treatment regimens for high-risk and relapse neuroblastomas (NB), which are often resistant to the current chemotherapeutic agents, remains a high priority. Heat-shock proteins (Hsp) are molecular chaperones assisting proper protein folding and guarding against protein misfolding, aggregation and promiscuous binding. Chaperone proteins have been shown to be overexpressed in many tumor types, and are thought to facilitate tumor cell survival. Many small-molecule drugs targeting Hsp90 have been identified as anticancer agents, including geldanamycin (GA) and its derivatives, 17-AAG and 17-DMAG. The water soluble analog, 17-DMAG, is promising since it can be administered orally, and is less toxic in animal studies. We have previously found Hsp90 mRNA expression to be elevated in primary NB from patients who later experienced relapses.

Methods: The anti-proliferatory effects of Hsp90 inhibitors alone or in combination with cisplatin were tested on a panel of NB cell lines in monolayer culture and spheroid cultures *in vitro* or xenografts in nude mice. Client protein expression was assessed using western blotting.

Results: In the micromolar range, 17-DMAG was as effective as GA, and more effective than 17-AAG, at inhibiting NB cell proliferation *in vitro*. Treatment with 17-DMAG further increased the anti-proliferative effect of cisplatin treatment in NB cells, regardless of *MYCN* status or prior resistance to cisplatin. Treatment with 17-DMAG also reduced proliferation of NB cell lines grown as spheroids.17-DMAG induced apoptosis in NB cells, and increased the expression of co-chaperone proteins. We identified known Hsp90 client proteins which were NB cell line-specific. Treatment with 17-DMAG inhibited the growth of NB xenografts in nude mice without observable side effects.

Conclusions: Thus, 17-DMAG may be a well tolerable option for novel multi-agent chemotherapeutic strategies.

TR77 Methionine Depletion Synergizes with Microtubule-Depolymerizing Agents in Neuroblastoma

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Background: Methionine starvation inhibits protein synthesis, modulates gene methylation, and impedes cell cycle transition, affecting multiple pathways necessary for tumor DNA repair. Low methionine levels were previously shown to enhance the effect of chemotherapy. Methionine depletion using recombinant methioninase (rMETase) has proven safe and effective in non-human primates. Its therapeutic potential in neuroblastoma (NB) has not been tested.

Methods: The effect of rMETase alone on cell cycle in ten NB cell lines was tested using PI staining. SK-N-LD and SK-N-BE(1)N established at diagnosis, and LAN-1 [p53 deleted] and NMB-7 [p53 wild type] established at relapse were further tested in combination with 18 common chemotherapeutic agents using WST8 chromogenic proliferation assay. Combination index (CI<1= synergy) was calculated by CompuSyn software. Combinational effect on cell cycle was assayed by PI and MPM-2-cy5 staining. *In vivo* effect was tested in NB xenografted in athymic nude mice.

Results: *In vitro* studies showed that rMETase arrested one NB cell line in G1 phase and the other nine in G2. rMETase synergized with a broad spectrum of cytotoxic agents (alkylators, topoisomerase inhibitors, hsp90 inhibitor, and retinoids) in SK-N-LD and SK-N-BE(1)N, but not LAN-1 and NMB-7. In all 4 NB cell lines tested, microtubule depolymerization agents (vincristine, vinorelbine, vinblatine, and mebendazole), but not microtubule stabilizing agents (paclitaxel, docetaxel, and fludelone) synergized with rMETase (CI=0.39-0.67). No interactions were observed in M and G2 phase arrest between rMETase and vincristine. In LAN-1 and NMB-7 xenograft models, methionine depletion (rMETase iv at 100 Units/dose bid ×2d/wk×3wks) rendered vincristine (0.8mg/kg) iv qwk ×3wks more effective than vincristine alone (p<0.01).

Conclusions: rMETase synergizes with microtubule depolymerization agents *in vitro* and *in vivo*. The synergism between rMETase and other chemotherapeutic agents (including those stabilizing microtubules) appeared to depend on whether the NB cell line was established at diagnosis or at relapse.

TR78 Development of PI Polyamides Targeting N-myc as a Gene Silencer and a Gene Probe for Neuroblastoma

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Objective: Pyrrole-imidazole (PI) polyamides is a novel gene silencer which can bind to specific nucleotide sequences in the minor groove of double-helical DNA with high affinity and specificity to inhibit expression of target gene. We established original sutomatic synthesize method and have developed PI polyamides as novel gene silencers. This study shows development of PI polyamides targeting N-myc as a gene silencer and a gene probe of the molecular imaging for neuroblastoma.

Methods: PI polyamides were designed to around SP-1 site of the human N-myc promoter. Binding of PI polyamide to the target DNA was confirmed by gel mobility shift and BiaCore assays. We investigated pharmacokinetics and safety of PI polyamides in vivo. We examined effects of polyamides on expressions of N-myc mRNA and protein, and proliferation of NB-9 cells. We synthesized PI polyamide with PBr and examined Kd by BiaCore assay as a gene probe for the molecular imaging.

Results: PI polyamides showed strong, fast and specific binding to the target DNA. PI polyamides showed usual pharmacokinetics and easily distributed in several organs without any vectors, and its safe dose was under 20 mg/kg. PI polyamides inhibited expressions of N-myc mRNA and protein, and proliferation of NB-9 cells. PI polyamides with PBr were distributed in human N-myc gene-induced tumor in NOD-SCID MOUSE.

Conclusion: PI polyamides targeting N-myc will be feasible for the gene silencer and a gene probe of the molecular imaging by PET for neuroblastoma.

Poster

TR79 A Novel Therapeutic Combination for Neuroblastoma: The VEGFR/EGFR/RET Inhibitor Vandetanib with 13-cis-Retinoic Acid

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Background: Neuroblastoma is the most common extracranial solid malignancy of childhood. High-risk cases of neuroblastoma have extremely poor long-term survival rates, and novel therapies are needed. Vandetanib (ZD6474, ZACTIMA™) is a multi-kinase inhibitor with activity against VEGFR, EGFR, and RET kinase pathways that have been implicated in neuroblastoma pathogenesis. We hypothesized that the combination of 13-*cis*-retinoic acid (CRA) and vandetanib would be effective against neuroblastoma.

Methods: We evaluated the effects of vandetanib and CRA separately and in combination on differentiation and survival in human neuroblastoma cell lines *in vitro*. Using a NOD/SCID/IL2R-gamma subcutaneous xenograft model of human neuroblastoma, we analyzed treated tumors for growth, gross and histologic appearance, vascularity, apoptosis, and proliferation.

Results: Vandetanib induced apoptosis in the majority of neuroblastoma cell lines at 2.5-10μM. CRA induced morphologic differentiation and cell-cycle arrest without apoptosis. Vandetanib plus CRA resulted in significantly reduced neuroblastoma cell viability. In a mouse xenograft model, CRA alone did not have a significant effect on tumor growth. In contrast, vandetanib alone significantly inhibited tumor growth and vascularity, and the combination of vandetanib with CRA demonstrated significantly more growth inhibition than vandetanib or CRA alone.

Conclusions: Vandetanib, an inhibitor of VEGFR, EGFR, and RET tyrosine kinases, induces neuroblastoma tumor cell death *in vitro* and reduces tumor growth and vascularity *in vivo*. The combination of vandetanib with CRA was more effective in reducing tumor growth than either treatment alone. The antitumor effects of vandetanib and CRA suggest a novel clinical combination for multi-pathway targeting in neuroblastoma.

TR80 Dendritic Cell-Based Immunotherapy Using Sendai Virus Vector - a Preclinical Efficacy Study against Neuroblastoma -

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Background: The outcome of patients with advanced neuroblastoma has not been efficiently satisfied, despite of the modern intensive treatment. We have recently reported that the induction of efficient antitumor immunity to highly malignant B16F10 melanoma associated with down-regulated MHC class I, using DCs activated by recombinant Sendai virus (SeV/DC). Importantly, the antitumor effect of SeV/DC was greatly enhanced in use of SeV expressing interferon-b (IFN-b), upregulating the expression of MHC class I antigen. The aim of the current study is to investigate the potential of SeV/DC to treat less immunogenic neuroblastoma, as a preclinical efficacy study.

Methods: Female A/J mice were subcutaneously inoculated with C1300 neuroblastoma (10^6cells/head). The established tumor-burden mice were treated with bone marrow-derived DCs which were activated with temperature sensitive mutant SeV with or without therapeutic gene (tsSeV/dF or tsSeV/dF-IFNb)). In case of combination with irradiation and tsSeV/dF/DC, tsSeV/dF/DC (10^6cells/head) were administered intratumorally after preirradiation of x-ray (4G/day for 3 days).

Results: C1300 neuroblastoma showed stronger resistance against immunotherapy than B16F10 malignant melanoma. Use of tsSeV/dF-IFNb/DC without pretreatment of irradiation showed some efficacy on established C1300 neuroblastoma, but antitumor effect against vascularized/established tumor (> 5 mm) was weakened. The combination with irradiation and tsSeV/dF/DC was effective against vascularized/established tumor (> 5 mm), and dramatically enhanced the ratio of complete elimination of established tumor (5/8=62%). Antitumor effect of tsSeV/dF/DC with pretreatment by radiotherapy was enhanced CTL activity and established specific long term memory against C1300 neuroblastoma. The result of the effector cell-depletion experiment comfimed that CD4+T cells were predominant effector cells in antitumor immunity for C1300 neuroblastoma.

Conclusions: These results indicate that less immunogenic neuroblastoma could be a potential target of SeV/DC-based immunotherapy. Therefore, we concluded that SeV/DC system is warrant to further investigation to treat patients with intractable malignancies including far advanced neuroblastoma in clinical setting.

TR81 High-throughput siRNA and Drug Screening in Neuroblastoma

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Background: High-risk neuroblastoma patients remain to have a poor prognosis. The use of a combination therapy of a drug that causes cell death, together with target screening of siRNAs of genes that causes suppression of cell growth synergistically may lead to a cure to combat this disease. This project aimed to establish an *in vitro*, high-throughput siRNA target screening method in neuroblastoma cell lines. The synergistic or protective effect of siRNAs in combination with Topotecan, a topoisomerase I inhibitor currently used as second-line therapy especially in the relapse setting, was investigated.

Methods: A set of 418 genes involved in the apoptotic pathway (Qiagen Apoptosis Set v1) were chosen for the preliminary screen, with two siRNA sequences per gene. A wet reverse transfection protocol was used in 96-well microplates. Topotecan doses varied from 0uM, 1uM, 5uM and 10uM were added 24 hours post transfection. Cell viability was measured at 72 hours after the drug was added.

Results: In the preliminary screen, 119/418 genes alone without drug caused more than two fold suppression of cell growth. 64 genes showed synergy to Topotecan and 178 genes inhibited the action of Topotecan. Hits were selected for secondary screening with two new siRNAs sequences per gene. 47 genes alone without drug continued to show at least two fold suppression of cell growth and 33 genes showed synergy to Topotecan. 61 genes inhibited the action of Topotecan, giving insight into the mechanism of how this drug induces apoptosis in this cell line. Functional studies including cytotoxicity assay, caspase activation and cell cycle analysis will be investigated further on the positive hits.

Conclusions: We have therefore identified a potential series of 33 genes whose inhibition synergizes with Topotecan to suppress cell growth. Once validated these represent novel targets for combination therapy in patients with neuroblastoma.

TR82 Combined Therapeutic Effects of Bortezomib and Fenretinide on Neuroblastoma Cell Growth and Apoptosis

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Background: The proteasome inhibitor bortezomib was able to inhibit cell growth and angiogenesis in neuroblastoma (NB) (Brignole et al., JNCI 98:16, 2006). Bortezomib has been shown to induce synergistic activity when combined with several other antineoplastic agents. Here, we assayed a putative increased antitumour activity of bortezomib if delivered to NB cells together with fenretinide (HPR), a synthetic retinoic acid used as potential therapeutic agent in a variety of cancers, including NB.

Methods: Different NB cell lines were tested for sensitivity to bortezomib and HPR, given alone or in different dose-and time-dependent combination schedules. Cell proliferation, cell viability and apoptosis were evaluated by measuring ^{3}H-thymidine incorporation, trypan blue staining, DNA fragmentation and western-blot analysis. Angiogenesis was assessed by the chick embryo chorioallantoic membrane (CAM) assay. A xenograft and orthotopic NB mouse model was set up to examine *in vivo* sensitivity.

Results: Treatment of NB cells with 2,5 μM fenretinide plus 5nM bortezomib at 24 hours caused >50% inhibition of cell growth: these values correspond to at least the half concentration necessary to have the same results when both drugs were administred alone. This inhibition was associated to a marked G1 and G2/M cell cycle arrest with a nearly complete depletion of S phase by the combined treatment. Besides, NB cell death occurs with apoptosis features via ER stress by the activation of specific genes (i.e. GRP78, GADD 153, p-JNK, caspase-4). Tumour-bearing mice treated with HPR plus bortezomib lived statistically significantly longer than mice treated with each single drug. CAM analysis and histological evaluation of primary tumors evidenced that the combined therapeutic effects are mainly due to the increased antitumor activity more than to the anti-angiogenic potential of each drug.

Conclusions: Our findings provide the rationale for design a new therapeutic strategy to treat NB based on this pharmacological combination.

TR83 Increased Expression of Sphingosine Kinase 1 Mediates Acquired Resistance to Fenretinide in a Neuroblastoma Cell Line that is Partially Reversed by the Sphingosine Kinase Inhibitor Safingol

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Background: Fenretinide [N-(4 hydroxyphenyl) retinamide, 4-HPR] increases ceramides in cancer cells and is synergistically cytotoxic for neuroblastoma cell lines when combined with the sphingosine kinase inhibitor safingol. Phase I trials in neuroblastoma of intravenous and novel oral formulations of 4-HPR are in progress and of 4-HPR + safingol are in development.

Methods: Multistep selection for resistance to 4-HPR was conducted with the 4-HPR-sensitive (LC_{90}=2.20μM) neuroblastoma cell line SMS-KCNR. Cytotoxicity was assayed with the fluorescence DIMSCAN system and gene expression by real-time TaqMan RT-PCR.

Results: 4-HPR- resistant SMS-KCNR cells (KCNR-FR) showed a 4.4 fold decrease in 4-HPR sensitivity (LC_{90}=9.69μM) Synthesis of ceramides in response to 4-HPR in KCNR-FR was significantly decreased (21 %, p=0.01) relative to SMS-KCNR. Basal and post-5 μM 4-HPR expression for ceramide related genes (acid ceramidase, serine palmitoyl transferase II, neutral sphingomyelinase I, II, acid sphingomyelinase, 1-acyl ceramide synthase, glucosyl ceramide synthase) showed no significant difference between SMS-KCNR and KCNR-FR. However, sphingosine kinase 1 (SphK1) expression decreased in response to 5 μM 4-HPR (0.5h, 0.44 fold, p<0.0001; 4h, 0.76 fold, p< 0.0001) in SMS-KCNR, while in KCNR-FR, SphK1 RNA was increased by 4-HPR (0.5h, 1.45 fold, p<0.0001; 4h, 1.33 fold, p< 0.0001). To confirm a role for SphK1 in 4-HPR resistance, a lentiviral vector (pCCL-SphK1-hB7) was used to overexpress SphK1 in SMS-KCNR, achieving a 70–fold increase in enzymatic activity relative to empty vector control, p<0.0001). SphK1-transduced cells displayed a 1.9-fold increase in 4-HPR resistance (LC_{90}=4.0 μM vs. LC_{90}=2.16μM), p < 0.001, and also showed decreased cytotoxicity to 4-HPR + safingol. Safingol still synergistically increased 4-HPR cytotoxicity (Combination Index at LC_{90}=0.56) and partially overcame 4-HPR resistance in both KCNR-FR cells and SMS-KCNR transduced with SphK1.

Conclusions: These data demonstrate that increased sphingosine kinase 1 expression, targeted by safingol, can confer resistance to 4-HPR in neuroblastoma.

TR84 4Ig-B7H3: A Target for Monoclonal Antibody (mAb) Therapy of Human Neuroblastoma (NB)

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Background: mAb 8H9 specific for cell surface glycoprotein gp58 is highly reactive with NB, whereas normal tissues have restricted expression. Our phase I study of intra-Ommaya ^{131}I-8H9 has shown promise both in its efficacy and tolerability in patients with central nervous system (CNS) relapse. We now describe the biochemical characterization of the gp58 antigen.

Methods: gp58 was purified from the extract of NB cell line LAN1 by 8H9-affinity chromatography. The 8H9 antigen-positive band from SDS-PAGE was analyzed by mass spectrometry. Transcript was measured by quantitative (q)RT-PCR. *In vitro* binding kinetics was investigated by surface plasmon resonance using Biacore 2000. Protein analyses were performed using Western blots and immunohistochemistry.

Results: Two different peptides from mass spectrometric sequencing unequivocally identified the 8H9-reactive molecule as 4Ig-B7H3, the long and principal form of B7H3 in human tissues. As further confirmation, both 8H9 and MAB1027 (anti-B7H3 mAb from R&D System) recognized a single band at ~90 KD on Tris-Glycine SDS-PAGE under non-reducing conditions, when either 8H9-positive cell extracts or recombinant human 4Ig-B7H3 protein was used. This band migrated to ~58 KD after N-Glycanase treatment. While B7H3 transcript was ubiquitously expressed in solid tumors and normal human tissues by qRT-PCR, 4Ig-B7H3 protein was found only in tumors, but not in most normal tissues by both Western blot and immunohistochemistry. Binding kinetics indicated that both mAbs had a very slow and comparable dissociation rate (k_{off}) ($5.3x10^{-4}$ sec^{-1} and $2.2x10^{-4}$ sec^{-1} for 8H9 and MAB1027, respectively), which was crucial for sustained *in vivo* drug efficacy. However, when compared to MAB1027, 8H9 had much lower non-specific background staining of normal tissues by immunohistochemistry.

Conclusions: mAb 8H9 targets the tumor-associated cell surface antigen 4Ig-B7H3, which is known to inhibit NK cells and T cells. It has promising therapeutic potential for patients including those with relapsed NB metastatic to the CNS.

TR85 Receptor Tyrosine Kinase (RTK) Inhibition of Human Neuroblastomas

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Background: Neuroblastomas (NBs) express a number of RTKs (TrkA, TrkB, EGFR) that contribute to the survival and growth of NBs. We investigated the inhibition of these TK receptors, or key nodal points in their signaling pathways, to determine their efficacy in treatment of NBs *in vitro* and *in vivo*.

Methods: We used SY5Y-TrkA, SY5Y-TrkB and NLF (high EGFR expression) cells *in vitro*. Cells were grown in serum-free medium, pretreated with CEP-2563 (100 uM), Iressa (1 uM), rapamycin (5 nM), LY294002 (20 uM) or U0126 (10 uM) alone or in combination. Then, cells were treated with either NGF or BDNF (100 ng/ml). Cell viability was assessed either by RT-CES system or MTT assay. NLF cells were xenografted into nude mice to test the efficacy of Iressa (EGFR inhibitor; 180 mg/kg/d PO) *in vivo*. We compared tumor size and survival rate between treatment and control group.

Results: There is no significant additive effect on NLF cells by combining either rapamycin, LY294002 or U0126 with Iressa. However, when LY294002, rapamycin and Iressa were added at the same time, there were significantly fewer viable cells compared to Iressa alone. Rapamycin has no effect either alone or combined with CEP4416 on SY5Y-TrkA and TrkB cells. However, the combination of rapamycin and LY294002 has significantly greater inhibition of SY5Y-TrkA and -TrkB cells than either alone (p <0.01). Iressa slowed the growth of NLF xenografts compared to vehicle controls. On day 17, the average tumor size was 0.67 cm^3 for Iressa group and 1.18 cm^3 for vehicle group (p=0.05). Survival rate at day 28 is 66.7% for Iressa and 20% for vehicle (p =0.067).

Conclusions: Iressa has shown effect in treating neuroblastoma xenograft. Novel combination of rapamycin and LY294002 is significantly better than single reagent. It also has additive effect on Iressa in treating neuroblastoma.

TR86 ALK Receptor is a Potential Therapeutic Target for Neuroblastoma

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Background: Full-length ALK (Anaplastic Lymphoma Kinase) is a 200kDa trans-membrane receptor tyrosine kinase normally expressed almost exclusively in embryonic and perinatal neural cells. ALK expression has been reported in a number of neuroblastoma (NBL)-derived cell lines and primary tumor samples. However, it is not clear whether ALK expression in NBL cells reflects the embryonic origins of the tumor or a real neoplastic hallmark. We have investigated ALK expression and activity both in NBL tumor specimens and in vitro derived cell lines in the attempt to clarify the oncogenic role and the clinical significance of ALK in NBL pathogenesis.

Methods: ALK protein expression and activation was examined in 82 paraffin-embedded tumor samples and two highly-expressing (UKF-NB3, IMR-32), one low-expressing (SK-N-SH) and one negative (NB5) NBL cell lines. mRNA abundance and gene copy number were quantified by TaqMan. Functional inhibition of ALK was achieved by siRNA.

Results: ALK immunoreactivity was found in 75 cases. Remarkably, ALK expression levels were significantly upregulated in advanced/metastatic (stage 3-4) compared to localized (stage1-2) NBLs. Protein levels not always correlate with the amount of ALK transcript and gene copy number in either NBL tumors or cell lines. ALK constitutive phosphorylation/activation was observed only in ALK highly-expressing UKF-NB3 and IMR-32 cells. No ALK phosphorylation was detected in low expressing SK-N-SH cells. ALK depletion in IMR-32 cells by siRNA correlated with a complete inhibition of cell growth, increase of cell death and inhibition of ALK-associated signaling molecules STAT3, AKT and ERK1/2.

Conclusions: Our results suggest that aberrant ALK overexpression can be considered an oncogenic hallmark for NBL malignancy and disease progression. Indeed, only ALK-overexpressing cells exhibit receptor kinase activation. ALK signalling is a rate-limiting factor of proliferation and survival and thus, its inhibition could represent a potential novel therapeutic strategy for advanced and metastatic NBLs.

TR87 *In Vivo* Molecular Imaging of Neuroblastoma Progression by Small-Animal PET in the TH-*MYCN* Murine Transgenic Model

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Background: Small-animal Positron Emission Tomography (PET) offers the ability to evaluate tumor metabolism and response to therapy *in vivo*. These procedures can be repeated, allowing the same animal to be followed up in a manner similar to a human patient in a clinical setting.
The transgenic mouse model in which the expression of *MYCN* is targeted to neural crest cells using the rat tyrosine hydroxylase promoter closely recapitulates human neuroblastoma for molecular, biologic and cytogenetic features, and is an immunocompetent model to evaluate new therapies (TH-*MYCN*--Weiss, EMBO 1997, Chesler, Cancer Res, 2007).
We tested the accuracy of ^{18}F-fluorodeoxyglucose (^{18}F-FDG) and ^{18}F-DOPA PET to detect early neuroblastoma and to evaluate the metabolic behavior of the tumor progression in mice transgenic for TH-*MYCN*.

Methods: Small-animal PET was performed in homozygous and hemizygous TH-*MYCN* mice on a 129/SvJ background, using ^{18}F-FDG or ^{18}F-DOPA.
Animals were studied from the fourth week of age. PET images were interpreted visually and semi-quantitatively by calculating the Tumor-to-Background-Ratio (TBR).
All animals were sacrificed and histology was performed to verify PET results.

Results: ^{18}F-FDG was more sensitive that ^{18}F-DOPA for early detection and for serial evaluation of malignant progression. Both image quality and TBR calculated with ^{18}F-FDG was on average superior to that using ^{18}F-DOPA in both homozygous and hemizygous animals. Furthermore, the urinary excretion of ^{18}F-DOPA was very high and urinary bladder radioactivity saturated the images, reducing the diagnostic power of the technique.
Small-animal PET with ^{18}F-FDG in TH-*MYCN* mice permitted the identification of neuroblastoma at an early stage, and offers a sensitive method to follow metabolic progression of these tumors.

Conclusions: The use of molecular imaging by small-animal PET in the TH-*MYCN* mouse model can be used to improve the accuracy of *in vivo* preclinical testing of new therapies for neuroblastoma, by ensuring that homogeneous conditions are applied in all the animals under study.

TR88 In Vivo Silencing of a Molecular Target by Short Interfering RNA Electroporation

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Background: Screening for a molecular target for cancers including neuroblastomas requires multiple steps, of which an important one is evaluation of the knockdown effect of the target molecule on pregrown xenograft tumors. However, methods currently used for local administration of knockdown reagents, such as short interfering RNA (siRNA), are not satisfactory as to simplicity and efficiency.

Methods: We established an electroporation (Ep) method involving a constant voltage and 'plate and fork' type electrodes, and used it for *in vivo* delivery of siRNA. We evaluated this method from various aspects including convenience, delivery efficiency, and biological efficacy as to pregrown tumors.

Results: The delivery efficiency correlated to the electric current. The electric current correlated to vascular density and vascular endothelial growth factor (VEGF) expression, and had a threshold that guaranteed efficient delivery. Consequently, the Ep effectively transfected siRNA into tumors of high microvascular density, but not ones of low density. VEGF was chosen as a model target. VEGF overexpresses in neuroblastoma cell lines such as SK-N-LO, SK-N-SH, LS, SH-SY5Y, IMR-32 and Kelly, and clinical specimens. VEGF siRNA Ep suppressed the growth of tumors exhibiting high VEGF expression to less than 10% of the control level, but it had no effect on low-expressing VEGF tumors. Notably, a long interval (20 days) of Ep was enough to obtain a satisfactory effect. This method was superior to atelocollagen-mediated siRNA delivery. Systemically injected siRNA could also be transfected into tumors by this method.

Conclusions: Our data provide the technical basis for *in vivo* Ep, and this simple and efficient method of siRNA delivery is applicable to *in vivo* comprehensive screening for a molecular target.

TR89 A Novel P53 Ubiquitination Pathway In Neuroblastoma

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Background: Ubiquitination of p53 is a vital tumor promoting event regulating p53 stability in p53 wild type tumors such as neuroblastoma. MDM2 is the primary p53 specific E3-ubiquitin ligase. We have shown previously that treatment with MDM2 inhibitors rapidly produces p53 stabilization and triggers apoptosis. Further kinetic analysis reveals early onset MDM2-independent p53 degradation in neuroblastoma cells surviving initial Nutlin treatment. We hypothesize that Nutlin treatment induces an additional E-3 ligase activity, inhibition of which would augment p53 dependent apoptotic responses with therapeutic benefit.

Method: The rate or apoptosis and level of p53 stabilization in response to Nutlin were compared across a panel of solid tumor lines and multiple neuroblastoma cell lines.

Results: We demonstrate a very fast onset of apoptosis in neuroblastoma lines relative to osteosarcoma, lung, breast and colon tumor cell lines. All neuroblastoma cell lines tested showed a large increase in p53 within 4 hours of treatment and then a remarkable drop in p53 levels within 12-24hours of treatment. This pattern of p53 degradation is in sharp contrast to all other cell lines tested which show a gradual increase in total p53 levels. In addition, in p53 pull-down experiments we detect increased mono and poly-ubiquitinated p53 at later time points after treatment (16-24 hrs). In the presence of cycloheximide inhibiting protein synthesis and ALLN inhibiting proteosomal degradation, p53 degradation was halted and p53 response to Nutlin was then similar to non-neuroblastoma cell lines.

Conclusions: Since these data are obtained in the presence of active MDM2 blockade, they strongly suggest an alternative E3-ligase activity is up regulated upon stress and MDM2 inactivation in neuroblastoma cells. Further characterization of this neuroblastoma specific response to Nutlin is vital, as it will likely provide an important target for apoptotic directed therapies.

TR90 A Better Neuroblastoma Xenograft Model for Pre-Clinical Drug Testing

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Neuroblastoma (NB) is the most common extracranial solid cancer in infancy and childhood. Long-term survival for children with advanced disease is poor despite aggressive multimodality therapy. Pre-clinical animal models of NB are valuable tools for characterizing tumor behavior and evaluating therapies. Current metastatic models can not resemble the typical metastatic patterns in patients, such as bone/ bone marrow metastasis. We isolated candidate NB tumor-initiating cells (TICs) cancer stem cells from a multi-relapse neuroblastoma patient sample, NB12, and used relatively smaller number of TICs in both localized and intravenous metastatic murine models. We tested the anti-tumor activities of two compounds, rapamycin and vinblastine. In localized model, our result demonstrated that rapamycin suppressed tumor growth by approximately 85% of control ($p<0.001$), and vinblastine suppressed tumor by around 45% ($p<0.05$). In metastatic model, mice developed bone marrow metastasis after 7 weeks approved by bone marrow primary culture, and all the treatments were started. After 9 weeks, mice in control group showed stress signs like fluffy furs and slow movement, and some of them appeared tumor mass on femur or facial bones. Histologically, most of mice in control group (4/5) developed massive bone, lymph nodes, or adrenal metastasis, while all treatment groups with rapamycin and/or vinblastine showed only micro-metastatic sites on adrenal glands. Several other compounds are being tested now in this xenograft model. The application of this model not only provides us a better and more reliable model for pre-clinical drug test, but also make it more practical for patient-specific therapeutics.

TR91 Enhanced *In Vivo* Anti-Tumor Efficacy of Erlotinib for Neuroblastoma

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Background: Targeted therapies inhibiting critical signaling pathways may improve outcomes for neuroblastoma. EGFR inhibition may be beneficial, based on pre-clinical studies and case reports. However, limited data are available regarding ERBB pathway expression or function in neuroblastoma.

Methods: ERBB expression in neuroblastoma tumor cell lines was assessed by flow cytometry and western blot. Western blot assessed the effect of erlotinib on phosphorylation of EGFR, Her-2, AKT and MAPK with and without EGF stimulation. Flow cytometry with propidium iodide assessed cell cycle. *In vitro* growth inhibition of erlotinib was assessed over 4 days by direct counting (ViCell) and by Alamar blue assay. *In vivo* tumor inhibition was assessed using SK-N-SH xenograft tumors in nude mice dosed with 10 mg/kg Tarceva daily beginning when tumors were >30 mm^3.

Results: All cell lines expressed EGFR, Her-3 and Her-4, with variable levels between lines. Her-2 was present only in SK-N-AS. EGF treatment increased the phosphorylation of EGFR and Her-2, facilitating assessment of pharmacologic inhibition. Erlotinib caused reduction of phosphorylation of EGFR and Her-2 beginning at 0.1 micromolar and becoming maximal at 1 micromolar. Phosphorylation of AKT and MAPK was reduced in parallel with the loss of ERBB phosphorylation. Little inhibition of growth was observed: *in vitro* IC50 for erlotinib exceeded 5 micromolar. However, >60% reduction in tumor growth was observed in mice treated with erlotinib for 18 days (p=0.026).

Conclusions: *In vivo* anti-tumor efficacy of erlotinib greatly exceeded the *in vitro* growth inhibition, suggesting that erlotinib is impeding neuroblatoma growth by interfering with interactions with stromal components and/or vascular elements. Erlotinib inhibits EGFR phosphorylation in neuroblastoma at higher concentrations (1 micromolar) than published in cell-free kinase assays (0.01 micromolar), suggesting that neuroblastoma cells use active mechanisms such as MDR/pgp-1 to reduce the effective drug concentration intracellularly. Further pre-clinical and clinical studies are warranted.

TR92 MSO (Methionine Sulfoximine) Enhances Cytotoxicity of L-asparaginase in Neuroblastoma Cell Lines

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Background: L-asparaginase (L-asp), which is known as a key drug in childhood ALL, is not commonly used in pediatric solid tumors. Recently, it has been reported that L-asp has cytotoxicity in pediatric solid tumors, which is enhanced by using methionine sulfoximine (MSO), a glutamine synthetase inhibitor. We recently reported that reducing environmental glutamine significantly enhanced cytotoxicity of L-PAM, and reduced cell growth via glutathione (GSH)-depletion in neuroblastoma cell lines. Accordingly, we hypothesized that L-asp, together with MSO may be effective in neuroblastoma cell lines.

Methods: The growths of cells treated with L-asp (5 IU/ml) alone, MSO (1 mM) alone and L-asp + MSO were compared with control (untreated) in 9 neuroblastoma cell lines by MTT assay. We quantified the intracellular GSH by DTNB-GSSG reductase method, and also quantified the cellular content of amino acids by HPLC method. Apoptosis was determined by PI staining by flow cytometry.

Results: After 7 days of culture, the growth in cells treated with L-asp was significantly reduced to 9.6-72.4 % (mean 44.8 %) of control ($P<0.01$ in all cell lines). The cytotoxicity was significantly enhanced by using MSO in combination with L-asp in 7 of 9 cells ($P<0.01$). MSO alone did not effect on cell growth in any of the cells. In the SK-N-RA cell line, GSH was reduced in L-asp treated cells ($P<0.01$) and in MSO treated cells quantified on day 2 ($P<0.05$). Quantifying amino acids, glutamine, glutamic acid, asparagines and aspartic acid significantly reduced in cells treated with L-asp ($P<0.05$), while MSO alone did not make any differences compared to the control. Apoptotic cells significantly increased in cells treated with L-asp or L-asp+MSO ($P<0.01$ for both).

Conclusions: L-asp was cytotoxic in all 9 neuroblastoma cell lines, which was enhanced by using MSO. Though MSO alone did not effect on cell growth and apoptosis, L-asp alone or in combination with MSO was cytotoxic in neuroblastoma cell lines, which may be of therapeutic value in neuroblastoma.

TR93 HDM2 Attenuates Neuroblastoma Apoptotic Cell Death via Regulation of p53-Dependent Pro-apoptotic Effector Noxa Expression and Localization

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Backgrounds: Recently, we reported that a BH3-only member of Bcl-2 family proteins, Noxa, is one of the important pro-apoptotic effectors in neuroblastoma (NB) cell death (Kurata et al, Oncogene, 2007, in press). Noxa was increased in mitochondria by p53-dependent stimulation in the sensitive NB cells meanwhile Noxa was accumulated but not induced in the resistant NB cells. In the present study, we studied the role of HDM2 in p53-dependent apoptotic NB cell death and in regulation of Noxa expression and localization.

Methods: p53 wild-type NB cells (SK-N-SH: sensitive, IMR32 and NB-19: resistant) and p53-mutated NB cells (SK-N-DZ) cells were subjected to gene-knockdown experiments and gene-expression experiments using retrovirus vector.

Results and Discussion: HDM2 was associated with the accumulated and inactivated p53 and its ubiquitin ligase activity was not diminished in the p53 wild-type, resistant NB cells, suggesting that proteasome machineries might be attenuated. Knockdown of HDM2 by siRNA improved sensitivity of Doxorubicin (Doxo)-resistant NB cells and resulted in up-regulation of Noxa at mRNA/protein levels and mitochondria localization.
Intriguingly, cell proliferation and resistance to Doxo were up-regulated in over-expression of HDM2 in the Doxo-sensitive NB cells. p53 was accumulated in mitochondria but not activated by Doxo; down-stream effectors of p53, p21Cip1/Waf1 and Noxa, were not induced by Doxo treatment in those cells. Noxa expression pattern of the HDM2-over-expressed cells was similar to the resistant NB cells.
Furthermore, high expression level of *HDM2* co-relates with increased expression of *Noxa* in primary tumor samples consistent with the results in NB cell lines. Although *Noxa* mRNA expression was low in the *p53*-mutated NB cells, knockdown of *p53* or *p73* in *p53* wild-type NB cells did not decrease Noxa mRNA. Taken together, our results may indicate the significance of HDM2/p53/Noxa pathway in NB cell death and the possibility that HDM2 is a suitable candidate for molecular-targeted therapy of chemotherapy-resistant NB.

TR94 Chalcone Constituents of *Angelica Keiskei* Induces Apoptosis in Neuroblastoma

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Background: *Angelica keiskei* (Japanese common name "Ashitaba") has been used traditionally in Japan as a diuretic, laxative, analeptic and galactagogue. Some chalcones isolated from it reportedly have inhibitory effects against tumor promoter activity, antitumor and antimetastatic activities. In the present study, we aimed to identify agents that induce apoptosis in neuroblastoma cells.

Methods: Neuroblastoma cell line, IMR-32, NB39, and primary culture of rat cerebellar granule cells were used. Cytotoxicity was evaluated by [3-(4,5)-dimethyl-2-thyazolyl]2,5-diphenyl-2H-tetrazolium bromide (MTT) method. Apoptotic nuclear morphology was observed by staining with Hoechst 33342. Caspase activation and apoptosis-related protein expression were detected by Western blot analysis.

Results: Six chalcones from *Angelica keiskei* exhibited cytotoxicity against neuroblastoma cells, and two of them (isobavachalcone and xanthoangelol H) had no effect on rat cerebellar granule cells even at high concentration (10^{-4} M) exposure. Typical morphologic features of apoptosis, including cell shrinkage, chromatin condensation, nuclear fragmentation and formation of apoptotic bodies, were observed in isobavachalcone-treated cells. Western blot analysis showed that isobavachalcone significantly reduced pro-caspase-3 and pro-caspase-9, and subsequently increased the level of cleaved caspase-3 and cleaved caspase-9 in both neuroblastoma cell lines (IMR-32 and NB-39). Moreover, Bax was markedly induced by isobovachalcone application.

Conclusions: Isobavachalcone induces apoptotic cell death in neuroblastoma via the mitochondrial pathway and has no cytotoxicity against normal cells. Therefore, isobavachalcone may be applicable as an efficacious and safe drug for the treatment of neuroblastoma.

Poster

TR95 The Tyrosine Kinase Inhibitor Imatinib Mesylate Potentiates the Cytotoxic Effect of Chemotherapeutic Drugs in Neuroblastoma in Clinically Achievable Concentrations

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Background: Platelet-derived growth factor (PDGF), stem cell factor (SCF), and their respective receptors, PDGFR and c-Kit, are expressed in neuroblastoma. The tyrosine kinase inhibitor imatinib mesylate (imatinib) recently showed limited clinical activity as a single treatment in children with relapsed neuroblastoma. We therefore investigated the effect of clinical achievable doses of imatinib in combination with chemoterapeutic drugs on neuroblastoma growth.

Methods: Cell viability- and clonogenic-assay were used to measure the effects of single and combined drug effects on neuroblastoma growth and tumourigenic potential. Immunoblotting was used to analyze the phosphorylation status of PDGFreceptors.

Results: Treatment of neuroblastoma cells with low-dose imatinib inhibit phosphorylation of PDGF-RB and suppress clonogenic capacity of neuroblastoma cells. For imatinib alone in clinically achievable concentrations (0,5-2,5 uM), prolonged drug exposure (more than 120 hours) was necessary to achieve maximum inhibition *in vitro*. The combination of imatinib with cytotoxic drugs synergistically inhibited survival of several neuroblastoma cell lines.

Conclusion: Imatinib should be tested as an adjuvant treatment option in high-risk neuroblastoma.

TR96 Detection of Circulating Tyrosine Hydroxylase mRNA in a *MYCN* Transgenic Mouse Model of Neuroblastoma is not an Early Predictor of Tumour Formation

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Background: The *MYCN* transgenic mouse (TH-MYCN) is a valuable preclinical model for human neuroblastoma. Detection of tyrosine hydroxylase (TH) mRNA in peripheral blood of neuroblastoma patients is a useful prognostic indicator of disease progression. We have adapted the well-characterised human TH mRNA reverse transcriptase PCR (RT-PCR) assay to investigate its use for the detection of asymptomatic tumours in TH-MYCN mice.

Methods: TH-MYCN mice were examined on alternate days after weaning for evidence of tumours and, when present, were sacrificed and the tumour removed for study. Mice were genotyped by quantitative PCR using human and murine MYCN primers and probes. Tale vein blood was sampled at fortnightly intervals and 50-100ng/µl RNA isolated using a Purescript RNA Extraction Kit (Gentra). One-step RT-PCR was performed using murine TH-specific primers spanning exons 4-7. Products were analysed for a 180bp product; a positive (*MYCN* homozygote), and a negative control (wild-type) were included.

Results: In this preclinical model, 100% of MYCN$^{+/+}$and around. 30% of MYCN$^{+/-}$ offspring presented with tumours by 6-10 weeks and 12-18 weeks of age respectively. Although clinical presentation varied, with small abdominal tumours and spinal cord involvement or large palpable abdominal masses, in all cases neuroblastomas were aggressive and mice sacrificed within 24 hours of tumour detection. TH mRNA was detected in all TH-MYCN homozygotes by 6 weeks and 11/11 hemizygotes by 10-12 weeks. TH mRNA could not be detected in wild-type littermates. The intensity of the 180 bp TH mRNA band was variable but did not increase with age or tumour development. Levels of TH RNA in tumour-bearing mice were not significantly different between homozygous and hemizygous animals.

Conclusions: The sensitivity of the assay was high but the specificity was low indicating that circulating TH levels are not a useful predictive marker to detect asymptomatic neuroblastoma in the *MYCN* transgenic mouse model.

TR98 A Novel Drug RKS-2-62 is Cytotoxic to Neuroblastoma In Vitro and In Vivo

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Background: Novel therapies are needed to help increase the survival and quality of life for children with neuroblastoma. Nifurtimox induces reactive oxygen species (ROS) and apoptosis in neuroblastoma. Similar to nifurtimox, RKS-2-62 is a thiazine analog with a coumarin group attached. The coumarin enhances the generation of ROS leading to DNA damage and apoptosis. We hypothesize that RKS-2-62 would be more cytotoxic to neuroblastoma.

Methods: Neuroblastoma cell lines (CHLA-90, LA1-55n, LA-N2, SH-SY5Y, SMS-KCNR) were grown in 48-well plates, treated with 2.5-10 ug/ml RKS-2-62 for 48 hours, and assessed by calcein AM assays. Formation of ROS was examined through DCF absorbance by flow cytometry. Western blots for caspase-3, HIF1alpha, Bcl-2 were done. In a xenograft model nude mice were injected with 10^7 SMS-KCNR cells. Mice received daily doses of either 0 or 150 mg/kg of RKS-2-62 for 21 days. Tumors were resected and evaluated by immunohistochemistry.

Results: After 48 hours of treatment with 10 ug/ml RKS-2-62, cell viability was decreased to 11.0% in CHLA-90, 15.2% in LA1-55n, 14.6% in LA-N2, 6.8% in SH-SY5Y, 7.8% in SMS-KCNR cell lines. The decrease was seen in a dose dependent manner in all the cell lines. RKS-2-62 treated cells showed an increase in caspase-3 and Bcl-2 activity, suggestive of cells undergoing apoptosis. An increase in ROS production was detected through DCF flow cytometry. The xenograft models showed a decrease in tumor weight in mice treated with RKS-2-62 and the mice did not exhibit any side effects or weight loss.

Conclusions: RKS-2-62 inhibits neuroblastoma growth both in vitro and in vivo at lower doses than nifurtimox. The mechanism of action involves the production of ROS which induces caspase-3 mediated apoptosis. RKS-2-62 is effective and well tolerated in the mouse model and may be a novel therapy for neuroblastoma.

TR99 Phage Display Technology for Novel Tumor- and Vascular-Targeted Therapies against Neuroblastoma

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Background: Disseminated neuroblastoma (NB) is refractory to most current therapeutic regimens and prognosis remains poor. The lack of chemotherapeutic drugs selectivity causes severe dose-limiting side effects and limits their clinical utility. We recently showed that the therapeutic index of anticancer drugs is increased by liposome encapsulation, and that improvements in the tumor selectivity of liposomes was obtained by coupling tumor-specific antibodies and/or peptides to the surface of the lipidic envelop. Moreover, tumor-associated endothelial and perivascular cells either over-express angiogenic markers or express unique antigens. Targeting cytotoxic drugs to these molecular markers leads to destruction of the tumor vasculature and, indirectly, to tumor cell death.

Methods and Results: Phage display technology, which is a powerful tool in the discovery of ligands specific to receptors on the surface of tumor and tumor endothelial cells, was used *in vivo* to isolate peptides binding specifically to the tumor blood vessel markers aminopeptidase N (APN) and A (APA). APN-targeted, liposome-entrapped doxorubicin (DXR) displayed enhanced anti-tumor effects and prolonged survival in NB-bearing mice. APA-targeted, liposomal DXR, alone, and in combination with APN-targeted formulations, are under investigation for their effectiveness in inducing tumor regression in clinically relevant animal models of human NB. Moreover, a protocol has been established for the isolation of heterogeneous cell populations by tissue fractionation of primary tumor and metastases from orthotopic NB-bearing mice. When the mouse tissues were screened with phage-display peptide libraries, we obtained more than 60 single peptides that bound to both tumor and tumor parenchyma cells; ten of these are being further validated.

Conclusions: The availability of more new ligands that bind to tumor-associated antigens, and to new targets on both endothelial and perivascular tumor cells, will facilitate the design of more sophisticated strategies using ligand-targeted liposomal anticancer drugs that exhibit high levels of selective toxicity for the cancer cells.

TR100 [^{131}I]MIBG Targeted Radiotherapy for NAT-Expressing Tumours: Improving Treatment by Combination with Cytotoxic Drugs

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Background: [^{131}I]meta-iodobenzylguanidine ([^{131}I]MIBG) is an effective single agent for the treatment of neuroblastoma. However, administration of [^{131}I]MIBG in combination with more conventional treatments could increase its effectiveness. Previously, we reported supra-additive toxicity to NAT-expressing cells and xenografts as well as DNA repair inhibition following combinations of [^{131}I]MIBG and topotecan (topoisomerase-I inhibitor). Here, the potential of PARP-1 and 26S proteosome inhibition to enhance the efficacy of [^{131}I]MIBG/topotecan combinations were assessed *in vitro*.

Methods: Combinations of topotecan and PJ34 (PARP-1 inhibitor) were assessed using three treatment schedules: topotecan administered 24h before [i], after [ii] or simultaneously with [iii] PJ34. Using similar scheduling, PJ34/[^{131}I]MIBG combinations, and topotecan/MG132 (26S proteosome inhibitor) combinations were also assessed. Three cell lines were analysed: SK-N-BE(2c) (neuroblastoma), EJ138-CMV/NAT (NAT gene-transfected bladder cancer cell line) and UVW-CMV/NAT (NAT gene-transfected glioma cell line). Cytotoxicity was assessed by clonogenic assay followed by combination-index analysis.

Results: Topotecan/PJ34 combinations: Schedule [iii] delivery induced supra-additive kill of UVW-CMV/NAT, EJ138-CMV/NAT and SK-N-BE(2c) cells. Schedules [i] and [ii] were less effective. PJ34/[^{131}I]MIBG combinations: Supra-additivity was observed in UVW-CMV/NAT cells following schedule [i] (PJ34 administered before [^{131}I]MIBG), but not schedules [ii] or [iii]. Conversely, only schedule [iii] (PJ34 and [^{131}I]MIBG administered simultaneously) induced supra-additivity in EJ138-CMV/NAT. All three schedules induced infra-additive responses in SK-N-BE(2c) cells. Topotecan/MG132 combinations: Schedule [iii] (topotecan and MG132 simultaneously) was most effective at inducing supra-additivity in UVW-CMV/NAT.

Conclusions: Supra-additive responses were achieved in NAT-expressing cells using combinations of topotecan and PJ34, PJ34 and [^{131}I]MIBG and topotecan and MG132, suggesting that combination therapy involving PARP-1 or 26S proteosome inhibition in addition to [^{131}I]MIBG/topotecan may improve the outcome of patients with NAT-expressing tumours. In each case, effectiveness depended on the order of administration. We will soon embark on three-way combinations of topotecan/PJ34/[^{131}I]MIBG, to investigate further the optimal scheduling of combination therapy.

TR101 Prospects of a Survivin-Based Tumor Vaccine in NB

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Background: Chemotherapy-based approaches against NB have reached limits of efficacy and tolerability. Novel T cell engineering approaches have made the first real tests of cell therapies directed against NB possible, but the ability to direct an effector response to NB remains a hurdle. Testing T cell-based therapies requires a relevant antigen and an effector attack against the tumor. MHC class I is frequently absent on NB cells, a potential mechanism of immune evasion. Manipulation of class I expression on NB may, therefore, enhance T cell targeting.

Results: 8/9 HLA-A2+pts with HRNB harbored T cells capable of cytotoxic and interferon-γ (IFNγ) responses directed against the universal tumor antigen survivin. Survivin-specific T cells kill NB, and the immunodominant response to NB by T cells is directed against survivin in CD107a assays. However, despite high-level survivin expression in 26/26 HRNB tumor biopsies we tested, we were unable to detect tumor infiltration by T cells, suggesting immune evasion. Treatment of 5 NB lines with IFNγ significantly increased class I expression in vitro. Using human NB cell lines in a xenograft model, we observed that 3 daily doses of IFNγ successfully upregulated class I in vivo as well. To directly evaluate the influence of enhanced class I expression on the infiltration of T cells into NB tumors, we injected $50x10^6$ CD3/28 expanded human T cells iv into immunodeficient mice with developing flank tumors. Expansion of injected T cells is seen in these mice, after which they received IFNγ and the tumors evaluated by flow and IHC for T cell infiltration and class I upregulation. Increased class I was again detected on NB from IFNγ-treated mice, which correlated with the presence of tumor-infiltrating T cells.

Conclusions: Our data, taken together with successful vaccine responses seen in NB pts in MRD immediately post SCT, suggest a study design where T cell infusions restore cellular immunity, IFNγ is used to upregulate class I and allow T cell targeting, and a survivin peptide vaccine currently in Phase I is used to induce anti-NB immunity. All components of this design are available.

TR102 Enhancing Specificity of Cisplatin to Neuroblasts Employing Evolutionary Microarrays

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Background: Initial treatment for advanced neuroblastoma usually involves chemotherapy. While chemotherapy can be highly effective, the main problems associated with chemotherapeutic agents are the dose-dependent adverse effects occurring primarily through interactions with non-tumour related cells, and the risk of emergent drug resistant tumours. Most chemotherapeutic agents targeting cell division are non-selective. Our research interests are to utilise these cytotoxic substances as a basis to design bespoke anti-neuroblastoma agents. We will be initially focusing on platinum-based chemotherapeutic agents.

Methods: We are currently testing agents based on cisplatin, which are currently used in neuroblastoma chemotherapy, and to change these cytotoxic substances by evolutionary optimization and employing high throughput binding assays to generate enhanced specificity for target tumour (aneuploid) associated gene sequences. Our scheme will identify patient risk categories and reduce adverse effects whilst increasing the potency and dosage within neuroblastoma cells. The region 2p24, including *MYCN*, overall is amplified in 20% of cases, relates to aggressive phenotype and is ideal to target for generating the first cisplatin analogue with enhanced binding specificity to this oncogene-associated chromosomal region.

Results: At present, we have been successful in analyzing the DNA binding profile of the cisplatin molecule, utilizing a bespoke tiling microarray portraying the *MYCN* oncogene coding DNA sequence. Consequently, we have identified the specific regions of the *MYCN* oncogene which bind most avidly to cisplatin. This data is presently employed to generate optimized DNA sequences which possess enhanced cisplatin binding properties.

Conclusions: The outcome of our study will ultimately and rapidly lead to the design of novel platinum-based chemotherapeutic agents with selective binding properties for specific oncogene sequences in the neuroblast.

TR103 Starvation-Dependent Differential Stress Resistance Protects Normal but not Cancer Cells against High Dose Chemotherapy

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Background: Resistance to oxidative stress is observed in yeast cells lacking RAS2 and SCH9, and in worms and mice with reduced activity of IGF-IR homologs. Stress resistance is also observed in model systems subjected to calorie restriction (CR). The role of Ras2 and Sch9 in the negative regulation of antioxidant system together with the association between mutations that activate IGF-IR, Ras or Akt and many cancers prompted us to hypothesize that normal but not cancer cells would respond to starvation or down-regulation of Ras/Akt signalling by entering a stress resistance mode. Here we tested the hypothesis that short-term starvation (STS) or low glucose/low serum can protect mammalian cells but not or less cancer cells against oxidative damage or chemotherapy.

Methods: Primary glia, glioma or neuroblastoma cells were incubated in restriction conditions (0.5 mg/mL glucose and 1% serum) treated with cyclophosphamide and analysed by MTT/LDH assay. Resistance to high dose chemotherapy was evaluated in A/J, CD-1 and athimic mice, starved or not for 48-60 hours, and systemically treated with 80-110 mg/Kg etoposide. For cancer injection studies, A/J mice were injected intravenously with murine neuroblastoma NXS2 cell line, starved for 48 hors and treated with 80 mg/Kg etoposide. Survival time was used as the main criterion for determining the efficacy of each treatment

Results: Low glucose or low serum media protected primary glial cells but not glioma and neuroblastoma cell lines against cyclophosphamide. *In vivo* experiments showed that STS induces complete protection of mice treated with oxidative stress/chemotherapy and provided complete protection to mice but not to injected neuroblastoma cells against a high dose of chemotherapy. Multiple treatments with high dose chemotherapy in combination with STS are now currently investigating.

Conclusions: These studies describe a novel starvation-based strategy to enhance the efficacy of chemotherapy maximizing the differential toxicity to normal and cancer cells.

Poster

TR104 Metabolic Dysregulation Via the Combination of the Novel Glycolysis Inhibitor 3-BrOP and Rapamycin is a Promising Therapeutic Approach for Neuroblastoma

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Background: Neuroblastoma is the most common extra-cranial tumor in children. Half of patients have high-risk disease characterized by rapid tumor growth, resistance to chemotherapy, and high likelihood of metastasis. Many cancer cells exhibit dependence on glycolysis for ATP generation (Warburg effect). We have previously shown that a novel glycolysis inhibitor, 3-BrOP, is effective against glioblastoma, colon carcinoma, and leukemias. We hypothesized that inhibition of glycolysis may be an effective treatment strategy for neuroblastoma, and that combination with the mTOR inhibitor rapamycin would lead to metabolic dysregulation and cell death.

Methods: We determined the IC50 of 3-BrOP by the use of Alamarblue assay (MTT) and cell cycle analysis. The cell lines included SH-SY5Y, IMR-32, SMS-KCNR, and SH-EP cells. Synergy was determined by serial dilution and combination with rapamycin.

Results: All cells were sensitive to 3-BrOP. Single agent IC50s ranged from 17-21mcM, suggesting a uniform effect not dependent on NMYC/1p36 status. At 40mcM of 3-BrOP, viability by MTT was <20% for all cell lines. In combination with 100nM rapamycin, using lower doses of 3-BrOP (25mcM), we found <1% viability in SH-SY5Y and IMR-32 cells. This combination is highly synergistic with a combination index of 0.001 at 12-25mcM of 3-BrOP.

Conclusions: Inhibition of glycolysis via 3-BrOP induces growth arrest and death in human neuroblastoma cell lines. We observed significant synergy with rapamycin, suggesting dysregulation of multiple metabolic pathways is a promising therapeutic approach.

TR105 Optimization of NB Cell Response to Chemotherapeutic Drugs by Combining Epigenetic Modifiers and Various Other Drugs to Increase Caspase-8 Expression and Cell Death – Comprehensive Preclinical Studies

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Background: Loss of expression of the CASP8 gene, which occurs in the majority of NB patients, decreases the responsiveness of NB cells to chemotherapy. Caspase-8 expression can be restored by a variety of agents that alter epigenetic modifications and/or transcription factor expression. Given that multiple clinical protocols using these agents for the treatment of NB are being developed and/or in progress we preformed a systematic study to examine the effects of these compounds as single agents and in combinations on NB cell lines to establish a foundation for future patient treatment protocols.

Methods: A panel of NB cells were treated with multiple concentrations of a variety of agents including 5'aza-2'deoxycytidine, IFN-γ, HDAC inhibitors (valproic acid, SAHA and sodium butyrate) and drugs currently used to treat NB patients (doxorubicin, retinoic acid and topotecan) both as single agents and in all possible combinations to determine the optimal conditions for maximal caspase-8 expression and tumor cell death. The duration of caspase-8 expression and the effects of these treatments on chemotherapeutic responses were monitored by immunoblotting and using apoptotic assays.

Results: Combinations of these agents increased caspase-8 expression in an additive and/or synergistic manner and also increased cell death in response to chemotherapeutic agents. Furthermore, combination therapy provided optimal responses at lower drug concentrations than those need for similar responses with single agent, potentially decreasing detrimental side effects in patients. These studies also allow comparison of the various agents to determine their relative effectiveness in inducing caspase-8 expression and more importantly in inducing NB cell death.

Conclusions: The fact that combining multiple agents that induce caspase-8 expression with chemotherapeutic drugs results in additive or synergistic increases in caspase-8 expression and increased cell death demonstrates that combination therapy represents an attractive therapeutic strategy for the treatment of NB patients.

TR106 Celecoxib Potentiates the Cytotoxic Effect of Retinoids in Neuroblastoma Cells *In Vitro* Independently of COX-2 Expression

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Background: Retinoids induce differentiation and/or apoptosis in neuroblastoma cells *in vitro* and *in vivo* and 13-*cis* retinoic acid is currently used in treatment of children with neuroblastoma. Cyclooxygenase-2 (COX-2) is expressed in neuroblastoma primary tumours and cell lines and inhibitors of COX, non-steroidal anti-inflammatory drugs (NSAIDs), induce apoptosis in human neuroblastoma cells *in vitro* and in established human neuroblastoma xenografts *in vivo*.

Methods: The aim of the present study was to investigate the role of COX-2 in responses to celecoxib and to ask if the COX-2 specific inhibitor celecoxib enhances the cytotoxic effect of retinoids *in vitro*. COX-2 cDNA was cloned into the pcDNA4/TO vector under control of a tetracycline-inducible promoter. This construct was stably transfected into SH-SY5Y neuroblastoma cells expressing the tetracycline repressor. Western blotting verified over-expression of COX-2 in response to the addition of tetracycline and increased levels of prostaglandins in these cells confirmed enzyme activity. Neuroblastoma cell cytotoxicity was measured by XTT and induction of apoptosis was analysed by flow cytometry.

Results: In normal SH-SY5Y cells, treatment with celecoxib significantly potentiated the cytotoxic effect of sub-lethal doses of all-*trans* retinoic acid. This response was not affected by over-expression of COX-2 at similar drug ratios. Using siRNA, a 75% knock-down of COX-2 at the protein level in normal SH-SY5Y cells did not affect sensitivity to celecoxib.

Conclusions: These results suggest that celecoxib may potentiate the anti-tumour effect of retinoic acid currently used in treatment of minimal residual disease in neuroblastoma. Furthermore, the effect of celecoxib seems to be independent of the level of COX-2 protein expression.

TR107 Omega-3 Docosahexaenoic Acid as a Potential Adjuvant to Conventional Neuroblastoma Therapy

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Background: Omega-3 fatty acids have recently been implicated in cancer development, treatment, and prevention. Neural tissue is especially rich in the omega-3 fatty acid docosahexaenoic acid (DHA), but neuroblastoma cells are depleted of it. Among Alaska Natives with an omega-3-rich diet, the neuroblastoma incidence is reduced tenfold. Resolvins and protectins are DHA-generated bioactive products with neuroprotective and anti-inflammatory properties. We have shown that DHA is cytotoxic to neuroblastoma cells and hypothesized that DHA supplementation could be a neuroblastoma therapeutic option, enhance effects of conventional therapy and provide novel means of prevention. Here, we report that DHA acts in synergy with chemotherapeutic drugs and affects neuroblastoma *in vivo*. We also suggest a metabolic pathway for DHA in neuroblastoma cells, and define cytotoxic intermediates.

Methods: Neuroblastoma cells incubated with DHA were investigated for apoptosis, cell proliferation, cell cycle distribution, mitochondrial membrane potential (MMP), production of reactive oxygen species (ROS), secretion of PGE2, and analyzed for resolvins and protectins with LC-MS/MS-based lipidomics. DHA was also combined with chemotherapeutic and other cytotoxic drugs. Nude rats with neuroblastoma xenografts received DHA via enriched diet or oral gavage.

Results: DHA-incubation caused cell cycle arrest and apoptosis by depolarization of MMP, PARP-cleavage, increased ROS and reduced PGE_2. Neuroblastoma cells converted DHA to monohydroperoxy and monohydroxy fatty acids, but not further to resolvins or protectins. The 17-monohydroperoxy-DHA showed the highest cytotoxic potency. DHA enhanced cytotoxic effects of vincristine, etoposide, doxorubicin, NSAIDs and arsenic trioxide. *In vivo*, DHA-supplementation increased plasma and tissue DHA-concentrations, delayed time to tumor establishment and reduced tumor size.

Conclusions: Omega-3 DHA is toxic to neuroblastoma *in vitro* and *in vivo* partly due to conversion of DHA to cytotoxic intermediates of the resolvin and protectin pathway. Combined with chemotherapeutic drugs, DHA enhances their cytotoxic effect. Our data suggest further studies of DHA in neuroblastoma therapy.

TR108 Valproic Acid Enhances Etoposide-Induced Cytotoxicity in Neuroblastoma Cells

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Background: Neuroblastoma is the most common extracranial solid malignancy of childhood. High-risk cases of neuroblastoma have extremely poor long-term survival rates, and novel therapies are needed. Etoposide, a topoisomerase II inhibitor, is used for treatment of neuroblastoma. Valproic acid (VPA) has histone deacetylase inhibitor (HDI) activity. We hypothesized that epigenetic alterations described in neuroblastoma tumors make VPA a good candidate to evaluate for potentiation of etoposide-mediated cytotoxicity.

Methods: Human neuroblastoma cell lines SK-N-AS and SK-N-SH were incubated with 1.5 mM VPA and increasing concentrations of etoposide from $1x10^{-6}$ to 1 mM. Cell viability was measured with MTT assays. Western blots were performed for topoisomerase II levels before and after treatment with VPA.

Results: We observed that VPA and etoposide independently decreased cell viability in a time- and concentration-dependent manner. The combination of both drugs resulted in greatly enhanced cytotoxicity. IC50 values for SK-N-SH cells treated with etoposide were approximately $1x10^{-3}$, $1.8x10^{-4}$, and $3x10^{-5}$mM at 24, 72 and 96 hours, respectively. With the addition of VPA, cell viability was reduced approximately 10 fold at each time point. IC50 values for SK-N-AS cells treated with etoposide were approximately $1.8x10^{-3}$ mM and $6x10^{-4}$ mM at 72 and 96 hours, respectively. With the addition of VPA, IC50 values were reduced approximately 5 fold at the same exposure times. This enhanced cytotoxicity was demonstrated to be synergistic by the fraction product method of Webb formula. Furthermore, VPA treatment resulted in increased expression of topoisomerase II levels in a time- and concentration-dependent manner.

Conclusions: Our results demonstrate that VPA potentiates the cytotoxic effects of etoposide on neuroblastoma cells. Increased levels of topoisomerase II induced by VPA suggest a mechanism for the synergistic effect. This data supports the use of this combination in a phase I trial in patients with neuroblastoma.

TR109 MYCN Amplification is Detectable from Sera of Metastatic Neuroblastoma Patients but Not those with Small Tumor Burdens

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Background: MYCN amplification (MNA) is used to stratify neuroblastoma (NB) treatment worldwide. We showed MYCN DNA sequences can be readily detected at diagnosis in the peripheral blood of NB patients and correlated with MNA (Combaret, 2002, 2005). The aim of this study was to determine whether the detection of MYCN DNA in the serum can reliably predict tumoral MYCN status in infants with reduced tumor burden or with stage 4 or 4S disease, as this may have clinically utility.

Methods: Serum from NB patients at diagnosis was obtained from the COG for 224 patients (208 cases <18-months) and scored blinded using Real-time Q-PCR. A two-sided Fisher's Exact test was performed to compare serum and tumoral MYCN status.

Results: High levels of MYCN were detected in 41 of 57 serum samples from patients with MNA tumors. Importantly, there was no false positive in any of 167 non-MNA patients ($P<0.0001$). Thus, the sensitivity and specificity of the assay was 71.9% and 100%, respectively, in this cohort. In patients with low tumor burden (stages 1 and 2, N=34), those with MNA tumors were poorly detected (1 of 10). In children <18 months with stage 4 NB (N=124, 41 with MNA) sensitivity was 85%. In stage 4S patients (N=66) MYCN was detected in 5 of 6 patients harboring MNA tumors and none of 60 with non-amplified tumors ($p<0.0001$).

Conclusions: Detection of MYCN from patient sera is a non-invasive and specific tool for the determination of tumoral MYCN status. This simple and reproducible assay may represent a safe and reasonably sensitive tool to assess tumoral MYCN status in infants and toddlers with stage 4 or 4S disease in whom tumor material is not readily accessible.

TR110 High Frequency of Minimal Disease Detected in Bone Marrow and Peripheral Blood from Children with High Risk Neuroblastoma by QRT-PCR. A SIOPEN Study

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Background: The clinical utility of detecting minimal disease (MD) in peripheral blood (PB), bone marrow (BM) and peripheral blood stem cell harvest (PBSC) by quantitative reverse transcriptase polymerase chain reaction (QRT-PCR) in children with high risk neuroblastoma entered into the European trial HR-NBL1/SIOPEN is under investigation.

Methods: Samples are collected and analysed according to standard operating procedures (SOPs) (Viprey et al, 2007 Eur J Can. 43: 341-350).

Results: The frequency of tyrosine hydroxylase (TH) mRNA detection by QRT-PCR in BM (87%; 88/101) and PB (89%; 104/117) at diagnosis is higher than anticipated from published literature. This increased frequency of detection is attributed to the use of SOPs for the collection and processing of samples to ensure stabilisation of RNA from clinical samples, and analysis for TH mRNA using QRT-PCR rather than a non-quantitative method. After induction chemotherapy the frequency of TH mRNA detection decreases to 55% (53/96) in BM and 24% (22/91) in PB, suggesting that clearance of neuroblastoma cells from PB is more rapid than from BM. This may in-part reflect the level of disease in BM compared to PB; the level of TH mRNA at diagnosis in BM is 1 000-10 000 times greater than that in PB at the same time. After megatherapy TH mRNA was less frequently detected (BM 33% (14/43), PB 7% (3/44)). Seventeen percent (14/81) of PBSC harvests were positive for TH mRNA.

Conclusions: TH mRNA is detected at high frequency in BM and PB from children with neuroblastoma. QRT-PCR for multiple targets, alone and in combination, is currently being utilised to develop the best model for detection of clinically relevant MD in BM and PB of children with neuroblastoma.

TR111 Methylation Analysis of DCR2 Gene Using Tumor and Serum DNA of Neuroblastoma Patients

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Background: Rapidly progressing neuroblastomas (NBs) have recently been associated with aberrant hypermethylation of the Tumor necrosing factor- related apoptosis-inducing ligand (TRAIL) decoy receptor 2 gene (*DCR2*) promoter. We developed a method to evaluate *DCR2* methylation status in tumor and serum DNA, and assessed its utility as a prognostic factor and indicator of therapeutic efficacy in NB patients.

Methods: Using DNA-based real-time PCR, we evaluated the ratio of a methylated-*DCR2* specific sequence (M) and a reference sequence (R) located in a part of the *DCR2* promoter that is unaffected by methylation in 5 NB cell lines and 53 NBs. Of these patients, 13 had MNA NB, and 40 had non-MNA NB. The association between *DCR2*-methylation and stage was evaluated by Fisher's exact test, and differences of event-free survival (EFS) were evaluated by the Kaplan-Meier method.

Results: *DCR2* aberrant methylation was detected in all 5 NB cell lines and 16 of the 53 tumor samples. *DCR2* methylation was associated with stage both in whole NB group ($n=53$; $p<0.001$) and in non-MNA group, ($n=40$; $p<0.001$), and patients with *DCR2* methylation showed significantly poorer 5-year EFS both in whole NB group (43% vs. 83%; $p=0.002$) and in non-MNA group (34% vs. 96%; $p<0.001$). In 10 patients for which data was available, a strong correlation was observed between the M/R ratios in tumor and serum ($r=0.825$; $p=0.006$). Among 5 *DCR2*-methylated patients whose clinical courses were followed, the M/R ratios in serum fell to undetectable levels in the patients that experienced remission (n=2), whereas they increased to high levels in the relapsed patients (n=3).

Conclusions: Our study indicates that *DCR2* hypermethylation is a useful biomarker for predicting a poor prognosis in NB, especially in non-MNA NB patients. Furthermore, detection of methylated *DCR2* in serum DNA might be an indicator of therapeutic efficacy in *DCR2*-methylated NB patients.

Poster

TR112 p38 Mapk Inhibition Restores Bortezomib-Induced Apoptosis in Resistant Neuroblastoma

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Background: Bortezomib is an inhibitor of the 26S proteasome with antitumor activity against a wide range of malignancies including neuroblastoma (NB). However, the molecular mechanisms by which bortezomib induces cytotoxicity in NB have not been analyzed. The aim of our study was to characterize proteasome inhibition-induced apoptosis.

Methods: The cytotoxic effect of bortezomib and SB202190 (a p38 MAPK inhibitor) on NB cell lines was evaluated using the Uptiblue assay. Apoptotic cells were identified by HOECHST 33258 staining. Caspase activation was determined using the caspase-Glo® 3/7 assay. Analysis of regulators of apoptosis was performed by Western Blot.

Results: Bortezomib induced apoptosis of NB cells by activation of caspases but its effect varied with the NB cell lines tested. Proapoptotic factors (*eg* p53, NOXA, Bad, PUMA, Bax or Bak) and antiapoptotic factors (*eg* Bcl2, Bcl-xl, Mcl-1, XIAP and surviving) were not involved in the differential NB responses to bortezomib. Analysis of heat shock proteins (HSP) (*eg* HSP27, phosphorylated HSP27, HSP70 and HSP90) revealed expression of phosphorylated-HSP27 before bortezomib treatment and its induction after treatment in resistant NB cell lines. Combined exposure to bortezomib and SB202190 led to enhanced apoptosis in NB cell lines defined as resistant to bortezomib. p38 MAPK inhibition prevented phosphorylation of HSP 27.

Conclusion: We have identified resistance mechanisms to bortezomib in NB cells. We have shown that SB202190 enhanced bortezomib-induced apoptosis by preventing phosphorylation of HSP27. Further clinical investigations combining bortezomib with an inhibitor of p38 MAPK are warranted in advanced or refractory NB.

TR113 Plasma Midkine Level is a Prognostic Factor for Human Neuroblastoma

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Background: Neuroblastoma is the third-most-common solid tumor of childhood. To date, no reliable blood marker for neuroblastoma has been established. The growth factor midkine is highly expressed in human carcinomas, and its knockdown leads to tumor growth suppression in animal models.

Methods: We evaluated the plasma midkine level in human neuroblastoma patients. Plasma samples were obtained from patients found through mass screening, as well as from sporadic neuroblastoma patients. The total number of cases examined was 756. Among them, prognostic information was available for 175 sporadic cases and 287 mass screening cases. An enzyme-linked immunoassay for human MK was performed.

Results: Midkine levels were significantly higher in neuroblastoma patients including both mass screening cases and sporadic cases than in non-tumor controls ($p<0.0001$). The midkine level was significantly correlated with the statuses of MYCN amplification, TRKA expression, ploidy, stage and age ($p<0.0001$, <0.0001, $=0.004$, <0.0001 and <0.0001, respectively), which are known prognostic factors for neuroblastoma. There was a striking correlation between high plasma midkine level and poor prognosis ($p<0.0001$). Within sporadic cases, the midkine level was also strikingly higher than in non-tumor controls ($p<0.0001$), and correlated with the statuses of MYCN amplification and stage ($p =0.0005$ and $=0.003$, respectively). There was a significant correlation between high plasma midkine level and poor prognosis ($p=0.04$).

Conclusions: The MK level will be useful in potentially establishing it as a new biomarker for other carcinomas.

TR114 Detection of GD2 Positive Cells in Bone Marrow Samples and Survival of Patients with Localized Neuroblastoma

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Background: an independent, easily applicable, prognostic marker able to identify patients with localized disease that would benefit from a more careful follow-up is currently lacking.

Methods: the impact of GD2 positivity on survival of patients with localized disease has been evaluated together with its combined effect with other known risk factors. Anti GD2 immunocytochemistry was performed at diagnosis in bone marrow samples from 145 Italian children diagnosed with localized NB by INSS criteria. Event-free (EFS) and overall survival (OS) analyses were performed according to the Kaplan-Meier method and compared by the log-rank test.

Results: Nineteen of the 145 patients (13.1%) were found BM GD2-positive with the number of positive cells ranging between 1 and 155 out of $1x10^6$ total cells analyzed. Seven/19 (38.8%) GD2-positive *vs* 12/126 (9.5%) GD2-negative patients relapsed. The 5-year EFS and OS of the GD2-positive patients was significantly worse than that of the GD2-negative ones (62.2% *vs* 89.9%, $P < 0.001$; and 74.9% *vs* 95.9%, $P=0.005$, respectively). GD2 positivity was not associated to other known risk factors, and in particular to *Myc-N* amplification and 1p deletion. Among *Myc-N* negative patients the EFS of those negative for both GD2 and 1p deletion was significantly better than in children positive for either one of these two markers (EFS = 96.9% *vs* 66.0%, $P < 0.001$).

Conclusions: GD2 positivity may represent a prognostic marker for patients with non metastatic NB without *Myc-N* amplification and its combination with genetic alterations might help identifying patients that require a more careful follow-up.

TR115 Notch Signaling Pathway and Calreticulin in Neuroblastoma: Interactions and Prognostic Significance

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Background: Notch signaling cascade and calreticulin (CRT) are related to neuroblastoma (NB) cell differentiation. They may act concordantly in the tumorigenesis of NB. The aims of this study are to define the interactions between Notch signaling and CRT in NB cells, and the prognostic roles of Notch1 expression in NB patients.

Methods: Notch1 expression in NB tumors was examined by immunohistochemical staining and compared according to the clinicopathologic/biologic characters of the patients. The interactions and regulating mechanism between Notch signaling and CRT expression were defined by in vitro cell lines study.

Results: Kaplan-Meier analysis showed that patients with positive or negative Notch1 expression had an overall survival rate of 30.4% and 76.9% ($P < 0.001$), respectively. Patients divided into three groups according to co-expression status of Notch1 and CRT also showed significantly different survival rates: Notch1 negative and CRT positive with 84.9%, Notch1 and CRT double positive or negative with 53.9%, Notch1 positive and CRT negative with 23.1%. The co-expression status of Notch1 and CRT is also an independent prognostic factor in multivariate analysis by Cox proportional hazard mode. In vitro study showed CRT mRNA level increased significantly after DAPT treatment in NB cells and CRT knock-down by siRNA could significantly inhibit DAPT induced neuronal differentiation in NB cells. Using luciferase reporter gene system, luminescence emitted was decreased in proCRT-pGL4.14-transfected N7 cells, in comparison with the pGL4.14-transfected mock N7 cells, and was increased when proCRT-pGL4.14-transfected N7 cells were treated by DAPT for 24 hours, in comparison with these cells treated by DMSO.

Conclusions: Notch1 expression is significantly found in NB tumors and associated to poor outcomes of patients. Co-expression status of Notch1 and CRT could be an independent prognostic factor in patients with NB and may provide additional information to determine more appropriate intensity of therapy. CRT is downstream to Notch signaling in NB cells and Notch signaling regulates CRT expression by altering transcriptions of CRT gene.

TR116 Detection of Tyrosine Hydroxylase and GD2-Synthase by QRT-PCR in Bone Marrow and Peripheral Blood from NB Patients with Localized Disease

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Background: a peripheral blood (PB) prognostic marker able to identify patients with localized disease that would benefit from a more careful follow-up is currently lacking.

Methods: QRT-PCR for tyrosine hydroxylase (TH) and GD2-synthase (GD2-s) were performed in bone marrow (BM) and PB samples from 198 Italian neuroblastoma patients. Relationships between TH and GD2-s $\Delta\Delta$Ct values and agreement between positive and negative cases were evaluated by means of Spearman's correlation (r_s) and the Cohen's kappa (**k**) coefficient, respectively. The relationship between test results at diagnosis and patient outcome was evaluated by the Kaplan Meier method and survival curves compared by log-rank test.

Results: Correlation between TH and GD2-s results was high in stage 4 patients both in BM and PB (r_s 0.97 and 0.86, respectively) but absent in patients with localized disease. Moreover, correlation between BM and PB results were present only in stage 4 patients both for TH and GD2-s assay (r_s 0.61 and 0.58, respectively). Similarly the agreement between positive and negative cases was substantial in stage 4 patients (**k** 0.66 and 0.86 in BM and PB, respectively) but small in patients with localized disease. Finally, agreement between BM and PB results was good for the TH assay regardless of the patient stage, while for the GD2 assay was good only for stage 4 patients. Survival analysis demonstrated that TH positivity, both in BM and PB samples, was associated with worse event-free and overall survivals. Also GD2 positivity in PB was associated to a worse overall survival. However, all these findings were dependent on the higher positivity of the QRT-PCR assays found in stage 4 patients than in patients with localized disease.

Conclusions: TH and GD2-s QRT-PCR assays, performed in either BM or PB samples, do not have any impact on survival of patients with localized disease.

TR117 DCX Expression in Bone Marrow after Induction Correlates Whit Reduced Survival in High-Risk Neuroblastoma Patients

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Background: Detection of minimal residual disease (MRD) in bone marrow (BM) and peripheral blood (PB) is crutial for follow-up in high-risk neuroblastoma patients and might impact on survival.

Methods: Relative quantification of DCX and TH was studied by quantitative reverse transcriptase polymerase chain reaction (QRT-PCR) using Assays on Demand from Applied Biosystems (Oltra et al., 2005, Diagn Mol Pathol. 14: 53-57). We studied DCX and TH expression in 87 high risk neuroblastoma patients (78 stage 4 and 9 stage 3) treated according cooperative national protocols in Spain.

Results: The frequency of DCX and TH detection at diagnosis in BM was 75,3% and 76,7%. After induction chemotherapy the frequency of both markers decreased to 37,8% and 28,9%. In PB samples the frequency of DCX and TH was 58,3% and 50%, respectively, at diagnosis and 12,5% and 15,6% after induction. Only the DCX expression in BM after induction chemotherapy showed a statistically significant predictive value. Five years overall survival (OS) and event-free survival (EFS) were significantly reduced in patients with DCX expression in BM after induction ($p<0,002$).

Conclusions: DCX expression in BM after induction chemotherapy showed a statistically significant impact on OS and EFS in high risk neuroblastoma patients. In contrast, DCX expression in BM or PB at diagnosis did not show a disease prognostic value. TH expression did not show prognostic value in PB and BM at these treatment points.

TR118 Isolated Tumor Cells are Routinely Detected by Immunohistochemistry in Histologically Negative Bone Marrow Biopsies in Patients with Neuroblastoma

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Background: Evaluation of bone marrow (BM) by aspirate and biopsy is a critical component of diagnostic staging and surveillance for recurrent metastatic disease in neuroblastoma. The use of routine immunohistochemical analysis of histologically negative BM biopsies has not been adequately evaluated. In this retrospective study, we identified the frequency in which isolated tumor cells (ITCs) are identified with immunohistochemical methods in histologically negative BM biopsies, and correlated the presence of ITCs with clinical outcome.

Methods: 51 patients with neuroblastoma were identified from the case files of Stanford University. Search of the pathology archives identified 325 BM biopsies from 180 visits. Hematoxylin and eosin (H&E) slides from all tissue biopsies were reviewed and clinical outcome was determined. Immunohistochemical studies using synaptophysin, chromogranin, and beta-catenin were performed on all biopsies. Primary neuroblastoma tumors were used as positive controls. Histologically negative biopsies with ITCs were identified and the frequency of immunoreactivity to histologically unequivocal metastatic neuroblastoma was determined.

Results: 92 individual marrow biopsies demonstrating ≥ 10% tumor involvement by H&E were evaluated by all three antibodies. Beta-catenin demonstrated immunoreactivity in 100%, synaptophysin in 89%, and chromogranin in 71% of the biopsies. Of 220 biopsies which were negative for metastatic disease by H&E, immunohistochemistry identified ITCs 10% of the time. When the bilateral biopsies of each surveillance visit were analyzed together as a single evaluation time point, ITCs were present in 19%. Patients with ITCs developed BM recurrences (31%) more commonly than patients without a history of ITCs (9%) ($p = 0.075$).

Conclusions: Immunohistochemistry using synaptophysin, chromogranin, and beta-catenin can identify ITCs in histologically negative BM biopsies in patients with neuroblastoma. Identification of ITCs may identify patients at increased risk for BM relapse. This is a readily available technique that may be clinically useful in identifying minimal amounts of BM metastases.

TR119 Implications of RARB Expression in Neuroblastoma

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Background: 13-cis-retinoic acid has been shown to improve survival of a subset of high-risk neuroblastoma patients when administered after high-dose chemotherapy or bone marrow transplantation. In this study, we examined the expression of transcripts encoding retinoic acid beta (*RARB*) in a cohort of 93 primary neuroblastomas to gain insight into *RARB* expression at diagnosis and outcome of neuroblastoma patients.

Methods: Quantitative RT-PCR was used to measure *RARB* expression. Differential expression of variables in given subgroups were compared by Student' s t-tests. Survival probabilities in various subgroups were estimated by the method of Kaplan and Meier. Survival distributions were compared using log-rank tests. Cox regression analysis was used to assess the prognostic significance of variables.

Results: The study cohort was verified by using established prognostic markers (age, stage, *MYCN* amplification). *RARB* expression was not predictive of NB disease outcome as a single variable, although low *RARB* expression was significantly associated with *MYCN* amplification ($p=0.0045$). When we combined *RARB* expression (high or low) and age at diagnosis (<1 or >1 year of age), an interesting trend was identified. In the neuroblastoma population over one year of age, those with high *RARB* expression did better than those with low *RARB* expression. This observation was further supported by the fact that these two variables were prognostically independent of each other as shown by the multi-variable Cox regression analysis ($p=0.011$ for age; $p=0.009$ for *RARB*). These results suggest that age and *RARB* expression are additively informative in predicting neuroblastoma disease outcome. Moreover, a similar analysis conducted between *RARB* expression and *MYCN* amplification showed that high *RARB* expression was protective to neuroblastoma patients with *MYCN* amplification.

Conclusion: Screening for neuroblastoma with high *RARB* expression among older patients or those with *MYCN* amplification at diagnosis could identify patients who may respond well to retinoid treatment.

Poster

TR120 Highly Specific And Sensitive Risk Prediction for Appropriate Therapeutic Intensity to Advanced Neuroblastomas

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Background; Even MYCN can detect only a half of NB with the poor outcome. The predictive sensitivity should be improved.

Methods; *Non-mass* 89 advanced NBs were enrolled into this study. The biology was evaluated at diagnosis with all followings, *"MYCN amplification"*, *" INPC histology"* and *"Ha-ras/Trk A expression"*.

Results; (I) Intensity & sensitivity of risk predictors: In 196 advanced NBs, "*MYCN amplification*" was a strong indicator to the aggressiveness ($p<0.0001$). The amplification, however, detected only 42% of NBs with clinical events. "*Unfavorable histology*" and "*Low expression of Ha-ras/trk A* " also indicated poor outcome of the patients ($p=0.007$, $p<0.0001$) but detected only 51%, 53% of the poor outcome, respectively. (II) Combination of the predictors: We classified 61NBs into "high risk" from the 89 advanced NBs according to following three predictors; first, 26 NBs with *"MYCN amplification,* second 23 NBs with "*Unfavorable histology*" from the 63 NBs without the amplification, third, 12 NBs with "*Low expression* of Ha-ras/trk A " from the 40NBs with "favorable histology" and without MYCN amplification. Seventeen NBs were classified into "low risk " , because they had not any of the risk predictors. (III) Sensitivity of the combination: The "high risk" stratification could detect 86% out of 44 deceased patients. Among 52 NBs with the clinical events, 83% could be detected by this procedure. On the other hand, the DFS in the 17 "low risk"NBs was 88%. (IV) Effect of therapeutic intensity to high risk NBs: Among 18 EFS in high risk NBs, 9 (50%) had mega-dose chemotherapy followed by HSCT.

Conclusion; Combination of these conventional predictors has cost advantage for risk evaluation with high clinical sensitivity and specificity. Appropriate therapeutic intensity based on the evaluation at diagnosis should improve the efficacy in high risk NBs but also minimize therapy-related sequelae in low risk NBs.

TR121 Identification of Tyrosine Hydroxilase Cell-Free mRNAs in Plasma Samples from Neuroblastoma Patients

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Background: The identification of tumor specific RNAs in plasma samples may represent an useful tool to monitor the disease status of cancer patients that do not have genetic abnormality in their tumors.

Methods: In this study two real time RT-PCR assays for tyrosine hydroxylase (TH) and GD2 synthase (GD2-s) were used to quantify the amount of NB-specific RNAs present in plasma samples of either NB patients or healthy blood donors as control group. Total amount of RNA present in plasma was quantified by 18S, GAPDH, HPRT1, and B2M qRT-PCR assays. Two-sided Student's t test and Mann-Whitney U test were used to compare measurements between the two groups.

Results: Using conditions that excluded recovery from contaminating cells, cell-free RNA was detected in plasma samples from Patients and Controls, without any significant difference between the two groups. Not surprisingly, the amount of RNA was low and measurable only with the 18S qRT-PCR assay. However, reproducible results from two different aliquots of the same plasma were obtained with this assay. With respect to the NB-specific mRNAs, the difference between Patients and Controls was significant, regardless of the method used to evaluate the results, in the TH but not in the GD2-s assay, confirming the higher specificity of the former marker in blood samples.

Conclusions: By using controlled experimental conditions and a proper NB-specific marker, the isolation of TH mRNAs may represent an useful tool to monitor disease status in neuroblastoma patients.

TR122 Ganglioside Content in Blood Serum of Neuroblastoma Patients

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Gangliosides are glycosphingolipids present on the surface of many eukaryotic cells, especially neural tissues, where they play an important role in cell signalling. During neoplastic transformation, gangliosides are over-expressed in solid tumours, including colorectal cancer, neuroblastoma and melanoma, and released into the peripheral circulation. Gangliosides shed by tumour cells have significance in progression of the disease. They inhibit a number of immune responses both in vitro and in vivo and the ganglioside level in blood serum is correlated with tumour progression in humans.

One of the markers of neuroblastoma is GD2 ganglioside (disialoganglioside), which is abundantly expressed on the surface of neuroblastoma cells. In our study we evaluate the level of gangliosides in blood serum of 30 neuroblastoma patients using high-performance liquid chromatography (HPLC). The technique was used to detect fluorescently labelled oligosaccharides derived from serum glycosphingolipids by enzymatic digestion with ceramide glycanase. HPLC analysis allows for detection of serum gangliosides at the picomole level. The results were confronted with the clinical data of the neuroblastoma patients, including the stage of the disease and the outcome of therapy.

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TR123 Pretreatment of Neuroblastoma Cells with Cytostatic Drug Combinations Does Not Impair Uptake of mIBG , Noradrenaline, and Noradrenaline Transporter Expression

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Background: [^{123}I]mIBG which is taken up by the noradrenaline transporter (NAT) is widely used for initial diagnosis and therapy control of neuroblastoma. Pretreatment of neuroblastoma cells with cytostatic drugs could influence mIBG uptake and therefore falsify mIBG imaging. In this study we investigated whether pretreatment of neuroblastoma cell lines with cytostatic drug combinations (N4-N6) used in the German neuroblastoma protocol influences NAT- expression and the uptake of noradrenaline and mIBG.

Methods: The neuroblastoma cell lines SK-N-SH and Kelly were treated with cytostatic drug combinations related to N4 (doxorubicin, vincristine, 4-hydroperox yifosfamide), N5 (vindesine, etoposide, cisplatin), N6 (vincristine, doxorubicin, 4-hydroperoxyifosfamide, dacarbazine). [^{3}H]noradrenaline and [^{131}I]mIBG uptake was analysed (1) in cells pretreated with these cytostatic combinations for 4 – 72 hours and (2) in cells which had survived former treatment with these drug combinations to 50-80%. Uptake of [^{3}H] noradrenaline and [^{131}I]mIBG was measured in cell culture suspensions. Expression of the noradrenaline transporter (RT-PCR) was analysed under the same conditions as described above.

Results: Pretreatment of SK-N-SH and Kelly cells with the three cytostatic drug combinations for 4 - 72 hours did not impair the uptake of [^{3}H] noradrenaline and [^{131}I]mIBG. Similarly, the uptake of [^{3}H]noradrenaline and [^{131}I]mIBG into the cell fractions that survived pretreatment with these combinations was also not negatively influenced. In accordance with these results, no changes in NAT-expression in cells treated with the cytostatic combinations was observed compared to untreated controls.

Conclusions: There is no evidence that pretreatment of neuroblastoma cells with cytostatic drug combinations used in the German neuroblastoma protocol influences the uptake of radiolabeld mIBG. Therefore, it can be concluded that mIBG is well suitable for correct follow up studies during chemotherapy in neuroblastoma.

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Poster

TR124 Modulation of the Therapeutic Efficacy of Irinotecan by Se-(Methyl)selenocysteine against Human Neuroblastoma Xenografts

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Background: Irinotecan (CPT-11) is highly effective against NB xenografts. However, treatment CPT-11 alone can not completely abolish tumor. It has recently been reported that Se-(methyl)selenocysteine (MSC), a Se-containing compound, possesses anticancer properties. On the other hand, MSC protects normal tissues from toxicity of DNA-damaging drugs in p53-dependent manner. In NBs, p53 is rarely mutated but inactivated in many cases. We evaluated antitumor effects of CPT-11 with or without MSC against three NB xenografts. We further examined the relation between modulation of therapeutic efficacy by MSC and gene expression involved in p53-dependent cell cycle arrest, DNA repair and apoptosis.

Methods: NB xenografts used were two drug-sensitive lines, TNB9, TS-N-5nu, and a multi-drug resistant line, TS-N-2nu. Five different treatment schedules were evaluated: MSC alone at 5 mg/kg for 20 consecutive days, low-dose (5.9mg/kg) CPT-11 ± MSC for 20 consecutive days, and high-dose (59mg/kg) CPT-11 in three doses at 4-day intervals ± MSC for 12 consecutive days. Tumor growth inhibition was evaluated for mean tumor doubling time. Gene expressions of xeroderma pigmentosum group C (XPC), ribonucleotide reductase M2B (p53R2), p21, growth arrest and DNA-damage-inducible 45 (GADD45) and Noxa were analyzed by RT-PCR.

Results: MSC alone had no antitumor activity against any NB xenografts. CPT-11 alone on a low-dose prolonged schedule was more effective than a high-dose intermittent one against TNB9 and TS-N-5nu. In TS-N-2nu, there was no significant growth inhibition between two schedules for CPT-11 alone. Among schedules for CPT-11 with MSC, that of low-dose CPT-11 + MSC against TS-N-5nu was significantly effective than the treatment with CPT-11 alone ($p<0.001$). In TS-N-5nu treated with low-dose CPT-11 + MSC, gene expression of Noxa was highly increased, while gene expressions involved in cell cycle arrest and DNA repair were not induced.

Conclusion: MSC is a possible effective modulator to therapeutic efficacy of CPT-11 against NB.

TR125 [^{18}F]6-FluoroDOPA and [^{18}F]6-Fluorodopamine for PET in Neuroblastoma: Comparative *in vitro* and First *in vivo* Experiments Using the Small Animal PET Model

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Background: [^{18}F]6-FluoroDOPA is frequently used for PET of neuroendocrine tumours. It is incorporated into cells by amino acid transporters. [^{18}F]6-Fluorodopamine (6-FDA) can be taken up more specifically by dopamine-and noradrenaline transporters expressed on pheochromocytoma/ neuroblastoma cells. We developed a method that allows a rapid transformation of [^{18}F]6-FluoroDOPA to [^{18}F]6-Fluorodopamine and analysed comparatively uptake and metabolism of both substances in neuroblastoma cells. The aim is to investigate in the nude mouse model whether [^{18}F]6-FluoroDOPA or [^{18}F]6-Fluorodopamine is better qualified for PET of neuroblastoma.

Methods: [^{18}F]6-FluoroDOPA (6-FDOPA) was synthesised by nucleophilic aromatic subsitution of a nitro-benzaldehyde derivative. [^{18}F]6-Fluorodopamine was generated by enzymatic decarboxylation of [^{18}F]6-FluoroDOPA. Uptake studies of [^{14}C]-labeled DOPA and dopamine, respectively, were carried out using the neuroblastoma cell lines Kelly and SK-N-SH. The metabolism of unlabeled 6-FDOPA and 6-FDA was analysed by HPLC. PET experiments using nude mice were carried out on an analyser from Siemens.

Results: A method was established which allows the conversion of unlabeled or [^{14}C/^{18}F] labeled 6-FDOPA to 6-FDA within 20 minutes. Kinetics of 6-FDOPA to 6-FDA - and DOPA to dopamine transformation proved to be identical. Rapid generation of [^{18}F]6-FDA is necessary due to the short half life time of fluoro-18 (110 minutes). Cell culture studies showed that 6-FDOPA is almost completely converted to 6-FDA immediately after its uptake in neuroblastoma cells. First PET analyses using nude mice indicated the expected distribution of [^{18}F]6-FDOPA and [^{18}F]6-FDA.

Conclusion: A method was established for a rapid generation of [^{18}F]6-FDA from [^{18}F]6-FDOPA. Due to the enzymatic decarboxylation the radiolabeled 6-FDA has the same specific acitivity as 6-FDOPA. This allows a direct comparison of both substances in uptake and PET-studies. Current experiments should clarify which compound is better suitable for PET in neuroblastoma.

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TR126 VIP-Secreting Neuroblastic Tumours: Two Distinct Entities According to the Time at Onset of the Diarrhea

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Background: Neuroblastomas are usually evidenced by a growing tumour-mass. In rare cases however, paraneoplastic syndrome such as opsomyoclonus and watery diarrhoea (WD) are the main symptoms. WD is due to a high secretion of Vasoactive Intestinal Peptide (VIP), and is commonly thought to be linked to a good prognosis. Since few cases have been reported with unfavorable evolution, we questioned the clinical homogeneity within this group of very rare tumours.

Methods: we conducted a national retrospective analysis of all neuroblastic tumours associated with WD and VIP overexpression and/or secretion from 1988. Clinical data, local histopathology and biochemistry were analysed.

Results: 21 patients enrolled the study within the past 15 years. Weight loss was found in 19 and hypokaliema in 15. WD was the first symptom in 16 cases with a mean delay of 3 months before the tumour was diagnosed. 15/16 of these tumours were localized, one exhibited a MYCN amplification. All tumours harboured a differentiated phenotype. No relapse was deplored. WD appeared after the beginning of chemotherapy in 5 patients. 4/5 tumours were metastatic and 2 showed MYCN amplification. One patient died of disease. In 2 cases, histology revealed a differentiating phenotype appeared upon chemotherapy. In all cases, tumour removal was the only efficicient procedure to stop the diarrhea.

Conclusions: VIP-secreting neuroblastomas with WD are rare. Metabolic disorders can be life-threatening and require early tumour removal. Two distinct entities should be discussed. Primary VIPomas are localized diseases with straight differentiatied features and favorable evolution. At the opposite, some more aggressive neuroblastomas secondarily present VIPoma-like features, with WD appearing upon chemotherapy. This evolution might reflect a pro-differentiating effect of the treatment with possible impact on the evolution. However, secondary VIPomas are strongly associated to high risk factors, which should distinguish them from the mostly indolent primary VIPomas.

TR127 Cholinergic Switch might be a Common Process of Differentiation in Neuroblastic Tumours

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Background: TH and DBH, the key enzymes for catecholamines synthesis, are markers of sympathetic differentiation in neural-crest-derived cells. In neuroblastoma, occasional mutations have been described in *PHOX2B*, a key transcription factor of TH and DBH. This prompted us to anlayse the genetic modulations linked to TH and DBH expression, likely to characterize neuroblastomas' differentiation processes.

Methods: RNAs were extracted from 54 primary stroma-poor neuroblastic tumours; 9 harboured differentiating features. Expression analyses were performed on GeneChip ® Affymetrix U2 microarrays. Lists of differentially expressed genes were obtained with SAM tests. Results were confirmed by immunohistochemistries on paraffin-embedded tumours.

Results: Three groups were identified: low TH and DBH (n=5, group I), high TH and DBH (n=43, group II), high TH and low DBH (n=6, group III). *PHOX2B* was highly expressed in the three groups. One 931del5 frameshift was observed in group I and one G299T missense *PHOX2B* in group II, with normal TH and DBH. In group III, no mutation was identified in *PHOX2B*. Interestingly, the two genes the most correlated to *DBH* expression were transcription factors *GATA2* and *AP2ß*, both implied in sympathetic differentiation. In group III, neuroblasts widely displayed a differentiated phenotype. Genes related to cholinergic features (*VIP*, *VACHT*, *ACHE*) and receptors *RET* and *IL13R* were more strongly expressed in group III than in group II. In vitro, cells cultured in GDNF and LIF enriched medium displayed significant morphological changes suggestive of differentiating effects.

Conclusions: Our results illustrate that regulation of *TH* and *DBH* expression in neuroblastoma is not strictly dependent upon *PHO2XB*. Furthermore, cholinergic switch with *VACHT*, *ACHE* and *RET* overexpression may be a common process of differentiation. A complete cholinergic switch is achieved in differentiated tumours exhibiting high *VIP* expression and dramatic decrease in *DBH*, for which down-regulation of *GATA2* might play a determinant role.

Poster

Poster Discussion: Clinical Study

C24 Phase I Study of the Combination of Anti-GD2 Antibody 3F8 and Yeast-Derived (1→3), (1→6)-β-D-Glucan for Resistant Neuroblastoma

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Background: Beta glucans, complex, naturally-occurring polysaccharides prime leucocyte dectin and CR3 receptors and enhance anti-tumor cytotoxicity of complement-activating monoclonal antibodies (Clin Cancer Res 8:1217). We conducted a phase I study to determine the safety of the combination of soluble yeast-derived (1→3), (1→6) -β-D-Glucan (SBG) and anti-GD2 murine monoclonal antibody 3F8 in patients with resistant neuroblastoma (NB).

Methods: Heavily pre-treated patients with recurrent or refractory stage 4 NB were treated with 3F8/SBG. Each cycle consisted of oral SBG dose-escalated from 10 to 140mg/kg/day x 17 days in seven cohorts of 3-6 patients each plus intravenous (IV) 3F8 at a fixed dose of 10mg/m²/day x 10 days. Patients without human anti-mouse antibody (HAMA) could be re-treated up to a total of four cycles.

Results: Thirty patients completed 76 cycles of therapy with 3F8/SBG. All patients were evaluated for toxicity and 28 for response. Maximum tolerated dose for SBG was not reached. One patient developed dose-limiting toxicity (DLT) at SBG dose of 120mg/kg/day: transient self-limiting hepatic transaminase elevation observed five days after starting SBG but before 3F8 administration. There were no other >grade 2 therapy-related toxicities. 9, 5, 3 and 12 patients completed one, two, three and four cycles respectively. Reasons for withdrawal in patients who did not complete 4 cycles were PD in 12, persistently elevated HAMA in 3, DLT in 1; one patient was lost to follow up. Based on INRC, there were 1 CR , 1 PR, 12 SD and 14 PD. 5/22 patients demonstrated improvement in skeletal MIBG. Complete remission of NB in bone marrow was observed in 3/5 patients. Responses did not correlate with SBG dose.

Conclusions: 3F8/SBG is well tolerated and shows activity against resistant NB. We plan to escalate the dose of BG to 200mg/kg/day prior to incorporating it in a phase II study.

C25 Neuroblastoma in Adolescents and Adults

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Background: Neuroblastoma in adolescents and young adults is a particular rare disease and may have special biological features. Presentation, course of the disease and outcome in this group of patients was investigated.

Methods: Data of adolescents (age at diagnosis 12 - <18 years) and adults (18 - <30 years) were compared to children (2 - <12 years).

Results: Of 2505 patients registered between 1990-2006, 67 were older than 12 years (2.7%) including 39 adolescents and 28 adults. 11 patients had stage 1, 11 stage 2, 11 stage 3 and 34 stage 4. The clinical presentation was very similar in all groups, however MYCN amplification (MNA) was found in only 2/52 ≥12 year patients (3.8%, both stage 4) and 1p aberrations without MNA in 8/36 patients (22%).
Localized disease was more frequent in adolescents (49%; 19/39) and adults (50%; 14/28) than in children (38%; p=0.09). The prognosis of stage 1-3 disease in adults was inferior (3-y-EFS 0.55+/-0.14) compared to adolescents (3-y-EFS 0.74+/-0.10; p=0.04) and to children (n=344; 3-y-EFS 0.73+/-0.03; p=0.003). Seven adolescents and eight adults experienced relapses, seven patients died. First relapses were localized in 13 patients and metastatic in two. Nine patients presented multiple locoregional recurrences over a long period of time, finally with the development of metastases in seven patients. Median time from first relapse to death was 6.1 years compared to 0.67 years in children (p=0.002).
Stage 4 disease was diagnosed in 20/39 adolescents and 14/28 adults. 30 experienced relapses and 25 died. The outcome of older patients (3-y-EFS: 0.10+/-0.06) was significantly worse compared to children (n=557, 0.33+/-0.02, p=0.02).

Conclusions: The prognosis of adolescents and young adults is poor due to molecular inconspicuous high stages and treatment resistant progression. In localized disease, tumor regrowth is slow, but often fatal.

C26 Determining the Prognosis of MYCN-Amplified Neuroblastoma in Infants Based on Results of the Japanese Infantile Neuroblastoma Cooperative Study

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Background: Infants with *MYCN*-amplified neuroblastoma are rare. How *MYCN* is related to the prognosis of infantile cases is not clear. Therefore, we assessed *MYCN*-amplified cases into prognostic factors, in order to choose the appropriate treatment for these cases.

Patients and Methods: 619 patients were treated in a Japanese prospective study from 1994 to 2004, and were examined for *MYCN* amplification (MNA). The Kaplan-Meier product limit method and the log-rank test were used to estimate the 5-year overall survival rates (OS) and to compare OS probabilities between subgroups. Furthermore, we examined the content of treatment in *MYCN*-amplified cases.

Results: The 29 *MYCN*-amplified cases (OS 68.8%) had poorer clinical outcomes than the non-amplified 590 cases (OS 99.2%, p<0.001). Of the 29 cases, 6 cases with Unfavorable Histology (UH) had poorer prognoses than 15 cases with Favorable Histology (FH) (OS 25.0%vs.92.9%; p=0.004). The OS of 10 cases with diploid tumor (48.0%) was not different from that of 5 cases with hyper-diploid tumors (50.0%; p=0.446). OS in the progressed cases (stages 3 and 4) (36.5%, n=13) was poorer than that in the early cases (stages 1, 2, and 4s) (100%, n=16) (p=0.001).In the early cases, most cases with less than 9 times MNA received only tumor resection, while most cases with more than 10 times MNA received stem cell transplantation. In the progressed cases, 7 cases received stem cell transplantation and 6 cases radiation therapy, excluding six cases without completing the treatment.

Conclusion: The *MYCN*-amplified cases with UH or with progressed stages, belong to an extremely high risk group, and further intensive treatment is necessary. The early cases with less than 9 times MNA had an excellent prognosis as a result of intensive treatment. Further studies are needed to determine whether such patients should be given reduced treatments.

C27 High-dose Carboplatin-Irinotecan-Temozolomide (HD-CIT) for Resistant Neuroblastoma (NB)

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Background: We retrospectively reviewed patients (pts) receiving salvage therapy for refractory or relapsed high-risk NB, focusing on a novel high-dose combination.

Methods: HD-CIT comprised carboplatin (500 mg/m²/day, x2)-irinotecan (50 mg/m²/day, x5)-temozolomide (250 mg/m²/day, x5). Only one course was planned. Peripheral blood stem cells (PBSCs) were infused after HD-CIT if bone marrow (BM) reserve was poor.

Results: Among 26 NB pts, non-hematologic toxicity was limited to readily manageable diarrhea and transient hepatotoxicity (grade 3 or 4 in 4 pts). The 15 pts who did not receive PBSCs had prolonged myelosuppression, with the ANC reaching 500/μL on day 20-31 (median, 25). Evidence of anti-NB activity against primary (n=8) or secondary (n=8) refractory NB included complete response (CR) by ^{123}I-metaiodobenzylguanidine (MIBG) scan ± BM histology (n=3); CR in BM with normal or unchanged minimally abnormal MIBG scan n=3); and improved MIBG scan (n=1). Among the eight pts treated for progressive disease (PD), three had stable disease, one had continued PD, and one each had partial response (by BM and MIBG findings), CR of MIBG osteomedullary findings (but stable soft tissue disease), and improved MIBG scan. HD-CIT was used for consolidation of second CR in three pts: two remain relapse-free with short follow-up, one received further consolidative therapy and remained relapse-free but died of viral infection at 15 months.

Conclusions: HD-CIT may have clinical utility as consolidative or retrieval therapy because of anti-NB activity, modest extra-medullary toxicity, and lack of need for stem-cell support.

C28 High-Dose Cyclophosphamide-Topotecan-Vincristine (HD-CTV): Utility for a Broad Spectrum of Resistant Neuroblastoma (NB)

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Background: We retrospectively studied a novel high-dose chemotherapy regimen, devised for patients with high-risk NB who are undergoing induction or receiving therapy for resistant disease.

Methods: HD-CTV included cyclophosphamide (70 mg/kg/day, x2)-topotecan (2 mg/m^2/day, x4)-vincristine (0.067 mg/kg). Only one or two courses were planned, aiming for treatment intensification, avoidance of cardio-, nephro-, and oto-toxicity, and prevention of human anti-mouse antibody (HAMA) with subsequent antibody therapy.

Results: Patients received one (n=75) or two courses (n=51). The only common major toxicity was myelosuppression; blood-borne infections were documented in 28 (16%) of 173 courses. 93 patients were evaluable for response (International NB Response Criteria) (Table 1). Among 49 patients treated for primary refractory NB, 17 had disease regression. HD-CTV used as initial salvage therapy achieved disease regression in 12/15 patients treated in first relapse, and in 2/6 treated in >2nd relapse. Among 28 patients already receiving salvage therapy for first (n=25) or >2nd (n=3) relapse when treated with HD-CTV, disease regression was noted in 7/11 evaluable for response. Disease regression was seen in 1/11 patients who received HD-CTV for progressive disease that developed during induction. HD-CTV prevents early HAMA, as previously reported (Clin Cancer Res 10:84, 2004; Pediatr Blood & Cancer 48:430, 2007)

Table 1:

		Response				
Disease status	No. of evaluable patients*	CR/VGPR	PR	MR	NR	PD
Primary refractory	49	6	0	12	31	0
New relapse	21	3	6	5	6	1
On salvage therapy	11*	1	2	4	4	0
PD on therapy	12	0	0	1	4	7

CR/VGPR, complete/very good partial remission; MR, mixed response; NR, no response; PD, progressive disease; PR, partial response
** not included are 16 patients treated in first CR/VGPR and 17 patients treated in 2nd CR/VGPR*

Conclusions: HD-CTV spares vital organs and has anti-NB activity, especially as initial salvage therapy for a new relapse.

C29 Clinical Features of Neuroblastoma in Patients with Opsoclonus Myoclonus Syndrome

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Background: Neuroblastoma tumors in patients with opsoclonus myoclonus syndrome (OMS) have been reported often as small, with differentiated histology, and lack of mIBG uptake. We reevaluated this hypothesis in a large cohort of patients with neuroblastoma and OMS.

Methods: The clinical data of patients with OMS registered in the German Cooperative Neuroblastoma Trials were compared to the data of patients with neuroblastoma, but without OMS.

Results: Out of 2561 patients with neuroblastoma registered since 1990, 44 were reported with OMS (1,7%). INSS stage 1 neuroblastoma was diagnosed in 20/44 patients with OMS (48% vs. 21% of the control group) and stage 4 in 2/44 (5% vs. 37%, p<0.001). Median age of patients with OMS was higher (19 vs. 15 months, p=0.02).
Tumors in OMS patients had a lower volume (median 17 vs. 75 ml, p<0.001) and were more often found in the paravertebral regions (80%), while 51% of the neuroblastoma without OMS were located in the adrenal (p<0.001). In correspondence to the lower tumor volume, catecholamine metabolites were elevated only in 39% of patients with OMS, but in 79% of patients without OMS (p<0.001). No difference between the groups was seen regarding mIBG uptake (83% vs. 88%, p=0.65). OMS tumors often showed more differentiated histology (ganglioneuroblastoma; 42% vs. 20%, p=0.003) and more rarely unfavourable molecular marker (MYCN: 0/38; 1p imbalance: 1/28 patients with OMS investigated).
Overall survival of patients with OMS was excellent (3-y-OS: 0.97+/-0.03 vs. 0.77+/-0.01, p=0.01).

Conclusions: Neuroblastoma tumors in patients with OMS were smaller, more often differentiated and mostly located in the paravertebral regions. mIBG uptake was not different compared to patients without OMS, justifying the regular use of mIBG scintigraphy to search for neuroblastoma in patients with OMS.

C30 Bone Marrow Infiltration at Diagnosis and after Chemotherapy as Early Prognosis Marker in Neuroblastoma Stage 4

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Background: The value of the amount of bone marrow infiltration at diagnosis and after chemotherapy is not established in patients with stage 4 neuroblastoma.

Methods: Bone marrow aspirates of 219 stage 4 neuroblastoma patients were collected from 4 sites and investigated by cytology (CY) and GD2 immunocytology (IC) according to INRG criteria. The mean observation time of the patients was 6.0 years.

Results: At diagnosis 85.8% of patients demonstrated cytological and 84.0% immunocytological bone marrow infiltration. 34.7% of patients had bone marrow infiltration >30%, 14.6% 10-30%, 22.4% 1-10%, 16.4% <1%, and 11.9% none. CY and IC were discordant in 14 patients (CY+IC- n=9; CY-IC+ n=5). Bone marrow infiltration was prognostically relevant qualitatively (CY+ vs. CY- 5yEFS 33±4% vs. 63±9%, p=0.001, 5yOS 41±4% vs. 76±8%, p=0.001) and quantitatively (no infiltration: 5yEFS 61±10%, 5yOS 72±9%, infiltration <10%: 5yEFS 48±6%, 5yOS 60±6%, infiltration >10% 5yEFS 23±4%, p<0.001, 5yOS 29±4%, p<0.001).

The bone marrow response to 4-6 cycles of NB97 chemotherapy was excellent: Residual neuroblastoma cells were detected by CY in 21.5% of patients and by IC in 24.8%. Discordant results at response were seen in 19 patients (CY+IC- n=8; CY-IC+:11). The outcome was similar for CY+ vs. CY- patients (EFS p=0.895, OS p=0.325) and IC+ vs. IC- (EFS p=0.982, OS p=0.616). MRD (<1%) after chemotherapy was prognostically irrelevant. mIBG scintigraphy was still abnormal at metastatic sites in 46.8% and catecholamines remained elevated in 27.3% of patients. Both were not discriminative in this group of patients.

Conclusions: The presence and amount of initial bone marrow infiltration is prognostically relevant, but not the bone marrow response including presence of MRD. Due to high chemosensitivity, the bone marrow site does not appear to determine the course of the disease. In the clinical setting, bone marrow assessment by cytology and immunocytology is equally useful.

C31 An Optimality Criterion for Prognostic Risk Groups in Neuroblastoma and Hodgkin Lymphoma: An Analysis of Data from the Children's Oncology Group

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Background: Physicians who treat cancer often attempt to identify patient subgroups that are homogeneous in their chance of recurrence or death as a way to target the more toxic and presumably more effective treatments to those with the worst prognosis. However, prognosis-based treatment assignment in pediatric cancer has not to date been based on a quantitative assessment of the risks and benefits of different treatment strategies, or on morbidity and efficacy outcome measures that are relevant to children.

Methods: We perform a quantitative analysis of the risk and benefits of prognosis-based treatment assignment in two examples from the Children's Oncology Group using a mathematical model of cancer cure and permanent treatment morbidity, and define an optimality criterion for assigning treatments to specific risk groups.

Results: In stage 4 MYCN unamplified neuroblastoma, age-based risk grouping distinguishes clearly between patients with high and low risk of recurrence. However, our analysis suggests the optimal age cutpoint depends profoundly on the morbidity of the treatments being considered, and agrees with current published recommendations only for treatments that add significant morbidity. In Hodgkin lymphoma, under our model, no clearly optimal risk groupings exist, and a compelling quantitative rationale for defining risks groups at all may not exist.

Conclusions: Our analysis illustrates the inadequacy of naïve application of statistical criteria for defining prognostic risk groups in pediatric cancer, and highlights the importance of quantifying treatment morbidity when defining risk groups or in deciding whether risk grouping is warranted.

Poster

C32 Outcome of Children with Relapsing or Resistant Neuroblastoma. A 25 Year Experience in Italy

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Background: Prognosis of children with neuroblastoma after disease progression or relapse is poor. However, there is scarce information on factors affecting the chance of cure and length of survival after the event has occurred.

Patients and Methods: The clinical course of children with neuroblastoma enrolled in the Italian Neuroblastoma Registry between 12.1979 and 12.2004 who developed disease progression or relapse was investigated. The main clinical and biological characteristics at diagnosis, time interval between diagnosis and event occurrence, type of event and era of diagnosis were taken into consideration for the analysis.

Results: Of a total of 1925 children enrolled, 781 experienced tumor progression (424 cases; 54%) or relapse (347 cases; 46%). The 10-year overall survival (OS) rate after occurrence of the event was 6.8% (SE = .01) in case of progression and 14.4% (SE = .01) in case of relapse. Median time to death was 3.5 months in case of tumor progression, and 10.4 months after relapse. Univariate analysis showed that for both progression and relapse OS was significantly better in relation age less than 18 months, stage 1,2 and 4s, non abdominal site of the primary, normal LDH serum level and *MYCN* gene status. An apparent better survival was shown for children diagnosed in the most recent era, but their follow-up is short.

Conclusions: Survival after progression or relapse in NB patients is poor. As expected, patients with favorable risk factors at diagnosis have a better outcome. New risk adapted salvage protocols are needed.

C33 Poor Neurological Outcome in Children with Opsoclonus-Myoclonus Syndrome with or without Neuroblastoma

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Background : Opsoclonus-myoclonus syndrome (OMS) is a rare paraneoplastic syndrome, characterized by ataxia, myoclonus, opsoclonus, and behavioral changes. A neuroblastoma (NB) is found in 50-80% of children with OMS, and in the remaining cases an unidentified NB may have been present.

Methods : In order to compare the clinical presentation, treatment and outcome of children with NB-positive and NB-negative OMS, we have performed a retrospective study of 33 children treated for OMS in two major pediatric neurology centers between 1988 and 2005.

Results : Twenty-two patients had NB-positive OMS (median age at diagnosis 17,6 months; range 9–33 months). In all cases, the NB had good biological and clinical prognostic features. Oncological treatment consisted of surgery for all, and chemotherapy for 6 patients, resulting in complete remission in 20, and partial remission in 2 patients, without any relapses. Neurological treatment was heterogeneous: one patient's OMS recovered spontaneously following surgery, whereas the other 21 patients received corticosteroids (median duration 24 months; range 7 days–8 years). Nine patients also received immunoglobulins, two received rituximab, and one was treated with cyclophosphamide. Eleven children had OMS without a NB having been detected (median age 22,1 months; range 7–24 months). Treatment consisted of corticosteroids for all patients, immunoglobulins for 2, and cyclophosphamide in 1 patient. Of the 21 patients with NB-positive or NB-negative OMS with a follow-up over two years, 11 have recovered completely, 7 have motor, praxic and/or language sequelae, and 3 have persistent ataxia. No difference in neurological outcome was observed between the two groups of patients.

Conclusion : In both NB-positive and NB-negative OMS neurological outcome is poor. Because of the rarity of this disease, international collaboration is required in order to improve the knowledge of OMS in both patient groups in a prospective trial with a homogeneous treatment strategy.

C34 The Prognostic Impact of Persisting 123I-MIBG Uptake in Patients with Stage 4 Neuroblastoma >1 Year

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Background: 123I-meta-iodobenzylguanidine (MIBG) scintigraphy is well established for staging and re-staging of children with neuroblastoma. The aim of this study was to correlate the persistence of any MIBG positive tumor tissue during and after induction chemotherapy with event-free survival (EFS) and overall survival (OS) of patients.

Methods: We included 113 patients >1 year with MIBG positive stage 4 neuroblastoma with MIBG scintigraphy repeated exactly after four and six cycles of induction therapy. After induction chemotherapy, the patients either underwent stem cell transplantation or oral maintance chemotherapy in the randomized trial. The final outcome was analyzed according to the MIBG status of primary tumor and metastasis using univariate logrank test and multivariate Cox regression analysis.

Results: After four cycles of induction chemotherapy, 71% of patients were still MIBG positive for the primary tumor and 61% were MIBG positive for metastases. After six courses, 39% still had MIBG uptake by the primary tumor and 45% had residual metastatic MIBG positive disease. The MIBG status of the primary tumor site after four (EFS p=0.790, OS p=0.847) and six induction chemotherapy courses (EFS p=0.638, OS p=0.753) had no impact on outcome. Residual MIBG positive metastatic disease after four cycles induction chemotherapy was associated with poor outcome (3-year-EFS 28+/-5% vs. 56+/-8%, p=0.009; 3-year-OS 50+/-6% vs. 65+/-7%, p=0.021) as well as residual MIBG positive metastatic disease after six cycles (3-year-EFS 28+/-6% vs. 47+/-6%, p=0.011; 3-year-OS 50+/-7% vs. 60+/-6%, p=0.031). Multivariate analysis confirmed an independent impact of residual metastatic MIBG uptake after four and six cycles on EFS and OS.

Conclusions: Metabolic imaging with MIBG scintigraphy can identify poor responders by detection of persistent metastatic MIBG uptake The MIBG response of the primary tumor site had no impact on the outcome.

C35 Disease Outcome May Be Predicted by Molecular Detection of Minimal Residual Disease in Bone Marrow in Stage 4 Neuroblastoma

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Background: RT-PCR for tyrosine hydroxylase (TH) is a highly sensitive method to detect minimal residual disease (MRD) in bone marrow (BM) in neuroblasotma patients. We have determined whether sequential RT-PCR could predict the outcome of patients with advanced neuroblastoma.

Methods: Bone marrow samples from 39 patients with stage 4 neuroblastoma treated in 10 institutes of Tokai Pediatric Oncology Group were sequentially examined for tumor cell contamination by detecting TH mRNA using RT-PCR. All patients received repetitive multi-agent chemotherapy including cisplatin, cyclophosphamide or ifosphamide, pirarubicin, and etoposide or vincristine. Successively, surgical resection of primary tumor was performed. Thirtyseven patients received myeloablative therapy followed by hematopoietic stem cell transplantation after achieving complete response or good partial response. Follow-up period of survivors ranged from 27 to 191 months (median 89 months).

Results: All BM samples at diagnosis except one were cytologically positive for neuroblastoma cells, and the follow-up samples became negative for neuroblastoma cells within 3 months by cytologic examination. Out of 14 patients whose BM samples became negative for TH mRNA within 4 months after the start of chemotherapy, 10 patients remained alive without evidence of disease (71% of disease free survival at 5 years). In contrast, out of 25 patients whose BM samples remained positive at 4 months after the start of chemotherapy, 22 patients developed relapse and 19 patients died from disease (16% of disease free survival at 5 years). There was a statistically significant difference in disease free survival between the two groups (p = 0.002). There was no significant difference of clinical characteristics by the positivity of MRD at 4 months after the start of chemotherapy.

Conclusions: Persistence of MRD in BM may predict poor outcome in advanced neuroblastoma.

C36 Glivec and Advanced Neuroblastoma

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Background: Glivec (Imatinib Mesylate) inhibits cell growth and induces apoptosis in several tumoral cell lines, including Neuroblastoma, both in vitro and in animal models. Thus, we designed a multicentre phase II study to assess the possible antineoplastic activity of Glivec in patients with Neuroblastoma.

Methods: We included patients with stage 4 Neuroblastoma who did not achieve complete-very good partial response after first or second line treatment. Glivec was administered orally at a starting dose of 340 mg/sqm/day for 4 weeks. If no major toxicity occurred, the dose was increased to 600 mg/sqm/day. The maximum daily dose was set at 800 mg/day.

Results: Between March 2006 - January 2008, 9 patients (5 males, 4 females) were enrolled. One patient with residual disease after first line treatment is in therapy with partial response after 2 months of Glivec.
Two patients had untreated relapse: one achieved complete response and is alive after 12 months of treatment and 4 off therapy; the other one reached stable disease and underwent radiotherapy after 6 cycles of Glivec.
Two patients were resistant after second line treatment; one has been receiving treatment for 8 months and obtained a partial response, while the other one died after progression during the third cycle.
Four patients were resistant relapsers after second line therapies: all progressed.
One patient had headache and one had pain in both legs which improved by reducing the dose.

Conclusions: Preliminary data seem to suggest that some clinical benefits could be had in patients with residual disease after chemotherapy or "initial", not overwhelming relapse.

C37 Intra-Ommaya ^{124}I-8H9 Positron Emission Tomography (PET) for Detection and Dosimetry of Neuroblastoma Metastases in the Central Nervous System (CNS)

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Background: Radioimmunoconjugates have potential for imaging and treatment of CNS tumors when delivered intrathecally. 8H9 is a murine IgG1 monoclonal antibody specific for 4Ig-B7H3, a 58 kD cell surface antigen homogeneously distributed on a broad spectrum of tumors. 8H9 can be radiolabeled with ^{124}I or ^{131}I, retaining immunoreactivity. We investigated the use of intra-Ommaya ^{124}I-8H9 for quantitative PET imaging and dosimetry prior to therapy with ^{131}I -8H9 in patients with CNS metastases including neuroblastoma.

Methods: Five patients with 8H9-reactive CNS tumors (choroid plexus carcinoma [1], metastatic rhabdomyosarcoma[2], and neuroblastoma[2] were studied. 8H9 was labeled with ^{124}I by the iodogen method. 1.7 to 2 mCi ^{124}I -8H9 per patient was injected. Three consecutive PET/CT scans were obtained at approximately 4, 24, and 48 hours post-injection. Regions of interest and time-activity curves were drawn over the cranial-spinal axis. Dosimetry estimates based on counts per minute were obtained by serial cerebrospinal fluid (CSF) samplings over the same 48 hour period.

Results: All ^{124}I-8H9 injections were well tolerated. PET scans demonstrated high resolution images of the distribution of ^{124}I-8H9 throughout the thecal space. Relatively close agreement was demonstrated between PET dosimetry estimates (range 10.3 cGy/mCi in the lumbar spine – 105 cGy/mCi in the ventricles) and the mean dose calculated by serial CSF sampling in ^{131}I-8H9 injections (range 12.8-63.9 cGy/mCi). When compared to magnetic resonance imaging, ^{124}I -8H9 successfully targeted known sites of active neuroblastoma.

Conclusion: This is the first human study of anti-B7H3 antibody injected into the CSF, labeled with ^{124}I and ^{131}I for dosimetry and therapy of CNS metastases. ^{124}I-8H9 PET/CT provides higher resolution and contrast images than SPECT with ^{131}I-8H9 for distribution, targeting and dosimetry. ^{124}I-8H9/PET is a promising tool that may aid in the treatment planning for radioimmunotherapy trials for CNS malignancies.

C38 To Treat (with Cytotoxics) or not to Treat: The Major Challenge Managing Patients with Neuroblastic Tumors

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Background: Neuroblastic tumors (NBT) are notable for their bipolar natural history. The goal of NBT treatment is to prescribe surgery alone for low-risk disease and cytotoxic therapy for progressing tumors. We report the experience of a referral center using a "therapy versus non-therapy" approach for NBTs.

Methods: Thirty-eight, new, consecutive NBT patients were managed at HSJD from 2002 to 2007 using a risk stratification criteria based on INSS stage, age, and biologic markers (*MYCN*, ploidy, and histopathology). Clinicobiological evaluation included thorough imaging tests (PET/CT and MIBG), molecular diagnosis (GD2 and MYCN) and minimal disease work-up (GD2 synthase). The (cytotoxic) therapy group included stage 4, and MYCN amplified or diploid locoregional (LR) tumors. The minimal (1 cycle if major clinical problem) or no-cytotoxic therapy group included the rest.

Results: Fifteen (40%) cases including 1 stage 1, 5 stage 3 and 9 stage 4 were treated with cytotoxics. All tumors were diploid and 4 (28%) MYCN amplified. Four patients were <18 months of age. All patients received 5 cycles of MSKCC N-7 chemotherapy and surgery. Radiotherapy was administered to all but 2 patients. Consolidation was carried out using 3F8 immunotherapy for patients achieving minimal residual disease status. Two stage 4 patients had primary refractory disease and died. Three patients relapsed and died of disease. Overall, 10 (66%) of 15 patients are alive, median follow-up 19 months.
Twenty-three (60%) cases, including 3 congenital non-biopsied adrenal lesions, 4 stage 4s, 10 stage 1, 2 stage 2 and 4 stage 3 cases, were managed without cytotoxic therapy. Two stage 4s received 1 cycle of chemotherapy to relief compression. None have progressed or relapsed, median follow-up 34 months.

Conclusions: In our experience, using a comprehensive clinico-biological risk assessment, 60% of NBT patients have low-risk NBT that can be managed with minimal or no- cytotoxic therapy.

C39 Sequential High Dose Chemotherapy (HDC) with Autologous Stem Cell Transplantation (ASCT) in Neuroblastoma Patients with a Poor Response to Induction Conventional Chemotherapy

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Background: Response to induction chemotherapy is a major prognostic factor in patients treated for a high risk neuroblastoma. We developed a programme of sequential HDC followed by ASCT in poor responders to induction chemotherapy

Methods: We present herewith the results in terms of toxicity and survival of 19 consecutive patients treated between 1994 and 2006 according to this strategy. Eight patients were planned to receive 2 courses of Melphalan (100mg/m²) and one course of Busulfan (600mg/m²)-Melphalan (140 mg/m²) (Bu-Mel), five patients 2 courses of Thiotepa (600mg/m²) and one course of Bu-Mel and six patients 1 course of Thiotepa (900mg/m²) and one course of Bu-Mel. Each course was followed by peripheral stem-cell transplantation with at least 3x106 CD34/kg.

Results: No major toxicity was observed after Melphalan or Thiotepa courses. Main toxicity was observed after the last Bu-Mel course and consisted in digestive toxicity (9 patients), hepatic veno-occlusive disease (10 patients) and pulmonary toxicity (3 patients) (alveolar hemorrage). Two patients died of treatment-related early complications (multi-organ failure).
Ten patients died of disease progression. Seven patients are alive with a median follow up of 6 (1 -13) years from ASCT. Long term complications consisted in Busulfan related ovarian failure.

Conclusions: Survival of these patients leads to consider sequential HDC strategy for very poor risk patients. The high toxicity observed should be improved in the future by the use of IV Busulfan and may be in association with Prociclide® (Defibrotide) for preventing endothelial-related toxicity.

Poster

C40 Clinical Outcome of Cord Blood Transplantation for Patients with Neuroblastoma in Japan

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Background Autologous stem cell transplantation (SCT) is established as a treatment modality for patients with advanced stage of neuloblastoma (NB), but the clinical effect of cord blood transplantation (CBT) is not well documented so far. We retrospectively analyzed the clinical outcome of CBT for patients with NB in Japan.

Methods: Thirty four patients with NB were transplanted until 2006 and 14 patients had previous SCT. The median age of patients was 4.0 (range 1-16) and the clinical stage at CBT was complete remission (CR) in 21 patients and non CR in 13 patients. Overall survival (OS) and event free survival (EFS) were analyzed using Kaplan-Meier estimates. Statistical differences were tested using a log-rank test and risk factors for EFS were assessed by Cox hazard model.

Results: The median number of transplanted nucleated cell dose was 6.82×10^7/kg (range, 1.89-14.2). Engraftment was obtained in 24 patients and the median days to engraftment were 21 days (range, 10-63). The cumulative incidence of grade II to IV acute GVHD was 21.4%. The treatment related mortality and relapse was observed in 27.8% and 29.6% of patients, respectively. The probability of OS was 50.0% and EFS in CR and non CR at CBT was 41.4% and 53.8%, respectively (p=0.58). Patients who received above median number of cell dose had significantly better EFS and this was confirmed by multivariate analysis (p=0.02).

Conclusions: Although CBT is a relatively new strategy for patients with advanced stage of NB, our analysis showed that it is feasible as a treatment modality. The EFS of patients in non-CR at CBT was better than previously reported and this would suggest the possibility of graft versus tumor effect in CBT for NB. In conclusion, CBT should be considered when patients are ineligible for autolougous transplant.

C41 Dendritic Cell Vaccination in Relapsed Neuroblastoma Patients

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Background: Preclinical data indicate that vaccination with ex vivo generated mature dendritic cells (DC) might overcome tumor induced suppression of the immune system. Therefore, we have established DC vaccination for patients with treatment resistant neuroblastoma.

Methods: Patients with recurrent neuroblastoma in remission or with stable disease after completed relapse treatment were eligible for DC vaccination. Monocytes, collected by peripheral apheresis, were differentiated to DC during a 6 day culture period. For the last 24h they were matured using IL1ß, TNF-alpha, IL-6 and prostaglandin E2 and pulsed with cancer germline antigen peptides (MAGE1, MAGE3, NY-ESO-1, according to the expression level on the tumor and patient`s HLA-type) or inactivated autologous tumor lysate, or both. Ten subcutaneous vaccinations with up to 3×10^6 DC per vaccination were planned, five every 2 weeks, then every 4 weeks. The tumor status was assessed prior to the first vaccination, after 5 and 10 vaccinations, or if progression was clinically suspected.

Results: A total of 12 patients were treated. The median age at first diagnosis was 3.6 years (1.8-37.1). The median number of previous chemotherapy cycles was 15 (range 10-33). The median age at first vaccination was 8.0 years (4.5-42.5). DC were pulsed in vitro with cancer germline antigens (n=5), autologous tumor lysate (n=4) or both (n=3). No toxicity WHO grade 2 or higher was observed. Three patients completed all vaccinations but progressed 2.1, 3.5, and 13.0 months after last vaccination. The others experienced progression after 1–6 vaccinations. The median time to progression was 0.35 years (0.0-1.6). By December 2007, ten patients have died after neuroblastoma progression, and two patients are alive with progressing disease.

Conclusions: The DC vaccination protocol was feasible and well tolerated. Unfortunately, the treatment was not able to prevent progression in intensive pre-treated neuroblastoma patients.

C42 Therapeutic Strategy for INSS Stage 3 Neuroblastoma: A Single Institution Experience with 26 Cases

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Background: The appropriate treatment for advanced regional neuroblastoma has been difficult to define because of the marked biologic heterogeneity. This study analyzed the clinical outcome of patients with INSS stage 3 neuroblastoma treated at a single institution to refine the therapeutic strategy.

Methods: Among 170 patients with neuroblastoma encountered between 1985 and 2007, 26 patients with stage 3 were classified into two groups by age, 12 cases less than 12 months of age (infants) and 14 cases greater than 12 months (children). Clinicopathologic findings and therapeutic outcome of the two groups were retrospectively reviewed. Chemotherapy consisted mainly of cisplatin, etoposide, vincristine for infants, and cyclophosphamide, THP-adriamycin, cisplatin, and etoposide or vincristine for children.

Results: In cases where Shimada classification was available, all infants had favorable histology while eight out of ten children had unfavorable histology. N-myc was amplified in one infant and five out of eight children. The event-free survival rate was 100% for infants and 50% for children with a median follow-up of 8.4 and 11.5 years. Ipsilateral nephrectomy was required in eight children due to local eradication or renal hypertension. Two infants who received incomplete resection remained disease-free. Irradiation was given to four infants and twelve children. Myeloablative therapy with ABMT or PBSCT was applied to ten children. Complications included renal hypertension (n=3), multiple organ failure after PBSCT (n=2), intraoperative bleeding (n=2), bowel obstruction (n=2), adrenal insufficiency (n=1), and a second cancer (n=1). Seven children died, in which local relapse occurred in five cases.

Conclusions: Treatment for infants with stage 3 neuroblastoma should be decreased to reduce the complications, especially if they are classified into intermediate risk group defined by COG, while children with unfavorable biology should be treated more intensely by aggressive local eradication with wide field radiation because of a high ratio of local relapse.

C43 Impact of Total Body Irradiation for Autologous Stem Cell Transplantation of High-Risk Neuroblastoma

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Background: Total body irradiation (TBI) as a part of autologous transplantation has been proved effective for the treatment of high-risk neuroblastoma. Long-term adverse effect of TBI, however, has been recognized increasingly, which include second malignancies. There is no direct comparison between TBI and non-TBI regimen. We retrospectively analyzed the impact of TBI for the treatment of advanced neuroblastoma in Japan.

Patients and Methods: Two hundred fifteen patients with stage 4 neuroblastoma over 1 year of age at diagnosis who were transplanted for the first time between 1995 and 2005 were analyzed based on the transplantation registry of Japanese Society of Pediatric Hematology.

Results: One hundred sixty-eight out of 215 patients were autologous transplants evaluable. The actuarial event-free survival (EFS) at 5 years was 32.2±3.7% and over-all survival was 38.4±4.3%. The usage of TBI (TBI vs non-TBI; relative risk=1.86, p=.0147), disease status at transplantation (CR vs non-CR; relative risk=2.05, p=.0003) and the use of Melphalan for the preconditioning regimen (Melphalan containing vs others; relative risk=2.25, p=.0037) were independent factors that influenced EFS with multivariate analysis by Cox proportional hazard model. The use of TBI also had lower relapse rate (RR) compared with non-TBI regimen in patients with complete remission at transplantation, which was not significant (60.4% vs 42.9%, p=0.121). Three patients with second malignancies were documented in non-TBI conditioning group, but none in TBI group.

Conclusions: Melphalan containing and TBI based preparative regimen has significant superiority in EFS for autologous stem cell transplantation of advanced neuroblastoma. We need to pay attention to the long-term risk of second malignancies not only in TBI preconditioning group but also in non-TBI based transplantation.

C44 Hyperfractionated Low-Dose (21 Gy) Radiotherapy (RT) for Cranial Metastases in Patients with High-Risk Neuroblastoma (NB)

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Background: We present our experience with RT used to prevent relapse in cranial bone sites where substantial metastatic NB is common.

Patients and Methods: Patients with high-risk NB plus measurable cranial disease at diagnosis or residual cranial disease after induction had those sites irradiated with hyperfractionated 21 Gy; a brain-sparing technique was used for an extensive field. When irradiated, the patients either had no evidence of NB (Group 1) or primary refractory disease (Group 2). Follow-up was from the start of cranial RT.

Results: At three years, the 38 Group 1 patients had a progression-free survival (PFS) rate of 51%, and control of their cranial disease was 79%. Two relapses involved irradiated cranial sites (one was an isolated relapse). Two other patients relapsed in irradiated cranial sites at 6 and 12 months after a systemic relapse. The 33 Group 2 patients included 18 who had residual cranial (± extracranial) disease. RT to thos cranial sites was followed by major (n=12), minor (n=2), or no response (n=4); five progressed in the cranial RT field at 10-to-27 months (one isolated). Group 2 also included 15 patients who had persistent NB in extracranial but not in cranial sites: two relapsed in irradiated cranial sites and elsewhere at 8 and 14 months. In Group 2, at three years, the PFS rate was 32%, and control of cranial disease was 56%. Cranial RT was well tolerated, with no grade ≥2 toxicities.

Conclusions: Hyperfractionated 21 Gy cranial RT may help local control of NB and is feasible without significant toxicity in children.

C45 Detecting Unsuspected Relapse of High-Risk Neuroblastoma (NB): Major Role for ^{123}I-Metaiodobenzy Iguanidine (MIBG) Scan

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Background: Relapse-free survival (RFS) is a powerful measure of treatment efficacy. We evaluated the utility of different monitoring studies for detecting unsuspected relapse of high-risk NB.

Patients and Methods: The subjects of this report were all in first or ≥2nd complete/very good partial remission (CR/VGPR) of high-risk NB, but routine monitoring revealed an unsuspected relapse. Disease assessments every 2-4 months included computed tomography (CT), ^{131}I-MIBG (through 11/99) or ^{123}I-MIBG scan, urine catecholamine levels, and bone marrow (BM) histology (bilateral posterior and bilateral anterior iliac crests). Bone scan was routine through 2002.

Results: ^{123}I-MIBG scan was the most reliable means for revealing unsuspected relapse in 116 patients (75 in first CR/VGPR, 41 in ≥ 2nd CR/VGPR), with an 84% detection rate which was superior to the rates with ^{131}I-MIBG scan (61%), bone scan (37%), and BM histology (36%). Patients whose monitoring included ^{131}I-MIBG scan were more likely than patients monitored by ^{123}I-MIBG scan to have an extensive osteomedullary relapse, as evidenced by higher rates of concurrent relapse detected by BM tests (57% versus 31%), bone scan (57% versus 27%), and urine catecholamine levels (45% versus 22%). ^{123}I-MIBG scan was the sole positive study indicating relapse in 26/93 (28%) patients compared to ≤5% for each of the other staging studies, including ^{131}I-MIBG scan (p=0.02).

Conclusions: ^{123}I-MIBG scan is essential for valid estimation of the duration of RFS of patients with high-risk NB. Without ^{123}I-MIBG scan as part of a periodic comprehensive assessment of disease status, caution must be invoked when comparing RFS between institutions and protocols.

C46 A Phase I Study of Nifurtimox in Patients with Relapsed Neuroblastoma

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Background: Nifurtimox is a nitroheterocyclic compound containing a nitro group that has been shown to exert its cytotoxic effect specifically by generating free radicals. The objectives of the study were to is to test the safety and pharmacokinetic profile of nifurtimox in children with relapsed /refractory neuroblastoma alone and in combination with cyclophosphamide and topotecan.

Methods: Nifurtimox dose began at 20mg/kg/day (3 patients/cohort) and escalated by 10mg/kg/day with each cohort until 2/6 patients in a cohort experienced dose limiting toxicities (DLT). Patients received nifurtimox for 21 days (cycle 1), then repeated 3 cycles in combination with cyclophosphamide and topotecan. Eligibility criteria included patients <21 years old with relapsed neuroblastoma and measurable disease. Pharmacokinetic analysis was done on Day 1 and 4 of both nifurtimox alone and during the first week of nifurtimox in combination with cyclophosphamide and topotecan. DLT was defined as grade 4 toxicity, grade 3 neurotoxicity, or grade 2 toxicity that persisted for >7 days requiring treatment interruption >7 days.

Results: Three patient were enrolled at 20mg/kg/day, 6 patients at 30mg/kg/day and 5 patients at 40mg/kg/day. Toxicities greater or equal to grade 2 included stomach pain (n=1), anorexia (n=6), neuropathy (n=6) grade 2 seizure (n=5). DLT' s occurred at 40mg/kg/day and included one grade 3 pulmonary hemorrhage and one grade 3 motor neuropathy. The recommended Phase II dose is 30mg/kg/day. Pharmacokinetic (PK) analysis show peak serum levels at 3 hours. Fourteen patients are evaluable for efficacy with nifurtimox; 10 stable disease, 4 progressive disease. Eleven patients are evaluable for efficacy after 3 cycles with nifurtimox, cyclophosphamide and topotecan; 4 partial response and 7 stable disease.

Conclusions: Nifurtimox can safely be administered orally alone and in combination with cyclophosphamide and topotecan. The results show promising signs of anti-cancer activity warranting further evaluation. Clinical and PK data will be presented.

C47 Tyrosine Hydroxylase mRNA Predicts Outcome for Children with High-Risk Neuroblastoma

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Background: Intensified multimodal therapy has improved outcome for children with high-risk neuroblastoma. Better ways to monitor therapy and identify responsive and resistant subsets of patients could further improve clinical therapy. Tyrosine hydroxylase (TH) is the rate-limiting enzyme in catecholamine biosynthesis and up-regulated in hypoxic neuroblastoma cells.

Methods:TH, dopa decarboxylase (DDC) and GD2 synthase (GD2S) mRNAs were analyzed with quantitative RT-PCR in 554 peripheral blood (PB) and bone marrow (BM) samples in a consecutive series of 58 children with neuroblastoma. 24 children with high-risk disease (INSS stage 4 >2 years and/or MYCN-amplification stage 2-4) were uniformly treated with COJEC induction, myeloablative therapy, stem-cell rescue, 13-cis RA and followed for 35-94 months (median 69).

Results: High TH expression/cell correlated to MYCN amplification, I-type stem-cell phenotype, and chemotherapy resistance *in vitro*. GD2S was less specific for neuroblastoma in PB and BM than DDC or TH. TH and DDC concentrations predicted outcome most significantly. Concentrations of TH in BM and PB at diagnosis was prognostic for high-risk patients, and children with TH below the median in BM had better survival (91% at 5 years) than those with TH above the median (33%, *P*=0.009). Similar discriminative effect was obtained at other cut-off levels. TH below or above the median in PB was almost as discriminative for high-risk patients (80% vs. 34% survival probability).

Conclusions: TH expression in PB and BM corresponds to metastatic neuroblastoma at diagnosis, residual disease, and poor outcome. We suggest that high TH mRNA in PB or BM not merely reflect tumor load but rather indicate the unfavourable presence of aggressive hypoxic neuroblastoma cells. TH mRNA concentrations at diagnosis predict outcome for high-risk neuroblastoma suggesting a way to stratify for different treatment strategies. Children with high-risk neuroblastoma and concentration of TH mRNA below the median seems to be curable with current therapy.

Poster

C48 I-131 Metaiodobenzylguanidine (MIBG) Treatment in Previously Heavily Treated Stage IV Neuroblastoma Patients

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Background: The outcome of advanced stage neuroblastoma patients with multiple relapsed or refractory diseases is very dismal. We therefore would like to study the clinical usefulness of I-131 MIBG treatment in this group of patients.

Methods: A retrospective study of ten stage IV neuroblastoma patients (6 males, 4 females), age at the diagnosis range 3 to 8 years from January 2005 to December 2007 were analyzed. Patients were previously treated with multiple regimens of chemotherapy, high-dose chemotherapy with autologous peripheral stem cells (PBSCs) rescue, salvage surgery, and local radiation. All these patients had relapsed or refractory disease at the time of I-131 MIBG treatment. The therapeutic dose of I-131 MIBG activity per course was given on the basis of weight, the hematological parameter, tumor response assessment and internal dosimetry calculation. The cycle of treatment was more 4 weeks apart. The treatment response was evaluated by imaging and neuron-specific enolase (NSE) levels.

Results: A total of 26 courses of I-131 MIBG were administered; four patients received one course, one received two courses, two received three courses, two received four courses and one received six courses. The median therapeutic dose of I-131 MIBG was 5 GBq (range, 1.1 - 7.4). Median response of duration was 8.5 months (range, 1-19). A partial response achieved in 1, and stable response in 8 patients. Pain relief was observed in 1/1 patients with bone pain.The most common side effect was thrombocytopenia which became more severe with increasing number of course, grade 2 thrombocytopenia occurring in 4/10 patients over 3 courses and one child developed grade 4 thrombocytopenia with lung complication. Four children were died where as six are still alive.

Conclusions: It can be concluded that MIBG treatment in combination with chemotherapy and hematopoietic stem cell rescue is effective and less toxic in patients with relapsed or refractory neuroblastoma and further studies of the role of I-131 MIBG treatment in advanced neuroblastoma in Thailand are needed.

C49 Neuroblastoma in Adolescents

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Background: Neuroblastoma is diagnosed rarely during adolescence. It has been suggested that the behaviour and clinical characteristics may be different in older patients than in younger children.
For this reason we investigated the presentation, biological features and outcome of adolescents with neuroblastoma included in the Spanish Neuroblastoma Registry.

Methods: Patients older than 10 years included in the Spanish Neuroblastoma Registry Files. Tumor samples sent to the National Reference Laboratory for Pathology and Biology studies (ENQUA). Clinical characteristics and outcome are presented.

Results: During the period 1992-2005, 548 neuroblastoma patients were registered and 20 were older than 10 years. 4 %. There were 9 boys and 11 girls with a mean age of 13.53y. (10.18-19.21). The primary tumor was abdominal in all cases.
INSS stages were 1 (n=2), 2 (n=1), 3 (n=5), 4 (n=12). LDH was high in 5/16. INPC was unfavourable in 14/14 reviewed successfully, MNA 0/17, MYCN gain 1/17; Deletion of 1p 0/14, Unbalanced 2/14; DNA index Diploid-Tetraploid 3/13. 5/20 patients were not included in the corresponding collaborative protocols because they were studied initially in adult´s services. With a mean follow up of 5.4y (3.4-7.3y CI 95%) OS is 0.4 and EFS is 0.35 at 5y.

Conclusions: Neuroblastoma in adolescents represented 4% of our patients. Compared with younger children, we found more advanced stage, no MNA, neither del1p, Histology was Unfavorable in all. An important part of the patients were not included in collaborative estudies. OS and EFS are worse than in younger neuroblastoma patients.

C50 Neuroblastoma Originating from Extra-Abdominal Sites: Association with Favorable Clinical and Biological Features

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Background: A variety of clinical and biological factors are associated with the prognosis of neuroblastoma. Site of tumor origin may be one of prognostic factors. In the present study, clinical and biological characteristics according to the site of tumor origin were investigated.

Methods: Clinical (age, sex and stage) and biological (N-myc amplification, Shimada pathology and level of lactate dehydrogenase, ferritin and neuron-specific enolase) characteristics of patients with newly diagnosed neuroblastoma from February 1997 to December 2007 were compared according to the site of tumor origin (extra-abdominal versus abdominal). Event-free survival rate (EFS) was also compared between two groups.

Results: Among 143 neuroblastomas, 115 tumors were originated from abdomen, 26 from extra-abdominal sites and 2 from unknown primary sites. Frequencies of stage 4 tumor and N-myc amplified tumor were lower in the extra-abdominal group than in the abdominal group (34.6% versus 60.0%, $P = 0.019$ and 4.2% versus 45.0%, $P < 0.001$, respectively). Levels of lactate dehydrogenase, ferritin and neuron-specific enolase were lower in the extra-abdominal group than in the abdominal group (median 679 versus 1,391 IU/L, $P < 0.001$; 36 versus 171 ng/mL, $P = 0.042$; 16 versus 103 ng/mL, $P < 0.001$, respectively). The 5-year EFS (± 95% confidence interval) was higher in the extra-abdominal group than in the abdominal group (94.4 ± 10.6% versus 69.4 ± 9.4%, $P = 0.026$).

Conclusions: Neuroblastoma originating from extra-abdominal sites was associated with more favorable clinical and biological characteristics and a better outcome than neuroblastoma originating from abdomen.

C51 Report of 39 Cases of Stage 3,4 Neuroblastoma Patients Treated with Small Dose Chemotherapy Combined with Chinese Herbal L3

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Background: To observe if the Chinese herbal L3 formulated in our hospital in combination with small dose chemotherapy could achieve immuno-function protection and get good response in Stage3, 4 neuroblastoma patients

Methods: VP or VCP small dose chemotherapy were used in combination with Chinese herbal L3 in our hospital to treat 39 post-operation Stage3,4 neuroblastoma patients, 7 days a course and 4 courses a cycle. Patients were treated 2-4 years, immuno-fuctions et al were observed and all the patients in this group were followed up for average of 5 years.

Results: 19 cases got CR, among which the oldest patient is 29 years now, 23 years post-operation (operated on age 6). Also there was a patient who got CR and got married after years of therapy and gave birth to a healthy child. There were also two patients in this group who were first diagnosed as NB by pathology biopsy before treatment and finally proved to be ganglioma after years of treatment. 14 cases died and 6 cases lose of follow-up.

Conclusions: Chinese herbal L3 could help to improve immuno-function of the patients and alleviate the toxic and side effect of chemotherapy.Chinese herbal L3 could help small dose chemotherapy to induce apoptosis and differentiation in NB patients.

C52 Characteristics & Outcome of Early Stage Neuroblastoma Treated with Surgery Alone or Low Intensity Chemotherapy

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Background: The treatment of INSS stage 1 & 2 neuroblastoma without poor prognostic features such as *MYCN* amplification or unfavorable Shimada histological pattern has not reached a consensus for the outcome has been reported to be excellent with different approaches. We reviewed our experience by adopting a "modified" approach from former POG regimen.

Patients & Method: This is a prospective cohort study. The patients were recruited from 5 regional hospitals which take care of all children with cancer locally. The data were collected by 2 data managers sponsored by Children Cancer Foundations. Children with INSS stage1 neuroblastoma were treated with surgery alone. Children with either stage 2a or 2b will be treated with the former POG low intensity chemotherapy protocol with cyclophosphamide & adriamycin. *MYCN* was evaluated by FISH in a University laboratory. Patient with residual tumor after 5 courses of chemotherapy were treated with 2nd look surgery if possible or just observed with no further treatment.

Results: From Jan 1996 to Dec 2006, a total of 25 patients were diagnosed and treated in the 5 centers. There were 13 stage1 patients, none received chemotherapy. There were 12 stage2 patients, 8 received POG protocol, the 4 patients who did not receive chemotherapy were stage 2a patients (tumor completely resected but with positive ipsilateral lymph nodes at diagnosis). The median age was 0.95yrs (range birth to 13.95yrs). Interestingly, 10/13 stage1 patients had normal spot urine catecholamines and 8 of these 10 patients also had negative MIBG scan at diagnosis. In contrary, all stage2 patients had increase spot urine catecholamines and 5 had normal MIBG scan. 2 stage2 patients had residual mass but the mass was not MIBG positive. All stage2 patients' urine catecholamines returned to normal. None of these patients had *MYCN* amplification and only one stage1 patient had unfavorable Shimada features. All patients survived without major complications with a median follow up of 4.9 yrs (range 1.26-10.45yrs). Neutropenic fever was uncommon in those treated with chemotherapy.

Conclusion: Early stage neuroblastoma without poor risk feature has extremely good outcome with low intensity therapeutic approach. The low positivity of urine catecholamine and MIBG scan in this group of patients emphasizes the importance of histological diagnosis.

C53 Stage 4s Neuroblastoma- Report of 10 Years Experience of AIIMS-NB 96 Study

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Backgroud: Neuroblastoma stage 4s(NB4s) has a relatively better outcome and known spontaneous regression. However some of these infants do badly. The aim of this study was to evaluate the outcome of infants with NB4s in relation to age, location of primary, and presence of symptoms especially respiratory distress(RD).

Methods: Prospective study at a tertiary care pediatric surgical center from June 1990 through December 2007. Outcome evaluation was done for disease progression or regression and for survival(OS)

Results: Thirteen patients with NB4s were treated in this period. Six(46.2%) of these were below 2 months of age. Five(38.5%) had an unknown primary while in 8(61.5%) the primary involvement of the adrenal. All presented with hepatomegaly. In addition 8(61.5%) had respiratory distress and 2 had intractable diarrhea. Subcutaneous nodules were present in 2(15.5%) and 3(20.1%) had positive bone marrow aspiration. The diagnosis of NB4s was obtained by liver biopsy in 9(69.2%) and FNAC of the primary in 4(30.8%). Three children(20.1%) were asymptomatic and in all there was a complete spontaneous regression. In 10(76.9%) symptomatic children, chemotherapy was administered; of these 7(70%) survived. 9 of 13 patients have achieved CR. All the 5 patients without RD are alive and in CR, while only 5 of 8(62.5%) patients with RD are alive. The OS was 76.9%(95CI 72.7-133.8) and the DFS was 69.2%(95CI 59.7-126.4) with the mean period of follow-up being 40 months. Two of the three patients who died were neonate and one was 65 days old. All the deaths were due to progressive disease with rapidly increasing hepatomegaly and RD unsalvageable despite creation of ventral hernia in two and radiotherapy in one.

Conclusions: Though OS of NB4s was 77%, there was a poorer outcome in neonates with severe RD. Early institution of chemotherapy was mandatory in about 75% of patients due to severe symptoms.

C54 The Incidence of Neuroblastoma in Shiga Prefecture, JAPAN.: Comparison of the Data in Mass-Screening Period and after Discontinuation of the MS

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Background: Unfortunately we have no precise registration system of childhood cancer in Japan. It is very difficult to obtain accurate registration data especially in Tokyo or other big cities. However, Shiga prefecture is locating very central area in Japan and small area enough to get accurate registration data of neuroblastoma(NBl). We compared the data of NBl before, during and after the MS.

Methods: We have been monitoring the incidence of NBl in Shiga prefecture from 1975 until now using childhood cancer registration in Shiga and telephone inquiry to medical institutions for further confirmation. We used the data from 1995 to the end of 2006. Among this period, MS was done 1995 to Dec., 2003(period A). MS was discontinued from 2004. The period B is from 2004 to Dec., 2006.

Results: Twenty four cases were found in A while only five cases in B. 18 patients in A were found through MS and 19 patients out of 24 were under age of 12 months. Only one patient was less than 12 months of age in B. Among 24 patients in A, only 3 patients (12.5%) had a stage 4 disease. But four out of five were stage 4 in B. Annual incidence was 2.66/year in A and 1.33/year in B period. From the discontinuation of MS, annual incidence of NBl in Shiga prefecture decreased at 50% in B. Patients younger than 12 months of age were naturally decreased($p<0.05$) significantly. Conversely patients with stage 4 disease increased significantly ($p<0.01$) after discontinuation of MS.

Conclusions: The discontinuation of MS will give a significant decrease of incidence of NBl and show a great change on the characteristics of patients with NBl in Japan.

C55 The Analysis of a 15-year Experience of Neuroblastoma Treatment in Lviv Region, Ukraine

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Background: Aim of the analysis was to evaluate the experince in the treatment of neuroblastoma in Lviv region, Ukraine

Methods: 43 children (24 boys and 19 girls) from Lviv region, Ukraine with established diagnosis of neuroblastoma were observed and treated in our clinic during the period of 1992-2007. The age was fom 0 to 14 years with 40% of infants. The proportion of disease stages was following: I – 19%, II (including IIa and IIb) – 23%, III and IV 21% of each, IVs – 16%.The primary tumour was localised in the adrenal gland in 17 patients, retroperitoneal space in 9, mediastinum in 5, neck region in 4, presacral region in 4, CNS in 1 case.
The metastatic sites were the lymph nodes, liver and bone marrow. The chemotherapy was held due to recommendations of Study Group of Japan, POG and SIOP using TOKIO (19% of patients treated), POG-8741/42 (9%) and NB-97 (63%) protocols. 9% of children received only surgical treatment.

Results: The overall mortality was 29%(12 patients). 7 patients died due to disease progression, 3 patients due to relapsed disease, 2 patients due to intercurrent infections. The 5-year disease-free survival was 42%.

Conclusions: Despite the poor financial situation the neuroblastoma treatment results are quite positive, the early diagnostics development is required for further improvement of survival results.

Poster

C56 Bone and Bone Marrow Metastasis in Pediatric Neuroblastoma Patients

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Background: To observe the extent of bone cortex destruction and predilection site before and after bone marrow metastasis in children neuroblastoma (NB) patients by X-ray examination.

Methods: From 1974 to 1991, 174 cases were diagnosed as neuroblastoma, and 56 cases of 111 patients examined by X-ray imaging presented bone metastasis. Between 1974 and 1983, 27 cases were diagnosed by marrow smear and X-ray imaging, from 1984 to 1989 neuroblastoma was confirmed in 66 cases by marrow smear, B-mode ultrasound tomography, CT and X-rays imaging. 81 cases were diagnosed by VMA, marrow cell single-clone examination, B-mode ultrasound tomography, CT, MRI and X-ray imaging between 1986 and 1991.

Results: Bone metastasis of NB tumor cells was earlier than bone marrow metastasis of them. Pelvic is the most common affected site in flat bones and thigh and vertebrae are the most common predilection sites in os longum and quadrate bone, respectively.

Conclusions: Neuroblastoma patients were easily misdiagnosed as rheumatic fever, theumatic arthritis and osteomyelitis due to bone and bone marrow metastasis symptoms. In most of the patients, bone metastasis was earlier than bone marrow metastasis.

C57 Expression of KIT and PDGFR is Associated with a Good Clinical Outcome in Neuroblastoma

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Background: The clinical course of neuroblastoma (NB) depends on age, stage, and *MYCN* amplification. Receptor tyrosine kinases (RTKs) promote cell growth, migration, and metastasis in cancer cells, including NB. However, the correlation of the expression profile of RTKs with prognosis in NB remains controversial.

Methods: Expression and mutation analysis of *KIT*, *PDGFR*, *FLT3*, *RET*, and *TRKA* mRNAs were performed in 24 NB cell lines and 40 tumor samples using RT-PCR followed by direct sequencing. Immunohistochemical analysis of *KIT*, *PDGFR*, and *ALK* protein expression was also examined in NB tumor samples.

Results: The expression of *KIT*, *PDGFRβ*, and *FLT3* mRNA was associated with NB in patients under 1 year ($P<0.02$) and *TRKA* expression ($P<0.001$). The loss of expression of these kinases was associated with *MYCN* amplification ($P<0.02$) and advanced stages of disease in patients over 1 year of age ($P<0.005$). *PDGFRα* mRNA expression was detected in all cell lines and tumor samples, and *RET* mRNA expression was not associated with any clinical parameters. Immunohistochemistry showed the similar findings. The expression of *ALK* protein was detected in 87.8% of clinical samples, and not associated with any clinical parameters. We did not find any activating mutations in *KIT*, *PDGFR*, *FLT3*, or *RET*. The mutation of *ALK* is under examination. Notably, the GNNK- isoform of *KIT* was predominant in all cell lines and clinical samples.

Conclusions: Expression of *KIT*, *PDGFRβ*, and *FLT3* was associated with a good clinical outcome in NB. The loss of expression of these RTKs might correlate to the disease progression of NB. Further, other RTKs including *MET* and *EGFR* are under examination.

C58 Better Outcome for Infants Younger than 12 Months Compared to Children 12 to 18 Months Old at Diagnosis with Neuroblastoma

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Background: The infant less than 12 months of age at diagnosis has favorable prognosis. Some reports extended the cut-off of age to 18 or 19 months. To identify prognostic implication of age, we retrospectively analyzed clinical data of children who have neuroblastoma diagnosed age younger than 18 months.

Materials and Methods: One hundred and twenty children were diagnosed as neuroblastoma and treated at Asan Medical Center between December 1989 and November 2007. Of 120 patients, 44 were diagnosed at younger than 18 months old. We analyzed the outcome of 44 patients according to diagnosis age, as of January 2008.

Results: Of a total of 44 patients, 20 were younger than 6 months (Group A), 16 were between 6 and 12 months (Group B) and 8 patients were between 12 and 18 months (Group C) of age at diagnosis. Among 44 patients, 7 patients experienced relapse or progression of disease (3 in A, 1 in B, 3 in C), and 3 patients died of causes other than progression or relapse. With a median follow-up of 5.7 years, the Kaplan-Meier estimates of EFS at 3 years were 76.0% (A), 87.1% (B) and 50.0% (C), respectively. The EFS of 81.3% for infant (A+B) was better than 50.0% for older children (C) ($P=0.068$). Of 3 patients who relapsed in Group A, 2 are alive without disease 9.3 years and 10.2 years after relapse, respectively, and the remaining one is on salvage treatment after relapse. Of 3 patients who relapsed in Group C, 2 died of disease and one survives without disease 7.3 years after relapse. The 3-yr OS were 95% (A), 87.1% (B) and 62.5% (C), respectively. Overall survival (OS) for patients less than 12 months old (90.7%) was significantly better than the OS for patients 12 to 18 months (62.5%) ($P=0.048$).

Conclusions: The outcome for infant was better compared to children 12-18 months of age at diagnosis. To clarify the prognostic contribution of age to the outcome in neuroblastoma, collaborative study including larger number of patients is warranted.

C59 Analysis of NB2000 Protocol Including Myeloablative Chemotherapy with Stem Cell Transplantation, Intraoperative Radiation and 13-Cis-Retinoic Acid Therapy for Children with High-risk Neuroblastoma

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Background: In January 1999, Tohoku Neuroblastoma Study Group (TNBSG) introduced NB2000 protocol for children with high-risk neuroblastoma (NB), which consisted of myeloablative chemoradiotherapy with autologous stem cell transplanation (MT/SCT), intraoperative radiotherapy (IOR) and 13-cis-retinoic acid (13CRA). We studied whether this protocol improved the outcome of children with advanced NB.

Patients and Methods: From January 1999 to September 2005, 27 children with advanced NB (4 at stage III and 23 at stage IV) were enrolled in this study. 14/27(51.9%) cases had amplified N-myc, and 21/27 (77.8%) cases showed an unfavorable histology. Median age of children was 4.2 years (range 1.0 to 14.3), and median follow up 31 months (range 8 to 84 months).

Results: As for January 2008, eight of 27 patients (39.1%) are alive, six in complete remission (CR), one in partial remission (PR) and one in progressive disease (PD). Of 15 dead children (44%), 10 died of progressive disease; five of regimen related complications (veno-occlusive disease, interstitial pneumonia, aspergillus, respiratory syncytial virus and cytomegalovirus infections). 5-year overall survival (OS) and event free survival (EFS) rates are 29 % and 27 %, respectively. The rate of CR/very good PR (VGPR) rate after induction chemotherapy was low (14/24, 58.3%), but improved to 13/17 (76.5%) after MT/SCT. Patients who received IOR at the dose of 10Gy or more had no local recurrence.

Conclusions: Treatment with MT/SCT following induction chemoratdiotherapy improved CR/VGPR rates in patients with advanced NB, but meanwhile the increased intensity of the treatment regimen was related to serious adverse effects. IOR might reduce the risk of local relapse, although therapeutic effect of 13CRA therapy following MT/SCT was not confirmed.

C60 Clinical Utility of QPCR-Based Prognostic Factors in Neuroblastoma is Highly Dependent on Stage and Age at Diagnosis

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Background: In neuroblastoma (NB), a number of prognostic factors have been shown to correlate with clinical outcome. At MSKCC, the initial management of NB patients stratified according to risk groups has been relatively consistent in the past two decades. Without "variable treatment" as a confounding factor, we undertook an analysis of the clinical utility of quantitative (q)PCR-based prognostic factors using diagnostic NB tumors.

Methods: 185 neuroblastoma tumors obtained at diagnosis (dx) were analyzed for *MYCN* amplification (MNA), 17q gain, and gene expression of MYCN and CAMTA1 using MYC as a control marker, and compared with Shimada histology and ploidy. These tumors belong to three clinical risk groups (gp), namely gp 1: stage 1, 2, 3 or 4S (median age at dx =12 months) [n=69], gp 2: stage 4, ≤18 months at dx [n=25], and gp 3: stage 4, >18 months at dx [n=91]. MNA and 17q gain were determined by qPCR; gene expression by qRT-PCR. Prognostic importance was tested by Kaplan-Meier analyses of progression-free survival (PFS) and overall survival (OS).

Results: When all patients were analyzed as a single cohort, histology, ploidy, NMA, 17q gain, and expression of MYCN and CAMTA1 were all prognostic for PFS and OS. PFS in gp 1 correlated significantly ($p<0.05$ to $p\leq0.001$) with histology, ploidy, 17q gain, and MYCN expression, whereas OS correlated only with 17q gain. In gp2, MNA, as well as expression of MYCN and CAMTA1 were strongly associated with both PFS and OS. However, there was no significant correlation of either PFS or OS with any of the prognostic markers in gp 3, the highest risk group.

Conclusions: The utility of prognostic factors is highly dependent on patient risk groupings based on stage and age. There may not be a universal PCR-based predictor in the context of current NB therapy.

C61 Paraneoplastic Limbic Encephalitis: a Rare and Serious Complication to Neuroblastoma

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Background: Paraneoplastic limbic encephalitis (PLE) is an autoimmune syndrome associated with circulating anti-Hu autoantibodies. The term is usually applied to adult patients and only a handful of similarly affected children with neuroblastoma (NB) have been reported. These patients were older at diagnosis than opsoclonus-myoclonus patients. PLE has a poor prognosis with high mortality and serious long-term sequelae.

Methods: A case report presenting clinical, tumor biological, immunological and radiological data.

Results: A male patient was diagnosed at 14 years of age with an adrenal stage 3 ganglioneuroblastoma. Unusual presenting symptoms and signs were severe abdominal neuralgic pains, severe gastrointestinal dysfunction necessitating long-term parenteral nutrition, and extensive peri-vascular lymphocytic tumor infiltration. Neuralgic pains persisted after treatment (non-radical primary surgery and chemotherapy). Epileptic seizures commenced after twelve months, followed by three months of rapid neurological deterioration leading to a semi-comatose state with multiple signs of brain stem dysfunction. Anti-Hu autoantibodies were detected in high titer. Maximal immunomodulatory treatment was attempted, combining courses of high-dose glucocorticoids, cyclophosphamide, rituximab and finally plasmapheresis. After treatment of a local tumor relapse, myeloablative treatment (MAT) was undertaken. A syngenic stem cell rescue was made, using a monozygotic twin brother as donor. Remarkable neurological improvements were seen after plasmapheresis sessions and after MAT. Neurological sequelae are still evident, particularly affecting short-term memory.

Conclusions: Although rare in NB, awareness of PLE is important for an early diagnosis and optimal immunosuppressive treatment. *Neuralgic pains*, *dysautonomic symptoms* (severe vomiting and diarrea), *extensive lymphoid infiltration of the tumor* and *epilepsia* were the major forerunners of a full-blown PLE in this case. Measurement of serum anti-Hu antibodies was of diagnostic help, but less useful in monitoring the disease. Plasmapheresis may lead to rapid temporary improvement. MAT with donor stem cell rescue should be considered for achieving long-term improvement, in view of the poor prognosis.

C62 Comparison of the FISH, MLPA and Array-CND Methods for Genetic Studies of Neuroblastoma Tumors in a Clinical Setting

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Background: Well-known genomics aberrations are present in primary neuroblastoma (NBL) tumor cells. These include regions 2p (MYCN amplification) 1p, 11q (deletion) and 17q (gain). These aberrations are strongly correlated to clinical outcome and their detection is used in the clinical diagnostic setting. FISH (fluorescence in situ hybridization) have been in use for more than 15 years, while more recently methods for the simultaneous analysis of several genomic regions or the whole genome have been developed. These include MLPA (Multiplex ligation-dependent probe amplification) and array- CND (copy number detection). Our laboratory in Gothenburg have provided a clinical biological analysis service for NBL tumor material for 15 years and more than 350 clinical analyses have been performed, mainly using the FISH technique. During the last two years we have introduced MLPA and arrayCND in the clinical setting. We present data on comparisons of the different methods and our experiences of their different advantages and disadvantages.

Methods: Thirty primary NBL tumors were subjected to genetic analyses by all three methods; FISH with probes for MYCN and distal 1p, MLPA, using the SALSA MLPA P251/P252/P253 kit from MRC-Holland and arrayCND using the Affymetrix 250k SNP arrays.

Results: Direct comparisons of analyzed regions showed no major differences. All used methods detect the aberrations they are directed to detect. Some fundamental differences between the three methods used: With the FISH technique the lab worker study single cells in the microscope, which enables detection of rare islands of cells, with unfavorable genetic pattern within a section of normal cells. In MLPA and arrayCND in contrast, DNA from a biopsy is used. Heterogeneity in samples was thus better visualized using FISH. With the MLPA kit several regions implicated in NBL genetics could be analyzed. Using the arrayCND method all structural aberrations could be readily detected, also those in regions not represented by the FISH probes or in the MLPA kit.

Conclusions: ArrayCND provided a detailed whole-genome-view of structural and numerical changes and it proved very useful and robust. In heterogeneous biopsies the FISH technique is useful as a complementary technique.

C63 Novel Treatment Approach for Relapsed/Refractory Neuroblastoma - An Evidence for Graft Versus Tumor Effect after Haploidentical Stem Cell Transplantation?

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Background: A pilot study was performed to evaluate feasibility of high dose iodine-131-metaiodobenzylguanidine (^{131}I-MIBG) followed by reduced intensity conditioning (RIC) and transplantation of T-cell depleted haploidentical peripheral blood stem cells (haplo-SCT) for refractory/relapsing neuroblastoma (RRNB).

Methods: Five patients with RRNB were treated after relapse (three patients relapsing after autologous stem cell transplantation, one after conventional treatment) or induction therapy failure (one child). All patients had residual disease at transplantation and received high dose ^{131}I-MIBG (median: 10.9 mCi/kg) on day -20, followed by fludarabine (125 mg/m^2), thiotepa (10 mg/kg), and melphalan (120 mg/m^2) given from day -8 to day -1. G-CSF mobilized T-cell depleted haploidentical paternal stem cells were infused on day 0 together with donor derived mesenchymal stem cells. A single dose rituximab (375mg/m^2) was given on day +1. Commencing cessation of short immunosuppression (mycophenolate mofetil, OKT3) all patients received donor derived T-cell infusion (DLI).

Results: The ^{131}I-MIBG infusion and RIC were well tolerated. All children engrafted. One patient rejected the graft and was rescued with autologous back-up. No primary acute GvHD was observed. Four children developed acute GvHD after DLI and were successfully treated. One child relapsed 7 months after haplo-SCT. Three children are alive and well 25-27 months after transplantation.

Conclusions: High dose ^{131}I-MIBG followed by RIC and haplo-SCT for RRNB is feasible. Three children achieved complete remission and are alive and well with no signs of disease >2 years after haplo-SCT. Further studies are necessary to evaluate the contribution of targeted therapy and the role of immune mediated tumor control.

Poster

C64 Radiosensitisation of High Administered Activity ^{131}I-Meta-Iodobenzylguanidine and Topotecan in Neuroblastoma (MATIN): Audit of Treatment Outcome in Five European Centres

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Background: MATIN (meta-iodobenzylguanidine and topotecan in neuroblastoma) is a schedule based on preclinical data showing synergy between ^{131}I-meta-iodobenzylguanidine (^{131}I-mIBG) and topotecan. It incorporates *in vivo* dosimetry to allow a safe and reliable increase in the administered activity of ^{131}I-mIBG. The intent is to deliver a total whole body absorbed radiation dose of 4 Gy from two administrations two weeks apart. It also incorporates the radiosensitising drug topotecan. MATIN is myeloablative, and stem cell rescue is used in all patients.

Methods: An audit of indications for, and feasibility and toxicity of, and response to this complex treatment schedule has been performed.

Results: The MATIN schedule has been piloted in 45 patients in 5 institutions across Europe between 2001 and 2007. The median age was 6 years, range 1-45 years. Thirty patients received MATIN for relapsed disease following treatment including high-dose chemotherapy. Fifteen patients received MATIN due to their inadequate response to initial chemotherapy. Despite anxieties that this schedule was too complex to deliver, 42 patients received treatment according to protocol, and MATIN was repeated in 6 patients. The treatment was well tolerated, and bone marrow suppression was the principal side effect. No unexpected side effects were seen. One patient died from toxicity. Thirty eight of 45 patients experienced symptomatic improvement, and objective responses were seen in 29 patients. Nine of 15 patients proceeded to potentially curative therapy as a result of MATIN. Median time to progression was 6 months in relapsed patients and 12 months in those treated with curative intent.

Conclusions: MATIN is a practical treatment option with acceptable morbidity and mortality rates. Good responses were achieved in a heavily pretreated group of patients, and this schedule will now be evaluated in a formal randomized phase II study comparing high administered activity ^{131}I-mIBG with and without topotecan.

C65 Oral Gefitinib, Topotecan, and Cyclophosphamide in Relapsed and Resistant Neuroblastoma

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Background: Experimental data have shown inhibitory activity of Gefitinib (G) on neuroblastoma (NB) cell proliferation in vitro and synergy of G with cytotoxic drugs, increasing anti-tumour activity in several tumour models. We evaluated the feasibility of an oral regimen including G plus topotecan (TPT) and cyclophosphamide (CPA) in heavily pre-treated stage 4 NB patients in a compassionate setting.

Methods: CPA (50 mg/m^2/day) followed by TPT (0.8 mg/m^2/day) plus G (fixed dose 250 mg/day) for 14 consecutive days were administered orally in the outpatient setting. Courses were repeated every 28 days until progression.

Results: Nine pts (3M/6F), median age 72 months (range 50-99), were enrolled since October 2004. All pts had relapsed or progressed after high-dose chemotherapy (HDC) followed by PBSC rescue. At inclusion, 5 pts had PR, one SD and one PD after second-line therapy, while 2 pts had experienced PD soon after HDC. Significant levels of EGFR mRNA were detected in 4/6 primary tumours. A median of 6 courses (range 1-24) were administered. A 25% dose reduction of TPT and CPA was required after the first course in 6 pts. After 4 courses, 3 pts were PR, 3 SD and 3 PD. After a median follow-up of 14 months (range 6-38), 4 pts are DOD and 5 are AWD. Of these 5 pts, 3 are still on treatment at 6+, 9+, 19+ months since inclusion and 2 pts have discontinued treatment due to PD. Median PFS is 8 months (range 1-27). Neutropenia CTC grade 4 was observed in 3 pts, thrombocytopenia grade 3/4 in 6, rash grade 2 in 2, and hepatic toxicity grade 3 in 1.

Conclusions: The regimen was feasible and well tolerated in this series of heavily pre-treated pts. PFS is encouraging, but deserves further evaluation in a phase II study.

C66 A Study for Children with Advanced Neuroblastoma with Peripheral Blood Stem Cell Supported Time Intensive Induction Chemotherapy, Followed by Myeloablative Therapy and Delayed Local Control Therapy

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Background: Neuroblastoma is the most common malignant solid tumor of childhood in Japan. Even though many clinical trials had been performed, the disease free survival rates of advanced neuroblastoma(ANB) is still low and unacceptable. In this study, we have evaluated the feasibility of time-intensive 6 courses initial induction chemotherapy with peripheral blood stem cell support(TI with PBSC support), immediately followed by myeloabrative therapy with autologous non-purged peripheral blood stem cell transplantation (SCT) and delayed local control treatment (DL)after SCT.

Methods: From March 1999 to April 2004, in Nihon university Itabashi Hospital, we had treated 6 newly diagnosed ANB patients with TI with PBSC support, SCT and DL therapy. 4 were male and 2 were female. The median age at diagnosis was 4 years old (range, 2 to 8). All patients were treated with induction chemotherapy with cisplatin(120mg/m^2 administered continuous infusion over a period of 120hours),doxorubicin(40mg/ m^2 on day3), vincristine(1.5mg/m^2 on day1) and cycloph osphamide(1,200mg/ m^2 on days 1 and 2). After the 3rd course of induction chemotherapy, every after chemotherapy, all patients were infused auotologous PBSC in order to support the recovery from the myelosuppression(1.12 – 21.90x10^{6}cells of CD34 positive). After 6 courses of TI with PBSC support,all patients were treated with myeloabrative therapy with carboplatin (1600mg/m^2), etoposide(800mg/m^2), melphalan(180mg/m^2), and total-body irradiation(10Gy). And after myeloabrative therapy, DL which consists of tumor removal and intraoperative radiation were performed. 13-cis-retinoic acid(160mg/ m^2/day) were administered for all patients after DL.

Results: The average duration between every induction chemotherapy was 32.8 days(28 – 50). No toxicity of PBSC apheresis was observed. Allergic reactions after PBSC infusion were observed in one. Five patients achieved CR after DL and one patient did not. The median follow-up time of all patients was 56 months (range,19 to 94 months). 5 are alive and free of disease. 1 had died of primary disease.

Conclusions: Our result suggest that TI with PBSC support, SCT and DL therapy is feasible for newly diagnosed ANB children.

C67 Iodine-131-Metaiodobenzylguanidine Therapy with Reduced-intensity Allogeneic Stem Cell Transplantation in Recurrent Neuroblastoma

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High-risk neuroblastoma has a poor outcome and new agents have been developed for recurrent neuroblastoma. Iodine-131-metaiodobenzylguanidine (MIBG) therapy is also a new approach for recurrent neuroblastoma. We describe the case history of a 6-year-old girl with recurrent neuroblastoma who received MIBG therapy with reduced-intensity allogeneic stem cell transplantation (RIST) because of an extensive bone marrow involvement. The post-transplant course was uneventful and complete chimerism was obtained. Neither acute nor chronic graft-versus-host disease (GVHD) was observed. The patient remained in remission for 3 months after RIST until the second relapse. MIBG therapy combined with RIST warrants further trials.

C68 The Impact of the Extent of Primary Site Resection on Event-Free Survival, Overall Survival, and Local Recurrence in High-Risk Neuroblastoma. A Report from the Children's Oncology Group A3973 Study

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Background: The need for complete primary site resection in high-risk neuroblastoma is controversial. We assessed the impact of extent of resection of the primary tumor by comparing event-free survival, overall survival, local recurrence, and surgical morbidity and mortality of patients entered into the Children's Oncology Group (COG) A3973 study stratified by extent of resection.

Methods: Of 488 eligible high-risk patients in this study, 238 had surgical data for this analysis. Extent of dissection was determined by review of surgical checklists and operative notes. The log-rank test was used to compare 2-year survival curves and cumulative incidence curves by extent of resection. Fisher's exact test was used to compare complication frequency.

Results: There were 28/238 (11.8%) stage 3, and 206/238 (86.6%) stage 4 patients. Median follow-up after resection was 33.3 months among 143 patients who were alive at last contact. ≥90% resection was accomplished in 69.4% and <90% resection in 30.6%. Surgical complications were observed in 27.3% with ≥90%, and 11.8% with <90% resection (p<0.02). There were no operative deaths and outcome is summarized below in Table 1.

Extent of resection	N	Event-free survival ± std error p=0.143	Overall survival ± std error p=0.228	Cumulative Incidence of Local recurrence p=0.002
≥90%	154	53%±4%	74%±4%	5%±1.7%
<90%	68	42%±6%	63%±6%	18%±4.8%

Conclusions: ≥90% resection was associated with significantly better local control, and event-free and overall survival appears higher although not statistically significant. The operative morbidity for patients undergoing more extensive resections was higher. These data support complete primary site resection in high-risk neuroblastoma.

C69 Consistent Evaluation of Image Defined Risk Factor for Localized Neuroblastoma by a Single Pediatric Radiologist at Two Major Hospitals in Osaka, Japan

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Background: Image defined risk factor (IDRF) has been developed to specify which neuroblastoma patients should be primarily operated safely. Currently new staging system using IDRF is under discussion at INRG. Mostly in large studies, evaluation of initial imaging was made at each hospital and the results were sent to data center in writing. Therefore, it seems to be difficult to evaluate IDRFs consistently. In order to evaluate IDRFs consistently by a single pediatric radiologist with direct access to imagings, we planed retrospective study at two major hospitals in Osaka, Japan.

Methods: 92 patients with localized neuroblastoma diagnosed between 1991 and 2007 were retrospectively evaluated. All images before treatment were directly accessed and evaluated by a single pediatric radiologist according to IDRFs (described as surgical risk factors in Cecchetto G et al. J Clin Oncol, 2005). There were 77 patients detected through mass screening and 15 patients found clinically.

Results: Of 92 patients, there were 58 patients (63.0%) without and 34 patients (37.0%) with IDRFs. Significantly fewer patients with IDRFs were observed in this cohort in comparison with the European study (37.0% vs. 50.0%, P < 0.05). An attempt at complete tumor excision was made in 53 patients; 42 of 58 patients without and 11 of 34 patients with IDRFs (72.4% vs. 32.4%). Complete excision was achieved in 37 of 42 patients without and 7 of 11 patients with IDRFs (88.1% vs. 63.3%). The type of excision varied significantly according to the presence or absence of IDRFs (P < 0.001). Nonfatal surgery-related complications occurred in 10 of 53 patients (18.9%) and were observed in fewer patients without IDRFs (16.7 vs. 27.3%).

Conclusions: IDRFs presence was associated with lower complete resection rate and greater risk of surgery-related complications. Mass screening may affect the lower incidence of patients with IDRFs in this Japanese cohort.

C70 Surgical Resections in Neuroblastoma Patients: Outcomes from AIIMS-Neuroblastoma-96 Trial

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Background: To correlate resection of primary tumor with overall survival in children with neuroblastoma (NB) on AIIMS-Neuroblastoma-96 protocol.

Methods: All children with NB registered from May 1996 through August 2005 were included. Chemotherapy consisted of cyclophosphamide, doxorubicin, etoposide and cisplatin. Radiotherapy was given to all stage-3 and 4.

Results: The overall survival(OS) for 86 children included in the study was 61.3% with a mean survival time of 55.5 months (95% CI 42.9-68). The 3-year disease free survival(DFS) was 31.4%. Forty-five (52.3%) underwent resection while 41 (47.6%) did not with an OS of 66.7% and 56% and 3-year DFS of 44.2% and 19.5% respectively. Among 23(26.7%) <1-year old patients, 7(30.4%) underwent resection and 16(69.6%) did not and the OS in these were 85.7% and 50% respectively. Among 63 (73.3%) >1-year old patients, 38(60.3%) underwent resection and 25(39.7%) did not and the OS in these were 63.1% and 60% respectively. Among 36(41.8%) stage-3 patients 22(61.1%) underwent resection and 15(68.1%) of these survived while 8 of 14(57.1%) who did not undergo resection survived. Among 41(41.6%) stage-4 patients 17(41.5%) underwent resection and 9(52.9%) of these survived while 13 of 24(54.2%) who did not undergo resection survived. Of the 53 tumors of adrenal origin 29(54.7%) underwent resection and 24(42.3%) did not and the OS in these was 62% and 37.5% respectively. Of the 33 tumors in other locations 16(48.5%) underwent resection and 17(51.5%) did not and the OS was 75% and 82.4% respectively.

Conclusions: Though the OS was not significantly different, the 3-year DFS was significantly better (44.2% vs 19.5%) among those undergoing resection and those not being resected. Survival rates were significantly better in patients < 1-year of age (85.7% vs 50%), stage-3 patients (68% vs 57%), and tumor of adrenal origin (62% vs 37.5%) when these were resected.

C71 The Implications of Surgical Intervention in the Treatment for Neuroblastoma

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Background: The role of surgical resection in the treatment for neuroblastomas is still controversial. In the present study, the implications of surgical intervention in neuroblastoms were assessed from cases in one institution.

Methods: In 123 neuroblastoma patients treated in our institution from 1985 to 2004, clinical characteristics, biology of tumor and extension of resection were analyzed.

Results: Of 82 neuroblastomas under 12 months, cases with stage 1,2,4S were 70 (85%), and cases with MYCN amplification were 2 (2%), cases with complete resection of primary tumor were 59 (72%), and case with dead of disease was only one (1%). Of 41 neuroblastomas over 1 year old, cases with stage 3,4 were 32 (78%), and cases with MYCN amplification were 15 (37%), cases with complete resection of primary tumor were 19 (46%), and cases with dead of disease was 26 (63%). Of 41 neuroblastomas over 1 year old, there were no significant difference of survival rate between 19 cases with complete resection and 22 cases with incomplete resection. No local recurrence was found in six cases with stage 4 over 1 year old who underwent complete resection of primary tumor and local irradiation, however, 4 of 6 cases died of metastatic recurrence.

Conclusions: In neuroblastomas under 12 months, the main treatment is the initial tumor extirpation, and initial tumor extirpation can avoid the chemotherapy. In advanced neuroblastomas over 1 year, local recurrence can be avoided by complete resection of primary tumor and local irradiation, however, the main treatment for matastasis is the systemic chemotherapy, and the surgeon should avoid the injury of main organ to prevent the administration of intensive chemotherapy at the radical operation of primary tumor.

Poster

C72 The Laparoscopic Biopsy for Abdominal Neuroblastoma

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Background: Neuroblastoma is one of the most common solid tumors, and the biopsy of this tumor is usually required as part of a planned multimodal treatment. In 1995, Holcomb and coworkers first reported endosurgical procedures for the diagnosis and treatment of pediatric malignancies, however the usefulness of laparoscopic biopsies for abdominal neuroblastoma is still unclear. The efficacy and complications of laparoscopic biopsy for abdominal neuroblastoma is evaluated.

Methods: Since November 1998, 25 laparoscopic biopsies (LB) and 6 open biopsies (OB) were performed for abdominal advanced neuroblastoma (Stage III or IV) at Saitama Children's Medical Center. After creating a pneumoperitoneum, the incisional biopsy using scissors was performed. The insufflation pressure was increased to 12 to 15 mmHg to prevent blood loss from the surface of the incised tumor. Operation time, blood loss, time to start postoperative feeding, and time to start chemotherapy were compared in both groups.

Results: LB significantly reduced the time to start postoperative feeding as well as the time to start chemotherapy. Operation time and blood loss were not different in both groups.

Conclusions: Precise indications of LB for the diagnosis of abdominal neuroblastoma provide better quality of life for infants and children.

C73 Laparoscopic Adrenalectomy in Children- 2 Centres Experience

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Background: Since 1992, when laparoscopic adrenalectomy (LA) has been first described, it become a treatment of choice in benign adrenal gland lesions. Treatment of malignant adrenal tumor attracts many of surgeons nowadays. Most of reports of laparoscopic adrenalectomy deals with adults, while using this method in children is quite rare, especially the role of LA in malignancy in pediatric population is still not well defined. We reviewed our two centre experience with laparoscopic adrenalectomy in children.

Methods: This retrospective two centre study included seven children (three boys, four girls), aged from 13 months to 15 years (mean 6 years) treated between September 2005 to August 2007. All lesions were unilateral (2: left-sided and 5 : right-sided) and they were detected postnatally by abdominal ultrasonography and then confirmed by computer tomography. The clinical diagnosis before surgery was neuroblastoma stage I in 5 cases and stage IV in 2 cases.

Results: All adrenal lesions were resected completely by transperitoneal approach. Three to six trockars were used and intraabdominal CO^2 pressure was 10-12 mm Hg during procedures. In one case conversion to open procedure was necessary due to technical problems. There were no postoperative complications. Blood transfusion was not required. Mean operative time was 120 minutes. Mean hospital stay was 6 days (5 to 8 days). All children are alive. Postoperative follow-up ranged from 4 to 29 months (mean 12 month). In all neuroblastoma cases no metastases or local recurrence occurred.

Conclusion: Laparoscopic adrenalectomy for neuroblastoma in children seems to be safe and feasible, especially in locally less advanced stages, but to assess its role collection of further multicenter experience is necessary.

C74 New Surgical Approaches in Neuroblastoma "Infant": Ten Years Experience

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Background: We present a review of last 10 years (1997-2007) cases of Neuroblastoma (NB) diagnosed and treated at Gaslini Children's Hospital (Genoa, Italy), as a contribution to an international guidelines workshop based on multiple clinical trials.

Methods: We revised the clinical notes of 180 patients: 149 patient resulted eligible for study purpose. Patients were divided in 2 clinical groups: group A included patient below 12 months of age ("infant") (53 cases, 36%), group B included patients older than 12 months of age (96 cases, 64%). Each group was analysed considering 1) Staging (International Neuroblastoma Staging System, INSS); 2) Localisation of NB; 3) Immediate surgery; 4) Delayed surgery; 5) Recurrence.

Results: Event-free survival (EFS) and overall survival (OS) curves and 5 years survival rates for each group were calculated: 5 years EFS resulted in 90% and 50% for group A and B respectively, 5 years OS resulted in 85% and 50% for group A and B respectively.

Conclusions: These results are in accordance with international NB outcomes, and once more we want to emphasize how important patient age is among other risk factors.

C75 Palliative Treatment in Neuroblastoma: Etoposide (VP16), an Effective Drug

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Background: No further treatment of relapse after high-dose chemotherapy (HDC) and stem cell transplantation (SCT) has been described yet. Patients treated at our centre can undergo phase I-II studies or palliative treatments. We present herewith our experience on VP16 in relapsing or progressing patients after HDC and SCT.

Methods: VP16 was administered orally at the dose of 100 mg/m²/day for 3 consecutive days for 3 consecutive weeks with a stop of one week between the courses. It continued until evidence of clinical progression. Patients who received 1 to 2 courses were considered non-responders, those who received 2-4 courses poor-responders and those who received more than 4 courses good-responders.

Results: From 1985 to 2006, 69 patients were treated with oral VP16 which was first-line relapse chemotherapy in 46 patients, second-line in 19, and third-line in 4. They received a total of 342 courses with a median of 3 (1-21) courses. 28 patients (40.5%) were non-responders, 17 (24.7%) poor responders and 24 (34.8%) were good responders and received a median of 8.5 (7-21) courses.
No significant toxicity were observed and this treatment allowed a good quality of life. The only factor displayed to have a significant impact on response and survival was the time to relapse.

Time to relapse from SCT (months)	NON-RESPONDERS	POOR-RESPONDERS	GOOD-RESPONDERS	Total
0-6	9 (75%)	3 (25%)	0	12
7-12	7 (41%)	6 (35%)	4 (23%)	17
>12	12 (30%)	8 (20%)	20 (50%)	40
Total	28 (40.5%)	17 (24.7%)	24 (34.8%)	

P= .006 (Fisher test)

Conclusions: Low-dose oral VP16 schedule should be considered as an effective palliative treatment. As time to relapse has been demonstrated to impact on response and survival, this should be taken into account for further studies evaluating new drugs.

Poster

C76 Outcomes of the Pediatric Oncology Group 9340/9341/9342 Trials for Children with High-Risk Neuroblastoma: A Report from the Children's Oncology Group

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Background: From 1993 through 1995, the Pediatric Oncology Group (POG) enrolled patients with high-risk neuroblastoma on 3 sequential, conjoined studies: a phase II induction window (9340), followed by intensive multiagent induction chemotherapy (9341), and subsequent myeloablative therapy with autologous stem cell rescue (9342). We report here the outcomes of patients treated on these studies.

Methods: Patients were between one and 21 years old with high-risk neuroblastoma. Phase II window therapy consisted of 2 courses of either paclitaxel, topotecan, or cyclophosphamide wth topotecan for the phase II window. Induction therapy consisted of at least five cycles of intensive chemotherapy, followed by myeloablative therapy with purged autologous stem cell reinfusion. Patient responses, treatment toxicities, and overall and event-free survival rates were calculated.

Results: 84% of patients responded to induction chemotherapy, with 39% achieving complete response. Toxicities were primarily hematologic. The 7-year EFS and OS rates for all eligible patients on POG 9341 were 23% +/- 4% and 28% +/- 4%, respectively. The 7-year EFS and OS rates for patients treated on POG 9342 were 27% +/- 6% and 29% +/- 6%, respectively.

Conclusions: These studies were the first attempt by POG to use autologous stem cell transplantation for neuroblastoma treatment in a cooperative group setting. Toxicities and outcomes were comparable to contemporary cooperative group studies. The phase II induction window had no detectable effect on outcomes. New strategies are needed to improve survival for this devastating disease.

C77 Low-dose Protracted Irinotecan as a Palliative Chemotherapy for Advanced Neuroblastoma

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Background: The management of refractory neuroblastoma remains a challenge. As intensive chemotherapy sometimes results in severe regimen-related toxicity and poor quality of life (QOL), palliative therapy for maintaining good QOL as long as possible should be given to these cases. However, palliative chemotherapy for refractory neuroblastoma has not been established yet.

Patients and Methods: Four cases (ages 2-12 years) of heavily pretreated refractory stage 4 neuroblastoma received a low-dose protracted schedule of irinotecan (20 mg/m^2/day for 5 days per week for 2 consecutive weeks). MYCN was amplified in 2 of the 4 cases. Three cases had suffered from intestinal paralysis and/or severe ematiation due to tumor involvement and regimen-related toxicity before irinotecan, and exhibited a poor performance status (PS). Normally, the administration of irinotecan was repeated every 3 weeks. However, when therapy-related toxicity affected the QOL, we suspended or delayed the irinotecan schedule. Informed written consent was obtained before starting irinotecan.

Results: A low-dose protracted schedule of irinotecan achieved not only disease stabilization but also dramatic improvement of the QOL for 12-22 months in 3 of the 4 cases. For the 3 cases with severe emaciation and poor PS, this regimen dramatically reduced their emaciated state, allowing them to stay home during the intervals between irinotecan courses. This regimen was tolerable except for one case whose 3 courses of irinotecan resulted in grade 4 hematologic toxicity. Mild gastrointestinal toxicity occurred in all cases, but it was manageable with supportive treatment.

Conclusions: This dosing schedule of irinotecan not only prevented disease progression, but also dramatically improved the QOL for significant periods. This regimen was tolerable even for the patients with poor PS or intestinal paralysis. Therefore, low-dose protracted irinotecan might be an attractive modality for palliative treatment for advanced neuroblastoma.

C79 Whole-Body Diffusion-weighted MR Imaging of Neuroblastoma Changes Disease Stage and Monitors Response to Therapy

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Background: Diffusion-weighted MR imaging (DW-MRI) has been reported to be useful to detect solid tumors such as breast cancer, rectal cancer and prostate cancer. The purpose of this study was to investigate whether DW-MRI is useful to evaluate the staging of neuroblastoma and monitor the tumor response to chemotherapy as compared to conventional oncological imaging methods.

Methods: We examined seven children with neuroblastoma at diagnosis and during the follow-up period using whole body DW-MRI, MIBG and bone scintigram. Diagnosis was established hitologically. The median age was four years old (range one to eight). Two were stage II, one was stage III and four were stage IV.

Results: The results of DW-MRI were not matched with either MIBG or bone scintigram in two out of seven cases. Three suspicious spine metastases were detected in one stage II patient only by DW-MRI at diagnosis. Since bone biopsy of the region histologically proved the metastasis of neuroblastoma, the stage was changed from II to IV. Another stage IV neuroblastoma patient had multiple bone metastases at diagnosis. After 6 courses of chemotherapy sacral region remained abnormal by DW-MRI but not by MIBG or bone scintigram. Since bone biopsy revealed viable tumor cells at the same region, local irradiation at the region was given with mega-dose chemotherapy.

Conclusions: DW-MRI was safer from irradiation than MIBG or bone scintigram. Sensitivity of DW-MRI was 3 mm in diameter while that of MIBG and bone scintigram was 10mm. Whole-body DW-MRI may be useful to detect the bone metastasis and assess the response to the therapy in neuroblastoma due to the small cell histology of the tumor.

C80 Favorable Response to Irinotecan and Temozolomide in a Case of Relapsed Neuroblastoma

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Background: Although treatment of advanced neuroblastoma (NB) has been improved in recent years, the prognosis is particularly dismal once patients with this tumor relapse after intensive treatment. It was in this spirit that we administered irinotecan and temozolomide in the following patient who had a relapse.

Methods: A 2-year-old Japanese boy was referred with an abdominal mass. He was diagnosed as stage 4 NB originating in the right adrenal gland with multiple bone metastases. Chemotherapy was started and excision of the tumor was carried out. Afterwards he received autologous stem cell transplantation with a conditioning regimen consisting of melphalan, etoposide, and carboplatin. Three years later he had recurrence in the bone, which was histologically the same as the initial specimen. Since then he has suffered from four relapses, either bone and/or bone marrow metastases. He received another stem cell rescue to overcome intensive chemotherapy. Nevertheless he again had multiple bone metastases at the age of eight. Chemotherapy consisting of irinotecan 50 mg/m^2/day and temozolomide 150 mg/m^2/day for 5 consecutive days was started.

Results: After two courses of irinotecan and temozolomide, improvement in tumor size was seen on MIBG scintigram. He received a total of six courses of therapy. MIBG showed almost complete resolution of bone metastases. The patient was then placed on higher doses of irinotecan regimen 180 mg/m^2/day for 3 consecutive days. The patient is scheduled to receive ^{131}I MIBG therapy for curative intent.

Conclusions: According to recent analyses, the 5-year survival rate was 41.1% for stage 4 NB in Japan, but there are no reports of irinotecan and temozolomide for these patients. The responses reported here in our patient suggest that irinotecan and temozolomide might be included as an active agent in the treatment of advanced NB.

C81 Presacral Cystic Neuroblastoma: A Case Report

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Background: Neuroblastoma is the most common extra-cranial childhood cancer, and is the most common malignancy in childhood. It is clinically and biologically heterogeneous, with very variable outcome. Cystic neuroblastoma is a rare variant, mostly arising from the adrenal glands, and usually presenting in early infancy. We report an unusual case of cystic neuroblastoma localized to the presacral region in a relatively older child.

Case Report: Our patient, N, was a 4-year-old Malay girl who presented with cough and fever for a week. A chest x-ray showed left lower lobe consolidation. She was tested positive for *Mycoplasma pneumoniae* and Influenza B. She however continued to have persistent fever despite antibiotic therapy. Incidentally she developed an episode of acute urinary retention during her admission. Ultrasound scan revealed a large cystic mass in the pelvis. Computed tomography (CT) scan showed that the mass was a large, midline and thick-walled cystic mass. The child underwent diagnostic laparoscopy, which showed a large tense pelvic mass stuck to the sacral periosteum. Laparotomy and tumour excision was done. The cyst ruptured on mobilization; the cyst fluid was chocolate-brown. The histology showed necrotic debris, with small foci of residual tumour consisting of differentiating neuroblastoma associated with focal calcification and extensive acute haemorrhages. Cytogenetic analyses could not be done because of the small amount of viable tumour tissue. We then proceeded to evaluate her disease. Her CT chest, bone scan, bone marrow aspiration and trephine biopsies, and urine vanillylmandelic acid were all normal. She also underwent Metaiodobenzylguanidine (MIBG) scan, which did not show evidence of MIBG-avid disease. She remained well and disease-free 1 year after her tumour resection.

Conclusion: This is, to our knowledge, the second reported case of presacral cystic neuroblastoma. Our case is unusual because it occurred in a relatively older child.

C82 Congenital Neuroblastoma with Multiple Sub Cutanous Nodul

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Background: Neuroblastoma is the most common malignant tumor of the newborn. Congenital neuroblastoma is rare and which can present as localized or metastatic diseases.

Methods: We report a case of congenital neuroblastoma with subcutaneous metastases in 10 day boy that admitted in our children hospital.Neonatal sepsis was diagnosed .
Antibiotic and respiratory support treatment was used.
Chest X- ray revealed pleural effusion in right lung; respiratory rate was 65/min. Abdominal sonography revealed adrenal mass lesion 17 × 26 mm and hepatomegaly.Nodular biopsy was performed.

Results: On histopathology examination nodular biopsy were diagnosed as neuroblastom abdominal was distended.
Multiple nodular distributed over his body. A biopsy was performed a nodular on arm. The histopathological picture confirmed the diagnosis of neuroblastoma. The patient died 44 day after birth

Conclusions: the first clinical manifestation usually result from complication of metastases disease, chemotherapy is unnecessary, but when there is evidence of disease progression chemotherapy is given.

C83 Encouraging Results of HLA-Mismatched Allogeneic Hematopoietic Stem Cell Transplantation for Advanced Neuroblastoma

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Background: Prognosis of advanced neuroblastoma (NBL), especially with bone metastases and/or MYCN amplification and/or poor response to chemotherapy is dismal, despite double megatherapy followed by autologous hematopoietic stem cell rescue in our experience. In order to overcome these issues we have been asking the role of allogeneic hematopoietic stem cell transplant (HSCT) especially from HLA-mismatched donor for high-risk advanced NBL.

Methods: We retrospectively analyzed nine of double transplant including HLA-mismatched allogeneic HSCT for high-risk advanced NBL performed between 1998 and 2004.

Results: The median age of the nine patients at diagnosis was two years and three months (range six months - six years). All patients were diagnosed as INSS 4 and all but one had bone metastases. MYCN was amplified in two patients. Six patients out of the nine received auto-allo double transplants and three patients received allo-allo double transplants. Seven patients received selected CD34 positive hematopoietic cells from an HLA haplo-identical parent and two patients received HLA-mismatched cord blood as an allogeneic transplant. One of three patients who underwent allo-allo double transplants received unselected hematopoietic cells as the second transplant from his HLA haplo-identical mother. Five patients (55.6%) out of the nine (auto-allo, 3/6; allo-allo, 2/3) are maintaining complete remission after double transplants for median 55 months (range, 36 – 70 months). One patient died of sepsis after the second transplant and no other severe transplant related complications were not observed.

Conclusions: Allogeneic HSCT has advantages compared to autologous transplant; no contamination of tumor cells in the graft and the presence of graft-versus-tumor (GVT) effect. Furthermore, HLA-mismatched allogeneic HSCT for high-risk advanced NBL may be superior to HLA-matched allogeneic HSCT under considerations of donor availability as well as GVT effects if transplant-related morbidity is well controlled.

C84 Molecular Diagnosis of Neuroblastoma: The Nation-Wide On-Line Report System in Japan

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Background and Purpose: The centralization of the molecular and pathological diagnoses is prerequisite for risk classification of neuroblastoma to choose a suitable therapeutic strategy. For the past 12 years, we have worked on establishing a system of molecular diagnosis as well as tissue bank of neuroblastoma in Japan. Our tissue bank has so far collected more than 2,500 neuroblastoma samples from 139 hospitals in Japan. Here we report our new system with an on-line report linked to the Japanese Neuroblastom Study Group (JNBSG).

Results and Discussion: In the historical process to establish biological diagnostic system of primary neuroblastomas, we employed to measure DNA ploidy (FACScan), responsiveness of tumor cells to nerve growth factor (NGF) in primary culture, *MYCN* copy number (Southern blot) and *TrkA* mRNA expression (Northern blot). However, the current molecular diagnosis has been focused on DNA ploidy (FACScan) and *MYCN* copy number measured by both quantitative real-time PCR and two-color FISH analyses. These data are reported on-line with password to each physician and in case to the JNBSG data center for the tumors registered. From January, 2006 to December, 2007, we analyzed 249 neuroblastoma samples. The number of patients with *MYCN* copy numbers of <2, 2-4, 4-10, and >10 were 140, 12, 0, and 35, respectively, in 187 samples. The combination of the above three tests gave a confirmative result. We are currently preparing to include pattern of genomic aberrations using customized array-CGH method. *TrkA* expression is included in the cDNA mini-chip which we established and customized in the company, SRL, Ltd., Japan. This mini-chip contains 200 genes top-ranked in our previous algorism to predict the prognosis of neuroblastoma, and it is under clinical validation. Thus, our molecular diagnosis system is practically well functioning by linking with the Pathology Center of Neuroblastoma.

C85 Neuroblastoma Screening at One Year of Age in Germany: There is No Remarkable Difference in Mortality between Screened and Unscreened Cohorts Seven Years after Completion of The Study

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Background: We previously reported overdiagnosis in children screened for neuroblastoma at one year of age (Schilling et al, NEJM 2002). Due to the short follow up at that time, the question whether neuroblastoma screening at one year of age may reduce mortality was not yet sufficiently addressable.

Methods: From 1995 to 2000, 2,581,188 children at one year of age were offered a urine test in six of 16 German states and 2,117,600 children in the remaining states served as contemporary controls. We compared the incidence of metastasized neuroblastoma and neuroblastoma related mortality at five and seven years after completion of the study.

Results: A total of 1,475,773 children were screened. Five years after the completion of the study, incidence in the screening area was found to be twice as high as in the control area (15.1 vs. 8.3 per 100,000 life births respectively). These additional, "overdiagnosed" cases detected by screening did not benefit from early detection or early treatment, but presumably would have regressed if not found by screening. Stage 4 incidence was used as early predictor for mortality and did not differ in both cohorts (4.1 vs. 4.6 per 100,000 investigated children). Mortality analysis after five years showed no difference between the screened and control cohorts (2.6 vs. 2.8 per 100,000). Analysis after seven years including subgroup analysis is currently underway and first results seem to support the observed trend. Detailed results will be presented.

Conclusions: Our results confirm previous findings, support our recommendation against implementation of neuroblastoma mass-screening and underline the importance of controlled, epidemiologic evaluation of this question.

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C86 Sexual Dysfunctions among Childhood Neuroblastoma Survivors in Developing Countries: Are We Truly Curing Them?

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Our success in pediatric oncology requires attention to the psychosocial consequences and quality of life. We must ask if the advances in pediatric oncology are resulting in what called a "truly cured child." It is incumbent upon oncologists to conceive of Neuroblastoma as more than just a disease that begins with diagnosis and ends when the treatment protocol ends instead, it initiates a life-long trajectory of survival having long-term implications for quality of life. Emotional needs are deeply frustrated in children, due to long hospitalization, separation from parents, friends, painful tests, anxiety and anguish, hours of loneliness without tenderness, this critical suspension from normal life and shift to emergency survival delays growth of autonomy and independence and further complicates e attainment of satisfying sexual identity and disrupts basic psychosexual maturity process. Adolescent sexuality is very complex, and even more when serious adverse event like cancer affects the individual well-being - sexual and non-sexual - in all its dimensions. Young survivors are significantly different in specific domains to healthy, they are less feminine in sexual identity have more restrictive and submissive images of sexuality, lower confidence with masturbation. They have less experience of intercourse. These effects can be better understood if put in perspectives with many changes and challenges young cancer survivors have to face and cope with in different emotional, affective, relational, cultural and existential domains. Cultural issues in our society, such as the myth that children are too young to be interested in sexuality and presumption that issues of survival overshadow sexuality, provide barriers to open communication about sexuality. Sexual function and fertility should no longer be regarded by oncologists as frivolous or irrelevant issues, the very privileged relationship that oncologist have with their patients should permit them to assist the patient with this aspect of health and recovery.

C87 Mass Screening for Neuroblastoma and its Mortality in Japan

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Background: In order to estimate the contribution of the mass screening for neuroblastoma toward reducing its mortality.

Methods: Using the data of the deaths of neoplasms in the adrenal glands (194.0 in ICD9; C74 in ICD10) in the vital statistics issued by the Japanese government (from 1981 to 2005), mortalities of neuroblastoma were calculated.

Results: For those aged 1-4 years, the mortality decreased in inverse proportion to the percentage of the children screened by HPLC within them. After around 1996, the mortality was stable, being 0.40 per 100,000 population per year. For those aged 5-9 years, the mortality decreased in inverse proportion to the percentage of the children screened by HPLC within them, too. After around 1999, the mortality was stable, being 0.25. The nation-wide mass screening program was stopped on March 2004. Virtually all of the children 0-1 year of age in the year 2005, therefore, were unscreened. In 2005, there were actually 6 deaths at these ages, which were more than 4.45 deaths expected from the mortality during 2001-04. To the contrary, most of the children 3-4 years of age in 2005 were screened. There were actually 7 deaths at these ages, though 10.1 deaths were expected from the mortality during 2001-04.

Conclusions: Through the mass screening the mortality became about 0.4 for the children aged 1-4 years, and 0.25, for those aged 5-9 years. These mortalities were about half of those before the start of the mass screening, respectively. It is possible that stopping the mass screening brought about the increase in mortality for those aged 0-1 year in 2005.

C88 Neuroblastoma Profile in Haji Adam Malik Hospital Medan Indonesia as a Top Referral Hospital in a Developing Countries

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Background: Neuroblastoma is one of the most common cancers affecting children today, forming 8%-10% of the total seen from birth through age 14 years. There is remain problem in developing countries to diagnose and management neuroblastoma regarding the lack of human resources and facilities. The incidence reported lower than their counterpart in developed countries may be due to underdiagnosis.

Methods: This study was retrospective, we review the medical chart of all children who visited Hematology-Oncology Division Out Patient Clinic and Pediatric Ward in Haji Adam Malik Hospital Medan-Indonesia during 2005-2007 period that have been diagnosed as neuroblastoma.

Results: In the study period, there were 65 children came to visit our hospital with abdominal mass. There were 22 of 65 cases that we diagnosed as neuroblastoma and enrolled to this study. As much as 10 cases refused when we suggested to follow some examination and in 15 cases were failed to diagnosis due to our limited facilities. Only 7 from 22 cases agreed to follow all diagnostic procedure and treatment plan, 15 cases did not follow our suggestion due to one and another reason. As much as 5 from 22 wanted to bring their child to traditional healer. Neuroblastoma affected group age 1-5 years 10 (22), followed by group age 5-10 years. The youngest patient in this study was 6 month old. Sex ratio 13 : 9 and almost all the sample were undernourished, some of them were suffered from severe malnutrition.

Conclusions: It is still remain problem to do screening for neuroblastoma in developing countries. The diagnostic tool and management are limited to the human resources and lack of facilities, and some of the deficit in many developing countries is likely to be due to under-diagnosis.

C89 Descriptive Epidemiology of the Neuroblastoma in Children in Kyrgyzstan

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Background: study the neuroblastomas incidence, sex, and ethnic differences.

Methods: there were 1955 registered with new diagnoses of cancer 1986-2000 in children, included 90 (9.2%) with neuroblastomas. Cancer registration in Kyrgyzstan is carried out by network of population-based regional registries, National Center of Statistic since 1983. Collected date from forms submitted along histological findings and death certificates. The population figures and cancer incidence rates were provided in for age, sex and ethnic groups. Counted crude, age-standardized rates (ASR) per 1 million. Estimated population relative risk in urban and rural areas.

Results: total ASR annual childhood cancer incidence was 74.8. The most frequent diagnostic groups were leukaemia's (20.8), brain tumours (7.3). Neuroblastomas were on the 4th place with ASR 4.3. Histological verification in neuroblastomas was 92%.
Of the 90 children 56 (62.2%) patients classified as ganglioneuroblastomas and 44 (48.8%) as sympathoblastomas. Boys (ASR 4.5) were affected 1.14 times as frequently as girls (4.1). ASR (6.8) was higher in the age youngest group (0-4). Analyses of geographical variations showed slightly highest incidence in urban (Bishkek, Chui) than rural (Naryn, Osh, Talas, Issyk-Kul) regions, but this not statistically significant (RR-1.1, 95% CI: 2.3-5.1; 2.4-4.1). Incidence rate in neuroblastomas was significantly higher in Uzbeks (6.1), Russians (5.4) compared with 3.8 in Kyrgyzs.
Between the 2 periods (1980-1989, 1990-1999) 5-year survival in neuroblastomas increased from 42% to 57%. There is evidence already that with new treatment regimens introduced during the 1980s survival in the 1990s will show increased gains.

Conclusions. Childhood neuroblastomas incidence in Kyrgyzstan especially in the native ethnic groups was low and similar to those reported from some Asian developing countries. The data could be use for a wide range of epidemiological and other studies. These include analyses of geographical variations in incidence, trends in survival, health of long-term survivors.

C90 The Effectiveness of Meetings of School Nurses for Study and Training (The Third Report) - For a Better Understanding of the School Children with Cancer –

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Background: In the earlier study, elementary schools teachers were surveyed regarding their attitudes toward children with cancer. Teachers find difficulty in dealing with these children and seek information on the disease and how to take care of the children. In response to such requests, meetings of the teaching staff for study and training were conducted and the effectiveness of these was confirmed. This study highlights the effectiveness and issues of the meetings of school nurses for study and training.

Method: The meetings were held twice. The meetings had the same contents as those for the teaching staff; (1) visit to an in-hospital classroom, and (2) a lecture regarding cancer diseases and the understanding of and response to the children and their families. In addition, before and after the meetings, a questionnaire survey was conducted that included 15 questions using a 4-point Likert scale as well as written comments regarding "the level of knowledge of pediatric cancer and the understanding of the response to the children and their families.

Results: 49 participants (39 valid responses). Compared with before and after the meetings, the points were significantly higher for all questions after the meetings. (Paired t-test, $P < .01$) School nurses were able to gain a better understanding of the educational environment during hospital stays, the mental state of children with cancer, responses to the children and their families and cooperation with medical practitioners.

Conclusion: Meetings of school nurses for study and training are effective for the promotion of a better understanding of school children with cancer and it is necessary to continue them in future.

C91 Sentiment of School-Age Patients of Pediatric Oncology and Family, and Their Care

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Background: To study the sentiment of patients around school-age of pediatric oncology and their families, their needs in the struggle against disease, and care in the school-age period.

Method: Five cases were studied. Content analysis was performed by semiconstitutive interview research. Approvals of the patients and families were obtained.

Results: What hurts them the most are the physical pains, such as inflammation brought on by chemotherapy, enema in an ileac state, drips and the collection of blood. What they enjoy are the playing and events. Some children cannot take off their mask of anxiety. Mothers are more deeply pained not by the disease name but in responding when asked, "Am I dying?" Cooperation with schools is insufficient. They felt that the world in TV programs has turned true. A point common to mothers was the difficulty to cope with the stress of the patients. They learned about the disease and therapy, and needed interaction among mothers having similar children. They now have a mutual gratitude.

Conclusions: The needs for care are:(1)Relief of children's physical pains and fulfilling their days(2)Support for independence of children with hopes(3)Sympathetic response to the affection of parents to children and relief of the fatigue of parents(4)Promotion of interaction among parents(5)Techniques of high quality of medical personnel and communication.

C92 Doctors' Perceptions of Promoting Autonomy in Children with Solid Tumors

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[1]Shukutoku University, [2]National College of Nursing, [3]Chiba University, [4]Chiba Rehabilitation Center

Back Ground: Progress of medical science has increased the survival rate for childhood cancer. More than 50% of children with cancer can return to daily life with their treatment continuing even after remission. In Japan, children with cancer should be hospitalized for treatment after 6 months, and they must undergo operation and other painful procedures. However, because of many adverse outcomes reported, it is natural to be passive and not have confidence through hospitalization.
As there are many experiences which they cannot but entrust, they are easy to be passiveness and to have no confidence.

Purpose: To examine doctors' perceptions of promoting autonomy in children with solid tumors.

Method: The subjects were pediatric oncologists and surgeons (n = 20). Self-report questionnaires developed by the authors were used to collect data. The subjects received the questionnaire directly or by mail. The number of questionnaires returned was 14.

Results: For promoting autonomy, the instances considered as most important by the pediatricians were "hospitalization," "discussing the disease with the child," and "operation." However, the pediatricians considered it very difficult to promote autonomy when "the treatment was ineffective and the child had to be shifted to palliative care," "the child refused any treatment," and "the child expressed unwillingness to undergo treatment.".
In case a child was of the age to understand the disease, all doctors considered direct discussion regarding the treatment with the child to promote autonomy. Moreover, for discussing the name of the disease, 7 doctors preferred discussing with the child, while 8 did not consider discussing this if the parents had an objection. Furthermore, for the adequate age of a child who could understand the disease and treatment, some doctors considered it to be more than 4-years, while others considered children in primary school as suitable for discussion.

C93 The Establishment of a Support Group for Caregivers of a Child with Neuroblastoma

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Background: About 30 children are diagnosed with neuroblastoma each year in Taiwan. There is a need to form a support group for caregivers of children with neuroblastoma to allow them to vent their emotions, share their experiences of raising a sick child, and gain peer support. This report: 1) shows the process by which a caregiver support group was set up; and 2) attempts to understand the needs of caregivers of children with neuroblastoma.

Methods: Responses from two parents in a pilot study suggested that a support group was needed for caregivers of children diagnosed with neuroblastoma. A group of multidisciplinary specialists including pediatric hematology oncologists, a pediatric surgeon, a pediatric radio-oncologist, and nurses was formed in a medical center in Northern Taiwan to discuss how to set up a support group. A list of parents with a child with neuroblastoma was compiled from medical records. A nurse specialist contacted each eligible caregiver via phone to invite him/her to participate in the first meeting of the support group. A 13-question survey was distributed to participants following the support group activity.

Results: 1). On October 16, 2007, a support group for caregivers of a child diagnosed with neuroblastoma was established. A total of 9 primary caregivers, 5 neuroblastoma children and two family members participated in the event. All 9 caregivers signed up to be the members of the support group. To date, a total of 16 primary caregivers have participated in this group; and 2). Their informational needs, in order of frequency of request, were prognosis, guidance about daily care, and explanation of treatment protocol and chemotherapy medicines, respectively. Some caregivers expect that attending a periodic support group meeting can provide them with the information they need.

Conclusion: The function of a support group is appreciated by the caregivers of a child diagnosed with neuroblastoma.

C94 Infant Neuroblastoma: Difficult Distinction Between Stage 4 and Stage 4s

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Department of Pediatrics, St. Luke's International Hospital, Tokyo, Japan

Neuroblastoma stage 4S occurs in childlen less than 1 year of age and metastases are confined to the liver, bone marrow, and skin but not in bone. We experienced a boy with neuroblastoma stage 4 which had most of characteristics of stage 4s. Four-month-old boy was admitted to our hospital because he had several spots on the skin. The general condition was good and his development was well. Diagnostic imaging studies revealed that he had a huge liver with multiple metastases, a-5cm-mass in the left adrenal and a-3cm-mass in the right adrenal. He had 4 subcutaneous lesions with purpura at 1-2 cm diameter. The plain X-ray film revealed bone lesion in the right calcaneus. MIBG scintigram showed uptake in the liver and bilateral adrenals and also in the right ankle. Bone scintigram showed a weak uptake in the right ankle. Bone marrow aspiration and biopsy from bilateral iliac bones revealed no cluster of tumor cells. The levels of VMA and HVA in the urine were very high and NSE in the serum was elevated (177 ng/ml), too. Based on these findings, the patient was clinically diagnosed as having neuroblastoma stage IV. Biopsy of skin nodules were performed. The pathology revealed undifferentiated neuroblastoma. Quantitative PCR analysis showed an amplification of MYCN around 5 copies and FISH also showed MYCN amplification around 10. DNA ploidy was diploid. We discussed with several authorities in the world and decided to observe him because he looked very healthy without decompensation. Furthermore, skin nodule disappered. Two months has past since he was diagonosed and we are still watching the patient. This baby has bone metastasis and MYCN is moderately amplified, that suggests an aggressive disease while skin lesions and his good general condition imply 4s disease. We would like audience to suggest a suitable treatment option.

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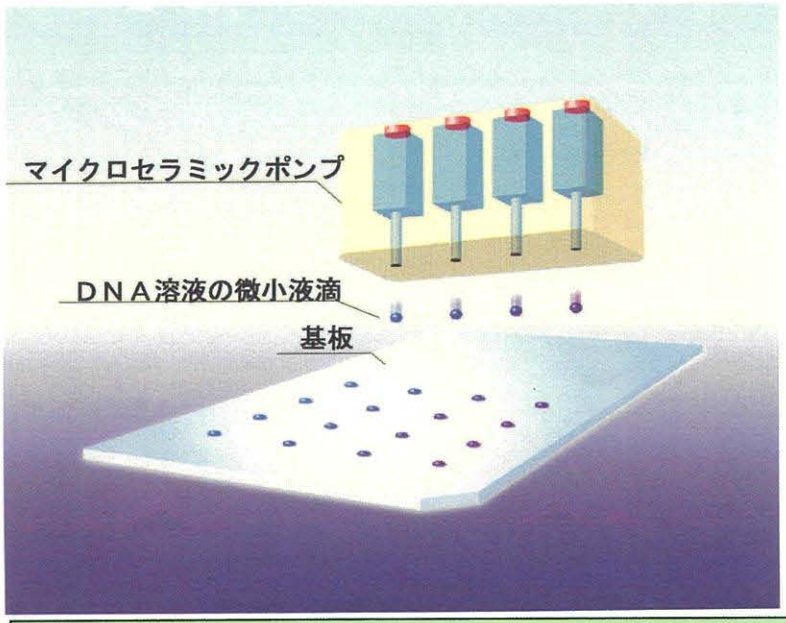
MEMO

DNAマイクロアレイの高品質カスタム製造サービス



非接触、ピコリットル単位で制御可能なスポット技術「GENESHOT^{R}」により、お客様の要望に応じたデザイン（サイズ／密度）でマイクロアレイを作成致します。

cDNA、オリゴDNAなど溶液を選ばず、確実にスポット致します。

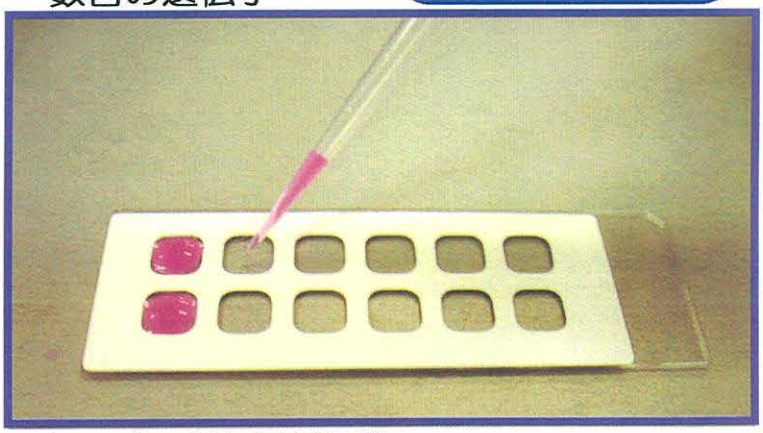
1，非接触、pl単位で制御可能の独自のマイクロセラミックポンプ方式！
2，スポット内のシグナル均一性が優秀！
3，チップ間の再現性に優れ、精度の高いデータ取得が可能
4，DNA溶液の変性や損傷がない
5，オリゴＤＮＡチップのＯＧＴ社特許ライセンス契約により製造権取得
6，12,000枚/年度の高実績！

・多検体アレイ

1基板で多検体処理可能
数十～数百の遺伝子

10$チップ実現！

搭載アレイ数（1基板当り）	搭載可能スポット数（1アレイ当り）
4アレイ	～4500スポット
6アレイ	～1000スポット
12アレイ	～500スポット
24アレイ	～195スポット
32アレイ	～64スポット



チップ間の再現性に優れ、精度の高いデータ取得が可能。
試薬類も少量化でき、アレイ実験のコスト低減にお役に立ちます。

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