SOCIETA ITALIANA DI CANCEROLOGIA

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Dangerous Liaisons

translating cancer biology
into better patients management

56th Annual Meeting of the Italian Cancer Society

Ferrara, 11-13 September 2014

Università degli Studi di Ferrara

56th Annual Meeting of the Italian Cancer Society

DANGEROUS LIAISONS

translating cancer biology into better patients management

CONGRESS VENUE
Università degli Studi di Ferrara - Via Luigi Borsari, 46 - 44121 Ferrara

OPENING CEREMONY VENUE
Azienda Ospedaliero Universitaria di Ferrara
Arcispedale Sant'Anna - Via Aldo Moro, 8 - 44124 Cona – Ferrara

SIC SECRETARIAT

SOCIETA ITALIANA DI CANCEROLOGIA

Società Italiana di Cancerologia
Via G. Venezian, 1 - 20133 Milan, Italy
Phone +39 02 2666895 - Fax +39 02 2664342
E-mail: sic@istitutotumori.mi.it
www.cancerologia.it

ORGANIZING SECRETARIAT

centro servizi congressuali

C.S.C. S.r.l.
Via L.S. Gualtieri, 11 - 06123 Perugia
Tel+390755730617 - Fax +390755730619
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56th Dangerous Liaisons

translating cancer biology
into better patients management

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Programme at a glance

Thursday September 11th - AULA MAGNA - OSPEDALE CONA	
10.00 - 13.00	Pre-Meeting SIC young investigators
14.00 - 14.30	Welcome Opening
14.30 - 15.15	Opening Lecture
15.15 - 18.30	Session 1: Cancer Microenvironment and Inflammation
19.00 - 19.15	Bus Transfer
19.15 - 22.00	Welcome Cocktail

Friday September 12th - POLO CHIMICO BIO MEDICO (Mammut)	
08.00	Registration and poster hanging
08.30 - 10.30	Session 2: Non Coding RNAs and Epigenetics
10.30 - 11.00	Coffee Break
11.00 - 13.00	Poster Viewing with Authors
13.00 - 14.00	Light Lunch
14.00 - 15.30	Poster Discussion (4 parallel sessions)
15.30 - 16.45	Session 3a: Cancer Stem Cells
16.45 - 17.15	Coffee Break
17.15 - 18.00	Session 3b: Cancer Stem Cells
18.15 - 19.15	SIC General Assembly
20.15	Social Dinner

Saturday September 13th - POLO CHIMICO BIO MEDICO (Mammut)	
08.30 - 09.15	Plenary Lecture
09.15 - 11.30	Session 4: “Translational” Therapy
11.30 - 12.30	Award Ceremony
12.30 - 13.30	Light Lunch
13.30 - 14.15	Giorgio Prodi Lecture
14.15 - 15.45	Session 5: Translational Diagnostics: NGS, Circulating Cells\Nucleic Acids
15.45 - 16.30	Closing Lecture
16.30 - 17.00	Closing Remarks

Thursday September 11th

Scientific Programme

Venue: AULA MAGNA - OSPEDALE CONA

10.00 - 13.00 **PRE-MEETING SIC YOUNG INVESTIGATORS**

Chairs: Marco Macagno (Turin, Italy), Selena Ventura (Bologna, Italy)

"Writing Grant Proposals: three techniques to present your idea with clarity and conviction"
Valerie Matarese Ph.D. (Vidor, TV)

Meeting of SIC young investigators

14.00 - 14.30 **WELCOME OPENING**

14.30 - 15.15 **OPENING LECTURE**

Causes and Consequences of microRNA Dysregulation in Cancer
Carlo M. Croce (Columbus, Ohio, USA)

15.15 - 18.30 **Session 1: CANCER MICROENVIRONMENT AND INFLAMMATION**

Chairs: Francesco Di Virgilio (Ferrara, Italy), Andrea Morandi (Florence, Italy)

15.15 - 15.45 **Immunosuppressive microenvironnment in neuroblastoma**
Vito Pistoia (Genoa, Italy)

15.45 - 16.00 **New players and targets in tumor vasculature**
V 9
Ugo Cavallaro (IEO, Istituto Europeo di Oncologia, Milan, Italy), *Elena Magrini, Fabrizio Bianchi, Andrea Doni, Francesca Angiolini, Alessandra Villa, Massimiliano Mazzone*

16.00 - 16.30 **The transcritpion factor STAT3 at the crossroad between cancer and inflammation**
Valeria Poli (Turin, Italy)

16.30 - 16.45 **Stable modulation of the glycolytic phenotype of tumor cells by anti-VEGF therapy**
V 17
Stefano Indraccolo (Istituto Oncologico Veneto, IRCCS, Padua, Italy), *Matteo Curtarello, Elisabetta Zulato, Giorgia Nardo, Silvia Valtorta, Giulia Guzzo, Elisabetta Rossi, Giovanni Esposito, Aichi Msaki, Andrea Rasola, Luca Persano, Francesco Ciccarese, Roberta Bertorelle, Sergio Todde, Mario Plebani, Henrike Schroer, Stefan Walenta, Wolfgang Mueller-Klieser, Alberto Amadori, Rosa Maria Moresco*

16.45 - 17.15 **The extracellular matrix at the interface between immunity and cancer**
Sabina Sangaletti (Milan, Italy)

17.15 - 17.45 **Matrix remodelling during cancer progression**
Janine Erler (Copenhagen, Denmark)

17.45 - 18.30 **PLENARY LECTURE**

Molecular pathways linking inflammation and cancer
Alberto Mantovani (Milan, Italy)

19.00 - 19.15 **BUS TRANSFER**

19.15 - 22.00 **Welcome Cocktail**

Friday September 12th

Scientific Programme

Venue: POLO CHIMICO BIO MEDICO (Mammut)

08.00 **REGISTRATION AND POSTER HANGING**

08.30 - 10.30 **Session 2: NON CODING RNAS AND EPIGENETICS**
Chairs: Massimo Negrini (Ferrara, Italy), Stefano Volinia (Ferrara, Italy)

08.30 - 09.00 **About Noam Chomsky, DNA patterns, Non-codingRNAs and Cancer patients**
George A. Calin (Houston, Texas, USA)

09.00 - 09.15 **Targeting aberrant HDAC4 activity in multiple myeloma (MM) through a miR-29b-based therapeutic strategy**
R 2
Nicola Amodio (Department of Experimental and Clinical Medicine, University Magna Graecia of Catanzaro, Catanzaro, Italy), *Marzia Leotta, Eugenio Morelli, Maria Angelica Stamato, Lavinia Raimondi, Anna Maria Gullà, Maria Teresa Di Martino, Teresa Calimeri, Marco Rossi, Antonino Neri, Pierosandro Tagliaferri, Pierfrancesco Tassone*

09.15 - 09.45 **EnhancerRNAs at the service of p53**
Reuven Agami (Amsterdam, The Netherlands)

09.45 - 10.00 **Mir-660 is downregulated in lung cancer patients and its replacement inhibits lung tumorigenesis by targeting MDM2-p53 interaction**
R 9
Orazio Fortunato (Fondazione IRCCS Istituto Nazionale dei Tumori, Tumor Genomics Unit, Milan), *Mattia Boeri, Massimo Moro, Carla Verri, Mavis Mensah, Davide Conte, Luca Roz, Ugo Pastorino, Gabriella Sozzi*

10.00 - 10.30 **The epigenetic role of microRNA-223 in hematopoiesis and leukemias**
Clara Nervi (Roma, Italy)

10.30 - 11.00 *Coffee Break*

11.00 - 13.00 **POSTER VIEWING WITH AUTHORS**

13.00 - 14.00 **Light Lunch**

Friday September 12th

Scientific Programme

Venue: POLO CHIMICO BIO MEDICO (Mammut)

14.00 - 15.30 **POSTER DISCUSSION (4 PARALLEL SESSIONS)**

14.00 - 15.30 **Poster Discussion 1** *Room E2*

CANCER GENETICS, EPIGENETICS AND CONTROL OF GENE EXPRESSION

Chairs: Marilena Iorio (Milan, Italy), Giuseppe Viglietto (Catanzaro, Italy)

B 18 **Searching for Oncogenic Drivers in the Stepwise Process of Hepatocarcinogenesis**
Patrizia Zavattari (Department of Biomedical Sciences, University of Cagliari, Cagliari, Italy), *Andrea Perra, Marta Anna Kovalik, Maria Maddalena Angioni, Silvia Menegon, Annalisa Petrelli, Luca Quagliata, Giovanna Maria Ledda-Columbano, Luigi Terracciano, Silvia Giordano, Amedeo Columbano*

B 8 **Involvement of ALK in Pediatric Rhabdomyosarcoma**
Patrizia Gasparini (Experimental Oncology Unit, Fondazione IRCCS Istituto Nazionale Tumori, Hospital, Milan, Italy), *Raffaella Villa, Michela Casanova, Paola Collini, Rita Alaggio, Roberto Caserini, Monica Tortoreto, Nadia Zaffaroni, Cristina Meazza, Maria Grazia Daidone, Maura Massimino, Andrea Ferrari, Gabriella Sozzi*

B 14 **Whole Exome Sequencing revealed a novel PALB2 mutation in a male breast cancer family**
Valentina Silvestri (Department of Molecular Medicine, Sapienza University of Rome, Rome, Italy), *Piera Rizzolo, Anna Sara Navazio, Virginia Valentini, Veronica Zelli, Giovanni Chillemi, Marco Montagna, Laura Ottini*

C 2 **Impairment of V-ATPase as a therapeutic modality targeting cancer stem cells of rhabdomyosarcoma**
Sofia Avnet (Orthopaedic Pathophysiology and Regenerative Medicine, Istituto Ortopedico Rizzoli, Bologna, Italy), *Manuela Salerno, Shigekuni Hosogi, Nicola Baldini*

C 13 **Hedgehog-Gli signaling pathway in lung cancer stem cells and its drug mediated targeting**
Agnese Po (Dipartimento di Medicina Molecolare, Sapienza Università di Roma, Roma, Italy), *Marianna Silvano, Danilo Cucchi, Adriana Eramo, Evelina Miele, Valentina Salvati, Giovanni Sette, Ruggero De Maria, Ann Zeuner, Enrico De Smaele, Alberto Gulino, Elisabetta Ferretti*

R 5 **miRNA and gene regulatory pathway of stage I epithelial ovarian cancer: reconstructing cancer circuits**
Enrica Calura (Department of Biology, University of Padova, Padua, Italy), *Gabriele Sales, Paolo Martini, Robert Fruscio, Eliana Bignotti, Antonella Ravaggi, Lara Paracchini, Mariacristina Di Marino, Laura Zanotti, Dionyssios Katsaros, Germana Tognon, Enrico Sartori, Sergio Pecorelli, Maurizio D'Incalci, Sergio Marchini, Chiara Romualdi*

R 12 **Regulation of microRNA processing by mutp53 in colon cancer**
Francesca Garibaldi (IFO-Istituti Fisioterapici Ospitalieri, Istituto Nazionale dei tumori Regina Elena, Rome, Italy), *Emmanuela Falcone, Gianluca Bossi, Daniela Trisciuoglio, Giulia Piaggio, Aymone Gurtner*

Friday September 12th

Scientific Programme

Venue: POLO CHIMICO BIO MEDICO (Mammut)

14.00 - 15.30 **Poster Discussion 2** *Room E3*

MICROENVIRONMENT AND TUMOR IMMUNOLOGY

Chairs: Enrico Iaccino (Catanzaro, Italy), Giulia Taraboletti (Bergamo, Italy)

D 4

An Angiopoietin-like Protein 2 autocrine signaling promotes EMT during pancreatic ductal carcinogenesis

Carmine Carbone (Digestive Molecular Clinical Oncology Research Unit, Università degli Studi di Verona, Verona, Italy), *Geny Piro, Matteo Fassan, Anna Tamburrino, Maria Mihaela Mina, Marco Zanotto, Paul J Chiao, Claudio Bassi, Aldo Scarpa, Giampaolo Tortora, Davide Melisi*

D 5

Endothelial podosome rosettes regulate vascular branching in tumor angiogenesis

Giulia Chiaverina (Institute for Cancer Research and Treatment, Department of Oncology, University of Torino, Turin, Italy, Candiolo, Italy), *Giorgio Seano, Paolo Armando Gagliardi, Laura di Blasio, Alberto Puliafito, Roberto Sessa, Claire Bouvard, Guido Tarone, Dominique Helley, Lydia Sorokin, Guido Serini, Federico Bussolino, Luca Primo*

D 15

Cadherin 6 and thyroid cancer: beyond a structural function

Valentina Sancisi (Laboratory of Translational Research, Arcispedale Santa Maria Nuova - IRCCS, Reggio Emilia, Italy), *Greta Gandolfi, Moira Ragazzi, Ione Tamagnini, Simonetta Piana, Alessia Ciarrocchi*

F 7

Metabolic control of YAP and TAZ by the mevalonate pathway

Giovanni Sorrentino (Laboratorio Nazionale CIB, Università degli studi di Trieste, Trieste, Italy), *Naomi Ruggeri, Valeria Specchia, Michelangelo Cordenonsi, Miguel Mano, Sirio Dupont, Andrea Manfrin, Eleonora Ingallina, Roberta Sommaggio, Silvano Piazza, Antonio Rosato, Stefano Piccolo, Giannino Del Sal*

V 1

The multifaceted anti-tumor activity of interleukin-27 in human lung cancer

Irma Airoldi (Laboratory of Oncology, Istituto Giannina Gaslini, Genoa, Italy), *Silvia Esposito, Maria Grazia Tupone, Giulia Barbarito, Marco Vincenzo Russo, Serena Di Meo, Carlo Sorrentino, Emma Di Carlo*

V 26

Mast Cell-Derived IL-8 Induces Epithelial-to-Mesenchymal Transition and Stem Cell Features in Human Thyroid Cancer cells

Carla Visciano (Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Istituto per L'Endocrinologia e l'Oncologia Molecolare del CNR, University of Naples, Federico II, Naples, Italy), *Federica Liotti, Nella Prevete, Gaetano Calì, Renato Franco, Francesca Collina, Amato de Paulis, Gianni Marone, Massimo Santoro, Rosa Marina Melillo*

U 2

The complement system in the immunosurveillance of HER-2 positive mammary cancer

Silvio Bandini (Molecular Biotechnology Center, University of Turin, Turin, Italy), *Marco Macagno, Albana Hisi, Laura Conti, Stefania Lanzardo, Amanda Bello, Manuela Iezzi, Federica Cavallo*

Friday September 12th

Scientific Programme

Venue: POLO CHIMICO BIO MEDICO (Mammut)

14.00 - 15.30 **Poster Discussion 3** *Room D6*

BIOMARKERS AND THERAPY

Chairs: Ymera Pignochino (Turin, Italy), Selena Ventura (Bologna, Italy)

L 8 **A specific c-MET inhibitor (JNJ-38877605) reduces bone metastases induced by kidney cancer stem cells in a human-in-mice model**

Ilaria Roato (CeRMS -Center for Research and Medical Studies- A.O. Città della Salute e della Scienza di Torino, Turin, Italy), *Livio Trusolino, Lucia D'Amico, Giorgia Migliardi, Roberta Pulito, Timothy Perera, Paolo M. Comoglio, Riccardo Ferracini*

L 2 **Establishment of a platform of patient-derived tumor xenografts (EOC-PDX) to study the biology and therapy of epithelial ovarian cancer**

Francesca Bizzaro (Department of Oncology, IRCCS-Istituto di Ricerche Farmacologiche Mario Negri, Milan, Italy), *Francesca Ricci, Marta Cesca, Federica Guffanti, Alessandra Decio, Carmen Ghilardi, Patrizia Perego, Robert Fruscio, Paola Ostano, Giovanna Chiorino, Maria Rosa Bani, Giovanna Damia, Raffaella Giavazzi*

P 16 **Evaluation by multicolor flow cytometry of circulating endothelial cells as prognostic and predictive biomarker in healthy donors and colorectal cancer patients**

Alessandra Leone (Istituto Nazionale per lo Studio e la Cura dei Tumori, 'Fondazione Giovanni Pascale'- IRCCS – Italia, Naples, Italy), *Maria Serena Roca, Carlo Vitagliano, Francesco Bianco, Silvia De Franciscis, Gianluca Rotta, Ernesta Cavalcanti, Rosa Azzaro, Annamaria Diodato, Carmela Cacciapuoti, Guglielmo Nasti, Antonio Avallone, Alfredo Budillon, Elena Di Gennaro*

P 3 **Activated d16HER2 homodimers and SRC kinase signaling axis is a predictor of Trastuzumab benefit**

Lorenzo Castagnoli (Fondazione IRCCS - Istituto Nazionale dei Tumori, Dept. Experimental Oncology and Molecular Medicine, Milan, Italy), *Manuela Iezzi, Valentina Ciravolo, Gaia Cristina Ghedini, Tiziana Triulzi, Patrizia Gasparini, Roberta Zappasodi, Patrizia Nanni, Elda Tagliabue, Serenella Pupa*

P 25 **Predictive biomarkers of trabectedin (TR) and PARP-1 inhibitor synergism in preclinical models of mesenchymal tumors (MTs)**

Ymera Pignochino (Candiolo Cancer Institute, IRCCS, University of Torino Medical School, Candiolo, Turin, Italy), *Federica Capozzi, Carmine Dell'Aglio, Marta Canta, Marco Basiricò, Annalisa Lorenzato, Lorenzo D'ambrosio, Danilo Galizia, Maria Serena Benassi, Massimo Aglietta, Giovanni Grignani*

P 26 **TAK1-regulated expression of BIRC3 is responsible for chemoradiotherapy (CRT) resistance in esophagogastric junction (EGJ) adenocarcinoma**

Geny Piro (Laboratory of Oncology and Molecular Therapy, University of Verona, Verona, Italy), *Simone Giacopuzzi, Maria Bencivenga, Giuseppe Verlato, Carmine Carbone, Valeria Merz, Anna Tamburrino, Giovanni de Manzoni, Giampaolo Tortora, Davide Melisi*

P 9 **Clinical relevance of circulating endothelial cells as prognostic and early predictive biomarker of response to anti-angiogenic therapy in rectal cancer patients**

Elena Di Gennaro (Istituto Nazionale per lo Studio e la Cura dei Tumori, "Fondazione Giovanni Pascale"- IRCCS – Italia, Naples, Italy), *Paolo Delrio, Alessandra Leone, Tania Moccia, Eleonora Cardone, Carmen Romano, Antonio Avallon, Alfredo Budillon*

Friday September 12th

Scientific Programme

Venue: POLO CHIMICO BIO MEDICO (Mammut)

14.00 - 15.30 **Poster Discussion 4** *Room D7*

DRUG RESISTANCE, SIGNAL TRANSDUCTION, TARGETED THERAPY

Chairs: Gaia Cristina Ghedini (Milan, Italy), Marco Macagno (Turin, Italy)

A 3 **GSK3A and GSK3B isoforms are redundant in modulating drug resistance and chemotherapy-induced necroptosis of p53-null colon cancer cells**

Marialuisa Lavitrano (Department of Surgery and Translational Medicine, University of Milan - Bicocca, Milan, Italy), *Emanuela Grassilli, Leonarda Ianzano, Fabio Pisano, Sara Bonomo, Carola Missaglia, Laura Masiero, Maria Grazia Cerrito, Gabriele Romano, Serena Bonin, Giorgio Stanta, Roberto Giovannoni*

I 1 **CDK6 and platinum drug resistance in Epithelial Ovarian Cancer**

Alessandra Dall'Acqua (CRO of Aviano, National Cancer Institute, Aviano, Italy), *Monica Schiappacassi, Maura Sonego, Ilenia Pellizzari, Gustavo Baldassarre*

I 8 **GENE expression profiling of Human Melanoma cell lines and clinical samples defines subsets differing in susceptibility to BRAFV600E and pathway-directed inhibitors**

Marialuisa Sensi (Fondazione IRCCS Istituto Nazionale dei Tumori, Hospital, Milan, Italy), *Matteo Dugo, Gabriella Nicolini, Gabrina Tragni, Ilaria Bersani, Antonella Tomassetti, Silvana Canevari, Andrea Anichini*

Q 1 **Multicomponent Nanovectors for Delivery of Therapeutic Molecules in Cancer**

Armando Cevenini (CEINGE-Biotecnologie Avanzate, s.c.a r.l., Naples, Italy), *Alessandro Parodi, SM Khaled, Iman K Yazdi, Matteo Ciancaioni, Xueavu Liu, Christian Celia, Nicoletta Peluso, Rosa Peltrini, Stefania Orrù, Luigi Del Vecchio, Ennio Tasciotti, Mauro Ferrari, Francesco Salvatore*

S 4 **Interaction between Sonic hedgehog and bombesin neuropeptide receptor pathways in small cell lung carcinoma**

Maria Domenica Castellone (Istituto di Endocrinologia ed Oncologia Sperimentale, Consiglio Nazionale delle Ricerche, Naples, Italy), *Mikko Olavi Laukkanen, Silvio Gutkind*

S 3 **The Double-Stranded RNA Adenosine Deaminase, ADAR1, Represents a Novel Substrate for AKT**

William Blalock (Institute of Molecular Genetics-National Research Council of Italy (IGM-CNR), Rizzoli Orthopedic Institute, Bologna, Italy), *Mirco Raffini, Manuela Piazzi, Irene Faenza, Alberto Bavelloni*

T 7 **Effects of Hedgehog signaling inhibition on epithelial-stromal interactions in triple negative breast cancer cells.**

Concetta Di Mauro (Department of molecular medicine and surgery, University of Naples Federico II, Naples, Italy), *Roberta Rosa, Valentina D'Amato, Luigi Formisano, Roberta Marciano, Lucia Raimondo, Alberto Servetto, Bianca Maria Veneziani, Sabino De Placido, Roberto Bianco*

Friday September 12th

Scientific Programme

Venue: POLO CHIMICO BIO MEDICO (Mammut)

15.30 - 16.45 **Session 3a: CANCER STEM CELLS**
Chairs: Silvano Capitani (Ferrara, Italy), Elda Tagliabue (Milan, Italy)

15.30 - 16.00 **Towards new therapy based on Cancer Stem Cell Targeting**
Ruggero De Maria (Rome, Italy)

16.00 - 16.15 **Surgery-induced wound response promotes stem-like and tumor-initiating features of breast cancer cells via STAT3 signaling**
C 18
Ilenia Segatto (CRO of Aviano, National Cancer Institute, Aviano, Italy), *Stefania Berton, Maura Sonego, Samuele Massarut, Tiziana Perin, Gustavo Baldassarre, Barbara Belletti*

16.15 - 16.45 **Dynamics of cancer stem cells in the pancreas**
Christopher Heeschen (Madrid, Spain)

16.45 - 17.15 *Coffee Break*

17.15 - 18.00 **Session 3b: CANCER STEM CELLS**
Chairs: Paolo Pinton (Ferrara, Italy), Gabriella Sozzi (Milan, Italy)

17.15 - 17.30 **Prolyl-isomerase Pin1 controls normal and cancer stem cells of the breast**
C 21
Alessandro Zannini (Laboratorio Nazionale cib, Dipartimento Scienze della Vita - Università degli Studi di Trieste, Trieste, Italy), *Alessandra Rustighi, Elena Campaner, Giovanni Sorrentino, Silvano Piazza, Giannino Del Sal*

17.30 - 18.00 **Connecting the machineries of tumor suppression and cell fate determination in stem cells**
Pier Paolo Di Fiore (Milan, Italy)

18.15 - 19.15 **SIC General Assembly**

20.15 **Social Dinner**

Saturday September 13th

Scientific Programme

Venue: POLO CHIMICO BIO MEDICO (Mammut)

08.30 - 09.15 **Plenary Lecture**

Restoration of anticancer immunosurveillance: a hallmark of successful antineoplastic therapies
Guido Kroemer (Villejuif, France)

09.15 - 11.30 **Session 4: "TRANSLATIONAL" THERAPY**
Chairs: Rita Falcioni (Rome, Italy), Antonio Frassoldati (Ferrara, Italy)

09.15 - 09.45 **State of the art and perspectives for the development of targeted therapies**
Giampaolo Tortora (Verona, Italy)

09.45 - 10.15 **Genomics of Hairy Cell Leukemia: biological and clinical implication**
Brunangelo Falini (Perugia, Italy)

10.15 - 10.45 **Next generation personalized medicine in colon and lung cancer**
Nicola Normanno (Naples, Italy) - **Sponsored by EACR**

10.45 - 11.30 **Companies lectures**
Chair: Massimo Negrini (Ferrara, Italy),

10.45 - 11.00 **Unlocking cancer with multiomic approach: from sample to data analysis**
Valentina Maran (Sales Channel Manager Genomics Italy Diagnostic and Genomics Group, Agilent Technologies)

11.00 - 11.15 **Droplet Digital PCR, new and enabling tool for cancer research**
Alessandro Martino (Field Application Specialist Southern Europe Bio-Rad Laboratories S.r.l. Life Science Group)

11.15 - 11.30 **The latest news in NGS Cancer clinical research with Ion Torrent**
Alberto Madini (Sales Rep., Life Sciences Solution, Thermo Fisher Scientific)

11.30 - 12.30 **Award Ceremony**

"Piero Trivella" and "EACR" Awards for the best posters

"Elena Cappannini" Award for the best 2013 publication

"Pezcoller Foundation" scholarship 2013-2014 winner award
Characterization of molecular mechanisms involved in MDSC mediated tolerance: paving the way to overcome tumor attack
Samantha Solito (Department of Surgery, Oncology and Gastroenterology, University of Padua)

"Pezcoller Foudation" scholarship 2015-2016 award

Travel Grants

12.30 - 13.30 *Light Lunch*

Saturday September 13th

Scientific Programme

Venue: POLO CHIMICO BIO MEDICO (Mammut)

13.30 - 14.15 **Giorgio Prodi Lecture**

Regulation of Self Renewal in Cancer Stem Cells
Pier Giuseppe Pelicci (Milan, Italy)

14.15 - 15.45 **Session 5: TRANSLATIONAL DIAGNOSTICS: NGS, CIRCULATING CELLS\NUCLEIC ACIDS**
Chairs: Mattia Boeri (Milan, Italy), Davide Melisi (Verona, Italy)

14.15 - 14.45 **Disseminated and circulating tumor cells as prognostic and predictive markers**
Simon Joosse (Eppendorf, Germany)

14.45 - 15.00 **A specific and sensitive method for the individuation of EGFR mutations in circulating free tumor DNA from NSCLC patients**

P 20

Raffaella Pasquale (Istituto Nazionale Tumori "Fondazione G. Pascale"-IRCCS, Cell Biology and Biotherapy Unit, Naples, Italy), *Anna Maria Rachiglio, Claudia Esposito, Alessandra Sacco, Riziero Esposito Abate, Simona Bevilacqua, Cristin Roma, Antonella De Luca, Nicola Normanno*

15.00 - 15.30 **Circulating tumour DNA as a biomarker of response in cancer therapy**
James Brenton (Cambridge, UK)

15.30 - 15.45 **Early relapse upon trastuzumab treatment is predictable by transcriptome analysis of primary HER2-positive breast cancer**

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Tiziana Triulzi (Fondazione IRCCS Istituto Nazionale dei Tumori, Department of Experimental Oncology and Molecular Medicine, Milan, Italy), *Loris De Cecco, Aleix Prat, Marco Sandri, Marta Giussani, Maria Luisa Carcangiu, Silvana Canevari, Serena Di Cosimo, Sylvie Menard, Manuela Campiglio, Elda Tagliabue*

15.45 - 16.30 **Closing Lecture**

Deconstructing the molecular genetics of human cancer and its therapeutic implications
Pier Paolo Pandolfi (Boston, Massachussets, USA)

16.30 - 17.00 **Closing Remarks**

Scientific Information

CME CREDITS

No. 3,2 Italian Ministry of Health CME (Continuing Medical Education) credits have been assigned for the following Professions and Disciplines:
Profession: Medical Surgeon (Disciplines: Haematology, Medical Genetics, Pathological Anatomy, Pharmacology and Clinical Toxicology, Clinical Pathology, Internal Medicine);
Biologist, Pharmacist, Chemist (Discipline: Analytical Chemistry); Physicist (Discipline: Health Physics); Veterinary Surgeon.

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Slides must be in English. Computer videoprojection will be available in Power Point. Please communicate to the Organizing Secretariat your different requirements as soon as possible. Slides have to be handed to the slide centre one hour before the session beginning.

PRIZES

Saturday September 13th 2014

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"Elena Cappannini" Award for the best 2013 publication

"Pezcoller Foudation" Ferruccio and Elena Bernardi scholarship Award

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General Information

DATES
Ferrara 11th -13th September 2014

CONGRESS VENUE
Università degli Studi di Ferrara - Via Luigi Borsari, 46 - 44121 Ferrara

OPENING CEREMONY VENUE
Azienda Ospedaliero Universitaria di Ferrara
Arcispedale Sant'Anna - Via Aldo Moro, 8 - 44124 Cona – Ferrara

OFFICIAL LANGUAGE
English. Simultaneous translation will not be provided.

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CERTIFICATE OF ATTENDANCE
Certificate of attendance is given to all registered participants at the end of the congress.

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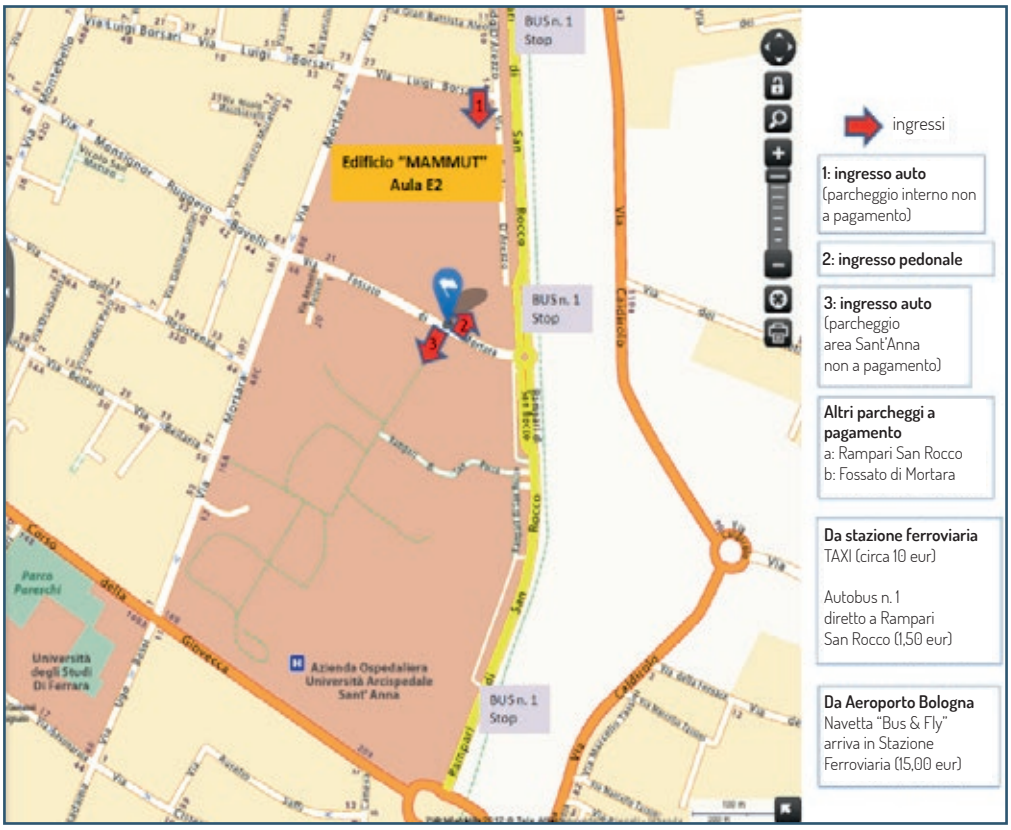

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By Car
Motorway A13, exit Ferrara South Parking area:
- Inside the "MAMMUT" (entrance 1)
- Inside the ex-hospital St. Anna (entrance 3)
- Along the Rampari of San Rocco or Fossato di Mortara (toll parking - 2,00 Euros for the entire day)

By train
Ferrara stop
From the station to the congress venue:
- by taxi
- bus N. 1 Rampari San Rocco stop, in front of the MAMMUT

By plane
From Bologna Marconi Airport to Ferrara Shuttle "BUS & FLY" (Time: 60 minutes. Cost: 15,00 Euros). It stops in the city center and at the train station.

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General Information

Opening Day Venue

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translating cancer biology
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Abstract Book

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Autophagy, Apoptosis, Necrosis And Senescence

A 1
TARGETING MUTP53 WITH ZN(II)-CURC COMPOUND INVOLVES BOTH REACTIVATION OF P53 WILD-TYPE CONFORMATION AND MUTP53 DEGRADATION THROUGH AUTOPHAGY

Alessia Garufi [1] - Maria Laura Avantaggiati [2] - Gabriella D'Orazi [1]

Department of Medical, Oral and Biotechnological Sciences, University 'G. D'Annunzio', Chieti, Italy [1] - Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University, Washington, DC, United States [2]

Introduction: TP53 oncosuppressor is frequently mutated in cancer contributing to tumor progression and resistance to therapies. Mutant p53 (mutp53) proteins are prone to loss of the Zn(II) ion bound to the core DNA binding domain and this in turn favours protein unfolding, aggregation and impairment of DNA binding and transcription ability of target genes. Mutp53 protein is also often highly expressed in cancers due to its increased half-life, contributing to tumor outgrowth. Therefore, targeting mutp53 protein for reactivation of wild-type function(s) and/or for mutp53 degradation may have therapeutic significance.

Materials and methods : Here we used a fluorescent curcumin-based Zn(II) complex [Zn(II)-curc] and assessed its ability to affect mutp53 conformation in cancer cells as well as mutp53 degradation through autophagy. Biochemical and molecular biology studies were carried out.

Results: Zn(II)-curc restored the folded conformation of mutp53 proteins (R175H), inducing wtp53 DNA binding and transactivation, as assessed by chromatin immunoprecipitation and reverse-transcription (RT)-PCR assays. Consequently, Zn(II)-curc triggered apoptosis in mutp53-carrying cell lines. In addition, Zn(II)-curc promoted mutp53H175 degradation through autophagy. Suppression of autophagy prevented Zn(II)-curc-induced mutp53H175 degradation and restoration of wild-type p53 oncosuppressor activities. On the contrary, the proteasome inhibitor MG132 failed to do so, suggesting that autophagy was the main route for p53H175 degradation. Mechanistically, Zn(II) restored the wtp53 ability to induce the expression of the p53 target gene DRAM (damage-regulated autophagy modulator), a key regulator of autophagy, leading to autophagic induction. Accordingly, inhibition of wtp53 transactivation by pifithrin-α (PFT-α) impaired both autophagy and mutp53H175 degradation induced by Zn(II)-curc

Conclusions: These results uncover a novel mechanism employed by Zn(II)-curc to reactivate mutp53H175 which involves, at least in part, mutp53 degradation *via* wtp53-mediated autophagy. We thus propose that due to its effect in reducing the levels of accumulated mutp53H175, together with the ability of ameliorating mutp53H175 misfolding, Zn(II)-curc may serve as a key lead compound for the development of anticancer drugs to effectively treat mutp53H175-carrying tumors.

A 2
THE MITOCHONDRIAL CHAPERONE TRAP1 PROMOTES NEOPLASTIC GROWTH BY INHIBITING SUCCINATE DEHYDROGENASE

Giulia Guzzo [1] - Marco Sciacovelli [1] - Paolo Bernardi [2] - Andrea Rasola [1]

University of Padua, University of Padua, Padua, Italy [1] - Univeristy of Padua, University of Padua, Padua, Italy [2]

Introduction: Cancer is a highly heterogeneous and complex disease, whose development requires a reorganization of cell metabolism. Most tumor cells downregulate mitochondrial oxidative phosphorylation (OXPHOS) and increase the rate of glucose consumption and lactate release, independently of oxygen availability (the Warburg effect). Tumor metabolic changes also increase the generation of reactive oxygen species (ROS), as OXPHOS complexes are the main sites of intracellular ROS production. Thus, neoplastic cells must reach a novel homeostatic redox equilibrium by a stimulation of anti-oxidant defenses to avoid oxidative stress.

Materials and Methods: TRAP1 expression was stably knocked down in two different tumor cell lines (SAOS and HeLa) or overexpressed in MEF. We used Amplex Red and MitoSox to measure ROS levels in basal or under stress condition. Calcium Retention Capacity Assay and flow cytometry were used to measure cell viability.

Results: Within this context, we have recently found that the mitochondrial chaperone TRAP1, whose expression is induced in several tumor types, is required for neoplastic growth following inhibition of succinate dehydrogenase (SDH) and the succinate-dependent stabilization of the transcription factor HIF1α, independently of hypoxic conditions. Here we observe that down-regulation of TRAP1 expression constitutively increases intracellular ROS by relieving SDH inhibition. Thus, down-modulation of TRAP1 sensitizes tumor cells to pro-oxidant stimuli. In cells without TRAP1, oxidative stress strongly induces the permeability transition pore (PTP), a mitochondrial channel whose opening irreversibly commits cells to death. The rise in mitochondrial superoxide levels prompted by the absence of TRAP1 completely abolished tumorigenicity in *in vitro* transformation assays.

Conclusions: We therefore propose a model whereby TRAP1 inhibition of SDH is twofold tumorigenic, both by inducing a pro-neoplastic transcription program mastered by HIF1α, and by shielding from oxidative stress and the consequent PTP mediated tumor cell death.

A 3
GSK3A AND GSK3B ISOFORMS ARE REDUNDANT IN MODULATING DRUG RESISTANCE AND CHEMOTHERAPY-INDUCED NECROPTOSIS OF P53-NULL COLON CANCER CELLS

Marialuisa Lavitrano [1] - Emanuela Grassilli [1] - Leonarda Ianzano [1] - Fabio Pisano [1] - Sara Bonomo [1] - Carola Missaglia [1] - Laura Masiero [1] - Maria Grazia Cerrito [1] - Gabriele Romano [1] - Serena Bonin [2] - Giorgio Stanta [2] - Roberto Giovannoni [1]

Department of Surgery and Translational Medicine, University of Milano - Bicocca, Milano, Italy [1] - Cattinara Hospital Surgical Pathology, University of Trieste, Trieste, Italy [2]

Introduction: Glycogen Synthase Kinase-3 alpha (GSK3A) and beta (GSK3B) isoforms are encoded by distinct genes, are 98% identical within their kinase domain and perform similar functions in several settings; however, they are not completely redundant and, depending on the cell type and differentiative status, they also play unique roles.

Materials and methods: stable and transient RNAi-mediated silencing or chemical inhibition followed by proliferation and cytotoxicity assays were used to assess biological consequences of GSK3B or GSK3A blockade; caspase activation assay, western blot, immunofluorescence were used to study mechanisms of cell death; immunofluorescence was used to study DNA repair; protein arrays were used to study the signaling pathways activated/inhibited upon GSK3A/B inhibition. In vivo xenograft studies were conducted to

18 confirm resensitization of drug-resistant cells to chemotherapyuponGSK3inhibition. Colon cancer samples

19 from a cohort of 50 chemotherapy-treated stage II patients were analyzed for active GSK3B expression.

Results: RNAi-mediated silencing or chemical inhibition of either GSK3B or GSK3A in various drug-resistant p53-null colon cancer cell lines abolished cell viability and colony growth after drug addition, without affecting cell proliferation or cell cycle in untreated cells. In particular, blocking either isoform impairs DNA repair upon exposure to DNA-damaging drugs. As a consequence, p53-null cells overcome their inability to undergo apoptosis and mount a necroptotic response, characterized by absence of caspase activation and RIP1-independent, PARP-dependent AIF nuclear re-localization. Studies are undergoing to characterize the signaling pathways activated/inhibited upon GSK3A/B inhibition in response to chemotherapy. In vivo studies showed that drug-resistant xenograft tumor mass was significantly reduced only when 5FU was given after GSK3B inhibition. Tissue microarray analysis of colon carcinoma samples from 5FU-treated patients revealed that GSK3B is significantly more activated in drug-resistant versus responsive patients.

Conclusions: GSK3A and GSK3B are redundant in regulating drug-resistance and chemotherapy-induced necroptosis and inhibition of only one isoform, or rather partial inhibition of overall cellular GSK3 activity, is enough to re-sensitize drug-resistant cells to chemotherapy.

A 4
A MITOCHONDRIAL PERSPECTIVE ON B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA

Veronica Orlandi [1] - Cosima T. Baldari [2] - Paolo Bernardi [1] - Andrea Rasola [1]

University of Padova, University of Padova, Padova, Italy [1] - University of Siena, University of Siena, Siena, Italy [2]

Introduction: B-cell chronic lymphocytic leukemia (B-CLL), the most frequent form of leukemia in adulthood, is characterized by defects in apoptosis regulation which induce a progressive accumulation of malignant B-cell in lymph nodes and bone marrow and lead to chemotherapy failure. At present, therapeutic approaches for B-CLL are missing, and the disease is generally considered incurable. We have demonstrated that a fraction of the kinases ERK and GSK3, whose activity is deregulated in tumors, localizes to mitochondria. Mitochondrial ERK and GSK3 contribute to the anti-apoptotic phenotype of neoplastic cells by inhibiting the permeability transition pore (PTP), a mitochondrial channel whose opening irreversibly commits cells to death. The PTP is also regulated by TRAP1 and cyclophilin D (CyP-D), and by the mitochondrial fraction of the protein p66shc. TRAP1 and CyP-D are target of mitochondrial kinases and also modulate the activity of oxidative phosphorylation (OXPHOS) complexes, contributing to the metabolic changes that characterize cells during neoplastic transformation.

Materials and methods: MEC1 cells stable transfected with p66shc were assessed for cells death induced by glucose starvation. The OCR was analyzed by XF24 flux analyzer. The $\Delta\Psi m$ was detected with TMRM. BN-PAGE assay was used to detect OXPHOS complexes. OXPHOS complexes activities were detected through spectrophotometric assays.

Results: We have found that p66shc expression increases the protein levels and the phosphorylation of mitochondrial ERK and GSK3. Analyzing cell viability in conditions of glucose starvation we found that p66shc expression protects cells from death. We also found that the PTP and CyP-D, TRAP1 and ERK were involved in the molecular circuitries that control the viability of B-CLL cells. We demonstrated that the p66shc expressing cells show a lower oxygen consumption rate and a lower mitochondrial membrane potential. These cells have lower level of assembled respiratory complex I, which is paralleled by a diminished complex I activity. We also found that TRAP1, CyP-D, ERK and GSK3 bind succinate dehydrogenase, and that this binding is increased in p66shc-expressing cells, where SDH activity is down-modulated.

Conclusions: These findings identify a multifaceted regulation of respiratory activity in B-CLL cells, through the assembly of multimers containing kinases and chaperones, and pave the way for the identification of novel anti-neoplastic targets.

A 5
N6-ISOPENTENYLADENOSINE, AN ENDOGENOUS ISOPRENOID DERIVATIVE, INDUCES MELANOMA CELLS DEATH ACTIVATING AUTOPHAGY VIA AMPK

Paola P [1] - Elena Ciaglia [1] - Chiara Laezza [2] - Patrizia Gazzerro [3] - Maurizio Bifulco [1] - Simona Pisanti [1]

Department of Medicine and Surgery, University of Salerno, Baronissi (SA), Italy [1] - Istituto di Oncologia ed Endocrinologia Sperimentale, CNR, Napoli, Italy [2] - Department of Pharmacy, University of Salerno, Fisciano (SA), Italy [3]

Introduction: N6-isopentenyladenosine (iPA) is a modified adenosine characterized by an isopentenyl chain derived by dimethylallyl pyrophosphate (DMAPP), an intermediate of mevalonate pathway. iPA is a promising isoprenoid derivative with pleiotropic biological effects, including a direct anti-tumor and anti-angiogenic activity. However, its mechanism of action is still unknown.

Materials and methods: Since angiogenesis is a key process for growth and spread of solid tumors, the effect of iPA on tumor angiogenesis was investigated employing A375 human melanoma cells, well known for their highly angiogenic phenotype, co-cultured with endothelial cells. Moreover, we analyzed the action of iPA on melanoma cells fate using proliferation assays, flow cytometry and WB analysis.

Results: iPA was able to affect angiogenic phenotype of melanoma cells. Noteworthy, iPA, in its active phosphorylated form iPAMP, inhibited the proliferation of melanoma cells, with a cell cycle arrest in G1 phase (2.5-10 μM). iPAMP, behaving as an AMP mimetic, was able to activate AMPK, leading to the induction of autophagy. In our cell system autophagy didn't appear as protective mechanism but as preliminary step to cell death induction. Indeed, the analysis of peculiar markers in time course suggested that the autophagy was set up before apoptosis. The monophosphorylation of iPA into iPAMP by adenosine kinase (ADK) is crucial for its biological activity, since the pretreatment with 5-Itu, selective inhibitor of ADK, reverted all the observed effects.

Conclusions: These results indicate for the first time that iPA exerted anti-melanoma activity, leading a concomitant induction of autophagy and apoptosis processes, both involved in cell death.

A 6
HARNESSING REACTIVE OXYGEN SPECIES AND CELL DEATH PATHWAYS FOR THE TREATMENT OF PEDIATRIC T-ALL

Micol Silic-Benussi [1] - Loredana Urso [2] - Ilaria Cavallari [2] - Francesca Rende [2] - Sonia Minuzzo [2] - Giuseppe Basso [3] - Stefano Indraccolo [4] - Vincenzo Ciminale [2]

Dipartimento di Scienze Chirurgiche, Oncologiche e Gastroenterologiche Sezione di Oncologia e Immunologia, Università degli Studi di Padova, Padova, Italy [1] - Dipartimento di Scienze Chirurgiche, Oncologiche e Gastroenterologiche, Università degli Studi di Padova, Padova, Italy [2] - Dipartimento di Salute della Donna e del Bambino, University of Padova, Italy, Università degli Studi di Padova, Padova, Italy [3] - Istituto Oncologico Veneto-IRRCS, 2Istituto Oncologico Veneto-IRRCS, Padova, Italy [4]

Acute lymphoblastic leukemia (ALL) is the most common haematological disease in pediatric patients. About 15% of newly diagnosed ALL are of the T-cell subtype. T-ALL represents an important clinical problem, as about 20% of the patients are resistant to the current prednisone-based treatment regimens. The present study describes new therapeutic strategies aimed at breaking prednisone-resistance of refractory T-ALL by manipulation of reactive oxygen species (ROS) homeostasis.

The study employed an animal model based on the growth of primary T-ALL cells in SCID-NOD mice. The xenograft cells retain the genetic characteristics of primary samples. The tumor cells can be reisolated from the mice for *in vitro* drug testing or transduced with a lentviral vector expressing luciferase, reinoculated into SCID/NOD mice, and tracked by noninvasive imaging during *in vivo* drug trials.

In experiments performed thus far, effective, long-term control of prednisone-resistant T-ALL *in vivo* was obtained through a "multimodal" strategy that combined drugs that increase mitochondrial ROS production, decrease the activity of ROS-scavenging pathways and inhibit survival pathways engaged by ROS. Individual T-ALL xenografts showed responses to distinct drug combinations, suggesting that more than one cellular pathway may lead to prednisone resistance.

A 7
LOSS OF NOTCH1-DEPENDENT P21WAF1/CIP1 EXPRESSION INFLUENCES THE NOTCH1 OUTCOME IN TUMORIGENESIS.

Claudio Talora [1] - Cialfi Samantha [1] - Rocco Palermo [1] - Sonia Manca [1] - Carlo De Blasio [1] - Paula Vargas-romero [1] - Saula Checquolo [2] - Diana Bellavia [1] - Daniela Uccelletti [3] - Saliola Michele [3] - Angelo D'alessandro [4] - Lello Zolla [4] - Alberto Gulino [1] - Isabella Screpanti [1]

Dept. Molecular Medicine, Sapienza University of Rome, Rome, Italy [1] - Dept. of Biotechnology and Medical-Surgical Sciences, Sapienza UNiversity of Rome, Latina, Italy [2] - Dept. Biology and Biotechnology "C. Darwin", Sapienza University of Rome, Rome, Italy [3] - Dept. Ecological and Biological Sciences, University of Tuscia, VIterbo, Italy [4]

Introduction: Notch signaling plays a complex role in carcinogenesis and its signaling pathway has both tumor suppressor and oncogenic components. In this study we investigated the effects of reactive oxygen species (ROS) on Notch1 signaling outcome in keratinocyte biology.

Results: We demonstrate that Notch1 function contributes to the Arsenic induced keratinocyte-transformation. We found that acute exposure to Arsenite increases oxidative-stress and inhibits proliferation of keratinocyte cells by upregulation of $p21^{waf1/Cip1}$. The necessity of $p21^{waf1/Cip1}$ for Arsenite-induced cell death was demonstrated by targeted downregulation of $p21^{waf1/Cip1}$ by using RNA interference. We further demonstrated that on acute exposure to Arsenite $p21^{waf1/Cip1}$ is upregulated and Notch1 downmodulated, whereas on chronic exposure to Arsenite malignant progression of Arsenite-treated keratinocytes cells was accompanied by regained expression and activity of Notch1. Notch1 activity in Arsenite-transformed keratinocytes inhibits Arsenite-induced upregulation of $p21^{waf1/Cip1}$, by sustaining c-myc expression. We further demonstrated that c-myc collaborate with Nrf2, a key regulator for the maintenance of redox homeostasis, to promote metabolic activities that support cell proliferation and cytoprotection. Therefore, Notch1-mediated repression of $p21^{waf1/Cip1}$ expression results in the inhibition of cell death and keratinocytes transformation.

Conclusions: Our results not only demonstrate that sustained Notch1 expression is at least one key event implicated in the arsenite human skin carcinogenic effect but also may provide mechanistic insights into the molecular aspects that determine whether Notch signaling will be either oncogenic or tumor suppressive.

A 8
ON THE MECHANISMS OF MITOPHAGY AND CANCER CELL SURVIVAL

Chiara Tommasino [1] - Lucrezia Gambardella [1] - Barbara Ascione [1] - Matteo Marconi [1] - Walter Malorni [1] - Anna Maria Giammarioli [1]

Department of Theraputic Research and Medicine Evaluation, Istituto Superiore di Sanità, Rome, Italy [1]

Introduction: Despite the large number of clinical studies on metastatic melanoma, it has not been defined an effective therapy yet, and this tumor commonly presents an acquired intrinsic resistance to chemotherapy and apoptosis induction. In multicellular organisms, apoptosis is considered the main mechanism of cell death whereas autophagy is the main survival process during metabolic stress conditions. Recent lines of evidence suggest that autophagy could play a critical role either in tumorigenesis or a relevant target for cancer therapy. Autophagy and apoptosis share common proteins and signaling pathways and exhibit some degree of mutual inhibition. In particular, tumor cells can activate autophagy as a compensatory survival mechanism to counteract apoptotic signals. Accordingly, autophagy has become one of the most attractive topics in cancer research although its role appears still contradictory.

Materials and methods: Metastatic Melanoma cell line Mel 501 was treated with pro-apoptotic agent Staurosporine (STS), a protein kinase inhibitor, at different concentrations (from 10nM to 1μM). High concentration of STS (1μM) are commonly used to induce mitochondria-mediated apoptosis in mammalian cells. Melanoma cells were investigated for the expression of proteins involved in apoptotic and autophagic mechanisms by flow cytometry, western blotting and immunofluorescence analysis.

Results: Surprisingly, we observed that STS induced cell death by apoptosis at high concentration whereas it induced survival by autophagy at low concentration (50nM). We also observed that the low dose of STS induced a selective form of autophagy, named "mitophagy", in which dysfunctional mitochondria are sequestrated and degraded in autophagic vacuoles, as demonstrated by autophagy and mitophagy protein expression levels.

Conclusions: STS can induce both cell death by apoptosis and cell survival by mitophagy depending on concentration. We hypothesize that this model system may be useful to elucidate the mechanisms underlying the crosstalk between autophagy, mitophagy and cell death.

A 9
ADMINISTRATION OF MDM2 INHIBITOR PLUS APO2L/TRAIL: A PROMISING STRATEGY FOR MALIGNANT PLEURAL MESOTHELIOMA TREATMENT.

Loredana Urso [1] - Micol Silic-benussi [1] - Vincenzo Ciminale [1] - Federico Rea [2] - Adolfo Favaretto [3] - Giulia Pasello [3]

Surgical Oncologic and Gastroenterological Sciences Department, University of Padua, Padua, Italy [1] - Thoracic Surgery Unit, University of Padua, Padua, Italy [2] - Second Medical Oncology Unit, Istituto Oncologico Veneto, Padua, Italy [3]

Background: Malignant Pleural Mesothelioma (MPM) is an aggressive tumor characterized by chemoresistance. MDM2 is an oncogene over-expressed in many types of cancers. The role of MDM2 is not only related to p53 regulation but it is also involved in the activation of other proteins, one of which is the Hypoxia Inducible Factor-1alpha (HIF-1alpha,) a transcription factor implied in VEGF regulation. The aim of our study was to investigate, in MPM cell lines, the efficacy of MDM2 inhibitor, Nutlin 3a, in association with APO2L/TRAIL for the treatment of Malignant Pleural Mesothelioma.

Methods: In vitro apoptosis assay was performed using Annexin-V-Fluos staining kit. MPM cell lines were treated with Nutlin 3a and /or Apo2L/TRAIL and Annexin V positive cells were detected by flow cytometry using a FACSCalibur apparatus. p53, p21 and survivin protein expression level were detected by western blot analysis using specific antibodies. TRAIL death receptors (DR4 and DR5) and decoy receptors (DcR1 and DcR2) expression levels were assessed by flow cytometric analysis. mRNA expression levels of VEGF was assessed by real-time PCR.

Results: Apoptosis assay performed in eight MPM cell lines (epithelioid- ZL55, H28, M14K; biphasic-MSTO211H, SPC111, ZL5; and sarcomatoid ZL34, MPM1801) revealed that there was a synergistic effect of Nutlin3a *plus* APO2L/TRAIL in inducing cell death with a greater effect in sarcomatoid ZL34 cell line. Interesting, ZL34 cells showed the highest resistance to APO2L/TRAIL and the highest levels of MDM2 mRNA. Experiments conducted in this sarcomatoid cell line showed that Nutlin 3a treatment provoked activation of p53 and p21, and inhibition of survivin in a dose dependent manner. Moreover, Nutlin 3a increased the expression of DR4/DR5 TRAIL death receptors but had no activity on DcR1/DcR2 decoy receptors. When we explored the effects of Nutlin 3a on mRNA expression levels of VEGF, our results demonstrated that MDM2 in-

hibition blocked the hypoxia-induced increase of VEGF mRNA.

Conclusion: *in vitro* results demonstrated that the simultaneous administration of Nutlin 3a and APO2L/TRAIL may be useful both in activating intrinsic and extrinsic apoptosis of tumor cells and in inhibiting angiogenesis with subsequent suppression of tumor growth in MPM. *In vivo* experiments in a ZL34 mouse model are currently ongoing.

Cancer Genetics

B 1
INTEGRATED BIOINFORMATIC APPROACHES FOR SOMATIC MUTATION CALLING IN MATCHED SENSITIVE-RESISTANT OVARIAN TUMOR PAIRS

Luca Beltrame [1] - Mariacristina Di Marino [1] - Luca Clivio [1] - Brad Chapman [2] - Enrica Calura [3] - Chiara Romualdi [3] - Maurizio D'Incalci [1] - Sergio Marchini [1]

Department of Oncology, IRCCS Istituto di Ricerche Farmacologiche "Mario Negri", Milano, Italy [1] - School of Public Health, Harvard University, Cambridge, United States [2] - Department of Biology, University of Padova, Padova, Italy [3]

Introduction: Epithelial ovarian cancer (EOC), is generally sensitive to first line platinum based therapy, however more than 80% of patients experience relapse within 18 months and become resistant to subsequent cycles, until the disease becomes incurable. Despite recent advances, the mechanisms underlying drug resistance in EOC have yet to be clearly identified. Earlier results from our laboratory, identified the EMT pathway as a key player (Marchini et al., 2013). To go deeper in detail on the genomic defects driving tumor resistance at relapse, we performed targeted DNA re-sequencing over 65 genes on a cohort of matched sensitive-resistant samples from 34 EOC pairs. Here we describe the development of an integrated bioinformatic approach aiming at idenfying potential somatic mutations in this cohort.

Materials and methods: We improved an existing, community developed, pipeline: bcbio-nextgen (http://github.com/chapmanb/bcbio-nextgen). Existing programs for somatic mutation calling in NGS samples were evaluated with regards to maintainability, specificity and compatibility with other tools. After the initial evaluation, three somatic variant callers were selected (MuTect, VarScan2, and FreeBayes) and the pipeline was modified to support them, including specific pre-processing steps according to commonly accepted best practices. After the run, several post-processing programs were run to filter and categorize potential mutations of interest. The pipeline was then tested on a high performance cluster computing platform (Cloud4CARE project). Tumor comparisons were performed using blood from the same patient as normal reference.

Results: Initial tests were made on reduced data sets from the Cancer Genome Atlas to assess the proper generation of the data and their correctness. Once the proper functionality was assessed, we ran the pipeline on the complete data set of EOC samples. The pipeline correctly discriminated germline mutations from somatic ones, and external validation confirmed the initial results.

Conclusions: Our results suggest that our bioinformatic approach is sensitive, robust, reproducible and viable for analysis of matched EOC samples.

B 2
FUNCTIONAL ROLE OF WILMS TUMOR SUPPRESSOR GENE (WT1) IN T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA (T-ALL)

Fulvio Bordin [1] - Erich Piovan [1] - Alberto Ambesi-impiombato [2] - Stefano Indraccolo [3] - Sonia Anna Minuzzo [1] - Valentina Agnusdei [1] - Giuseppe Basso [4] - Alberto Amadori [1] - Valeria Tosello [3]

Dipartimento di Scienze Chirurgiche, Oncologiche e Gastroenterologiche, Università di Padova, Padova, Italy [1] - Institute for Cancer Genetics, Columbia University, New York, United States [2] - Dipartimento di Immunologia e Diagnostica Molecolare Oncologica, Istituto Oncologico Veneto IRCCS, Padova, Italy [3] - Dipartimento di Pediatria, Università di Padova, Padova, Italy [4]

T-ALL is an aggressive hematologic tumor, resulting from the transformation of T-cell progenitors. *WT1* gene alterations have been reported in 10-12% of T-ALL and AML patients, but mechanisms downstream of WT1 have not been elucidated. The *WT1* gene encodes a zinc-finger transcription factor and is characterized by multiple alternative isoforms. The KTS(-) isoforms, lacking of three amino acids (lysine-threonine-serine or KTS) between zinc finger 3 and 4, is conserved throughout vertebrate evolution and have the capacity to bind DNA. Mutations are mainly heterozygous frame-shift in exon 7 that are predicted to produce a WT1 truncated protein that lack the property to bind DNA. Our hypothesis is that *WT1* alterations work as a loss of function of WT1 protein. Thus, our study focused on the analysis of WT1-deregulated targets following *WT1* loss in T-ALL cells and sought to understand the functional role of *WT1* alterations in the pathogenesis of T-ALL cells studying the effects of *WT1* loss. Finally, considering that *WT1* mutations are mainly located in exon 7, we evaluated a possible role of WT1 mutants as dominant negative or gain-of-function mutants. To define the structure of the transcriptional network activated by loss of function of WT1, we used a combination of approaches that included: (i) shRNA-knockdown technology and (ii) ChIP-on chip. Gene Set Enrichment Analysis identified targets that are bound and regulated by WT1 in the context of T-ALL. As demonstrated by luciferase assay, the KTS(-) isoforms induced significant luciferase levels in all the gene targets analyzed, while full length WT1 KTS(+) isoforms or mutants were not transcriptionally active. In particular, mutants in combinations with the KTS(-) isoforms did not modify the basal transcriptional activity of the KTS(-) isoforms excluding that WT1 mutants exert a dominant negative effect on the transcriptional activity of wild-type WT1 at least in our system. Overexpression of WT1 isoforms in T-ALL cells induced moderate apoptosis and cell cycle arrest while mutants and other isoforms did not. Finally *WT1* knockdown in T-ALL cells induced in-

creased survival following DNA-damaging conditions. In conclusion, analyzing WT1 downstream targets and function in T-ALL we identify new possible pathways of intervention for target therapy of T-ALL.

B 3
ANALYSIS OF THE ß-ARRESTIN 2 AND ITS FUNCTIONAL ROLE IN THE SDF-1/CXCR4 AXIS IN VIVO AND IN VITRO IN SEZARY SYNDROME.

Cristina Cristofoletti [1] - Maria Cristina Picchio [1] - Antonella Bresin [2] - Debora Pomponi [3] - Giandomenico Russo [1] - Maria Grazia Narducci [1] - Elisabetta Caprini [1]

Laboratory of molecular oncology, IDI-IRCCS, Rome, Italy [1] - Dipartimento di Morfologia, Chirurgia e Medicina Sperimentale Sezione di Patologia, Oncologia e Biologia Sperimentale, Università di Ferrara, Ferrara, Italy [2] - Laboratory of allergology, IDI-IRCCS, Rome, Italy [3]

Introduction: Sezary Syndrome (SS) is a rare form of cutaneous T-cell lymphoma (CTCL) characterized by erythroderma and presence of Sezary cells in skin, lymph nodes and peripheral blood (Olsen E. et al. *Blood*, 2007). Metastatic process has been greatly influenced by chemokines such as SDF-1 and its receptor CXCR4. The activation of this pathway modulates cell migration, survival, proliferation and gene transcription (Wong D. et al. *Clin. Cancer Research*, 2008). Recently, we have shown that CXCR4 is highly expressed by SS cells and that it is functionally active in response to SDF-1, a chemokine abundantly found in cutis of SS patients (Narducci M.G. et al. *Cancer Research*, 2008). Down-regulation of CXCR4 is mediated by primary regulators of G-protein coupled receptor protein, β-Arrestin 2 (ARRB2) (Cheng Z. et al. *Journal of Biol. Chem*., 2000), located in the most frequent lesion of SS patients (Del.17p13.2) (Caprini E., Cristofoletti C. et al. *Cancer Research,* 2009). In this study, we investigate the ARRB2 role in SS.

Materials and methods: ARRB2 genomic status and its expression level were evaluated by Genome-Wide Human SNP6.0 Array (Affymetrix) and quantitative RealTime-PCR (qRT-PCR) (Applied Biosystems). Purified SS cells from 13 patients were analyzed by Western Blotting (WB) for ARRB2 (Santa Cruz), CXCR4 and β-Actin (Cell Signaling). siRNA targeting human β-arrestin 2 and scrambled control (Thermo Scientific) were used to transfect HUT78 cells at [50 nM] (Polyplus). HUT78 chemotaxis assay was performed using SDF-1 at 50ng/mL (R&D).

Results: Cytogenetic characterization of ARRB2 locus, was conducted in 58 SS cases (40 patients plus follow-up and 3 cell lines derived from CTCL patients HUT78, H9 and HH). Heterozygous losses were observed in 73% of patients and in HH cell line, whereas HUT78 and H9 shown a reduplication of its locus. ARRB2 DNA deletion were validated by genomic qRT-PCR in 16/31 cases (51%) and mRNA down-regulation was observed in 21/31 cases (68%) by qRT-PCR. ARRB2 protein reduction was assessed by WB in 13/31 patients (42%). 24h after siRNA ARRB2 transfection, HUT78 revealed an increased migration index in response to SDF-1 respect to control of 3.5 fold change.

Conclusion: Our preliminary data suggest an inverse correlation between ARRB2 and CXCR4 expression. ARRB2 seems control the SS cells chemotaxis mediated by CXCR4 thus potentially represents a therapeutic target to treat this neoplasia.

B 4
CLONAL EVOLUTION IN CHRONIC LYMPHOCYTIC LEUKEMIA: LONGITUDINAL DATA ANALYSIS OF UNTREATED PATIENTS

Andrea D'argenio [1] - Angelo Veronese [1] - Cristian Bassi [2] - Manuela Ferracin [2] - Laura Lupini [2] - Felice Pepe [1] - Laura Z. Rassenti [3] - Veronica Balatti [4] - Francesco Autore [5] - Renato Mariani-costantini [1] - Thomas J. Kipps [3] - Luca Laurenti [5] - Carlo M. Croce [6] - Massimo Negrini [2] - Rosa Visone [1]

Aging Research Center (Ce.S.I.), Fondazione Università "G. d'Annunzio", Chieti, Italy [1] - Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Ferrara, Italy [2] - Department of Medicine, Moores Cancer Center, University of California at San Diego, La Jolla, CA, San Diego, La Jolla, CA, United States [3] - Department of Molecular Virology, Immunology, and Medical Genetics and Comprehensive Cancer Center, The Ohio State University, Columbus, OH, Columbus, OH, Italy [4] - Department of Hematology, Catholic University of the Sacred Heart, Rome, Italy [5] - Department of Molecular Virology, Immunology, and Medical Genetics and Comprehensive Cancer Center, The Ohio State University, Columbus, OH, Columbus, OH, United States [6]

Recent studies revealed that patients with chronic lymphocytic leukemia (CLL) undergo clonal evolution after chemotherapy. This process is characterized by (1) acquisition of CLL-specific genetic alterations, (2) tumor cell diversification and (3) leukemic cell sub-clone selection.

We investigated whether untreated CLLs could also experience clonal evolution. To address this question we studied the progression of somatic copy-number alterations in 20 CLL patients with either stable (n=9) or progressive (n=11) disease over two clinical time points (median between time points: 31 months). We observed by Genome-Wide SNP Array a significant decrease only in progressive disease patients: mean of the aberrations at the first time point=132; mean of the aberrations at the last time point=54 (p=0,046). Such decrease might be due to the loss of passenger aberrations and to a clonal selection of advantageous/driver genetic alterations.

Being the acquisition of driver chromosomal abnormalities during disease course commonly considered evidence of clonal evolution, we assessed the main CLL chromosomal abnormalities in an expanded cohort of 43 stable and 37 progressive disease patients (median between time points: 33 months). We focused on del(11)(q22–23), trisomy 12 and del(17)(p13) by analyzing the NTM, CPM and TP53 genes, respectively. Fourteen out of the 80 patients (17.5%) showed clonal evolution: n=6 (3 progressive and 3 stable disease) with del(11)(q22–23) and n=8 (6 progressive and 2 stable disease) with del(17)(p13).

To extend our longitudinal study of genetic alterations in CLL, we analyzed a subgroup of our cohort (20 progressive and 10 stable disease) by sequencing 30 genes implicated in CLL and comparing their alterations at the two time points. Eight out of 20 patients with progressive disease revealed a >0,15 increase in the frequency (sequencing reads with the variant/total number of sequencing reads covering the mutation site) of at least

one somatic mutation. Overall, more than half of the progressive disease patients analyzed (19/37) showed evidence of clonal evolution over time.

In conclusion, our study indicates that clonal evolution affects a high percentage of CLL untreated patients, a finding with relevant implications on the natural history of the disease.

B 5
EXPRESSION OF KRAS-4A ISOFORM IS MODULATED BY MOUSE PULMONARY ADENOMA SUSCEPTIBILITY 1 LOCUS

Alice Dassano [1] - Francesca Colombo [1] - Gaia Trincucci [1] - Elisa Frullanti [1] - Antonella Galvan [1] - Angela Pettinicchio [1] - Loris De Cecco [1] - Andrea Borrego [2] - Olga C. M. Ibañez [2] - Tommaso A. Dragani [1] - Giacomo Manenti [1]

Fondazione IRCCS, Istituto Nazionale dei Tumori, Milano, Italy [1] - Instituto Butantan, Biomedical Research Center, São Paulo, Brazil [2]

Introduction: The Pulmonary adenoma susceptibility 1 (*Pas1*) locus, mapping in the distal region of chromosome 6, is the major modulator of lung tumor susceptibility in mice. It contains six genes and, among them, *Kras* is the principal gene involved in lung tumorigenesis although its mechanism of action is still unknown. Moreover its role is further complicated by the existence of two isoforms: *Kras*-4A and -4B.

Materials and methods: To generate (A/J x C57BL/6)F4 population, (A/J x C57BL/6)F1 brothers and sisters were crossed for four generation. At weaning, F4 animals were randomly divided in two experimental groups: treated mice at 4 weeks of age, and untreated mice. Genotyping in lung tumor tissue was carried out using the Illumina SNP array, whereas in normal lung tissue 9 markers located on mouse chromosome 6 were genotyped by Pyrosequencing. Expression levels of *Kras* isoforms were evaluated by quantitative PCR. Then, genotypic and phenotypic data were combined in the expression quantitative trait loci (eQTL) analysis.

The allelic expression of *Kras*-4A and *Kras*-4B isoforms was analyzed taking into consideration two SNPs: rs30022167 and rs29968550 for both isoforms. Frequency of the two alleles of each SNP was measured by Pyrosequencing in cDNA and DNA of treated and untreated heterozygous F4 mice.

Results: We demonstrated that in an (A/J x C57BL/6)F4 population treated with urethane to induce lung tumors, *Pas1* locus controls tumor susceptibility (LOD score = 48) and acts, in lung tumor tissue, as an eQTL for *Kras*-4A (LOD score = 4.5), but not for *Kras*-4B. We observed higher expression of the A/J derived allele of *Kras*-4A isoform, attributable to variations in cis-regulatory elements mapping in the *Pas1* locus. We confirmed these data in normal lung tissue of the same F4 population (LOD score for *Kras*-4A isoform = 23.2), strengthening the expression QTL effect of *Pas1* locus observed in tumor.

Conclusions: Overall, these results indicate that the genetic control exerted by *Pas1* on lung tumor susceptibility mostly affects the expression levels of the *Kras*-4A transcript.

B 6
WHOLE EXOME SEQUENCING AND GENOME-WIDE HIGH RESOLUTION SNP ARRAY IN A PATIENT WITH PARTIAL ANDROGEN INSENSITIVITY SYNDROME AND MULTIPLE COLORECTAL CANCERS

Vittoria Disciglio [1] - Stefano Signoroni [1] - Paola Sala [1] - Massimo Milione [1] - Donata Penso [1] - Matteo Dugo [1] - Andrea Devecchi [1] - Mita Mancini [2] - Silvana Canevari [1] - Loris De Cecco [1] - Lucio Bertario [1]

Fondazione IRCCS Istituto Nazionale Tumori, Hospital, Milan, Italy [1] - Affymetrix Central Europe, /, /, United Kingdom [2]

Introduction: We report a 46,XY male patient with partial Androgen Insensitivity Syndrome (PAIS) who developed a rectosigmoid cancer (RSC) at 27 years. A testosterone therapy was provided to the patient from a 3 year age till the cancer diagnosis. 3 year after primary surgery, the patient developed a second primary rectal cancer relapsed 1 year later. The PAIS was diagnosed in a cousin who not underwent testosterone therapy. The two cousins have the same androgen receptor (*AR*) gene mutation inherited from their asymptomatic mothers. The family history of cancer is negative. Our aim is to explore the role of constitutional and somatic genetic events potentially responsible of early onset and progression of cancer in the index case.

Materials and Methods: We performed mutational and imunohistochemistry analysis of the major colorectal cancers on peripheral blood lymphocytes (PBLs) and tumor samples; Whole Exome-Sequencing (WES) and Copy Number Variations (CNVs) profiling on DNA extracted from secondary tumor tissue and PBLs of the patient. The analyses were extended to patient's relatives (parents, cousin and aunt). Exomes were captured from fragmented genomic DNA using the Ion TargetSeq Exome enrichment kit, and paired-end 50-base massively parallel sequencing was conducted on SOLiD 5500xl WF instrument and data analyzed with LifeScope software (LifeTechnologies) CNVs profiling was performed using Cytoscan HD Array and data analyzed with the Chromosome Analysis Suite (ChAS) software (Affymetrix).

Results: No mutations were detected on *MLH1*, *MSH2*, *MSH6, APC*, *MUTHY* (PBLs) and on *BRAF*, *KRAS, PI3KCA* (first RCS tissue) genes. Mismatch repair protein and microsatellite analyses gave normal results in both tumor samples. On average, 80% of reads aligned to the targeted regions and average depth of coverage within targeted regions was 80x. More than 60% of the target sequences were covered by ≥10 reads and 45% of sequenced gene regions had ≥20 reads. The quality control metrics of SNP arrays for each experiment were of high quality as indicated in ChAS Manual. The in depth analysis of both approaches and their integration is ongoing

Conclusion: To date published studies have reported that patients with PAIS and *AR* germline gene mutation have a predisposition to develop tumors different from colorectal cancer. The WES and CNVs data of the index case and his relatives and their integration might contribute to the identification of other genetic alterations different from *AR* gene mutation responsible for tumor development.

B 7
GENOMIC ALTERATIONS ASSOCIATED WITH DIFFERENT GROWTH PATTERNS AND AGGRESSIVENESS OF MELANOMA

Greta Gandolfi [1] - Valentina Sancisi [1] - Simonetta Piana [2] - Iris Zalaudek [3] - Giuseppe Argenziano [3] - Alessia Ciarrocchi [1]

Laboratory of Translational Research, Arcispedale S.Maria Nuova - IRCCS, Reggio Emilia, Italy [1] - Pathology Unit, Arcispedale S.Maria Nuova - IRCCS, Reggio Emilia, Italy [2] - Skin Cancer Unit, Arcispedale S.Maria Nuova - IRCCS, Reggio Emilia, Italy [3]

Introduction: The incidence of cutaneous melanoma raised in the past decades in all white- skinned industrialized countries and estimates predict a continuous increase over the upcoming years. This brings with it new clinical challenges and the urge to develop better tools to foreseeing the progression of the disease and new effective therapeutic approaches.

The clinical presentation and the pathological characteristics of melanoma are quite heterogeneous, most likely due to a complex etiopathological context. While some patients display lesions with a slow growth capacity, very low aggressive potential and a good prognosis, other patients present highly aggressive tumors whit high rate of growth, high metastatic potential, and a fatal outcome. In spite of the large amount of clinical and molecular works on melanoma we still do not know the determinants at the basis of the differential behaviour of this tumor.

Materials and methods: Fourty-two primary melanoma and four metastases were retrieved from our Institution and classified in fast growing and non-fast growing melanoma. Chromosomal aberrations were detected by SNP-array technology (OncoscanFFPE Express v2; Affymetrix). Data were analysed with Nexus Express (Biodiscovery). BRAF and NRAS mutations were analysed by direct sequencing. Evaluation of tumor interaction with the microenvironment was performed, by staining of intratumor and peritumor blood (podoplanin D2-40) and lymphatic vessels (CD31).

Results: Overall, the fast growing group was characterized by a higher incidence of chromosomal aberrations. All the fast growing melanomas showed major aberrations, including abrupt gains/losses of partial or full chromosomes. Chromosomal gains frequently involved 1q, 6p, 7q, 7p, 8q, 8p and 16p arms. Chromosomal losses frequently involved s 6q, 9p, 9q, 10p, 10q arm. Part of these alterations were conserved in matched metastasis. BRAF and NRAS mutations tend to be mutually exclusive and associated with aggressive behaviour. No differences were observed in the number of infiltrating blood and lymphatic vessels between the two groups.

Conclusions: Our data demonstrate that morphological and behavioural differences in melanoma are determined by specific genetic alterations and provide the basis for a novel classification of melanoma based on morphological and genomic features that should improve diagnosis and management of patients.

B 8
INVOLVEMENT OF ALK IN PEDIATRIC RHABDOMYOSARCOMA

Patrizia Gasparini [1] - Raffaella Villa [1] - Michela Casanova [2] - Paola Collini [3] - Rita Alaggio [4] - Roberto Caserini [1] - Monica Tortoreto [1] - Nadia Zaffaroni [1] - Cristina Meazza [2] - Maria Grazia Daidone [1] - Maura Massimino [2] - Andrea Ferrari [2] - Gabriella Sozzi [1]

Experimental Oncology Unit, Fondazione IRCCS Istituto Nazionale Tumori, Hospital, Milano, Italy [1] - Pediatric Oncology Unit, Fondazione IRCCS Istituto Nazionale Tumori, Hospital, Milano, Italy [2] - Department of Pathology, Fondazione IRCCS Istituto Nazionale Tumori, Hospital, Milano, Italy [3] - Department of Pathology, University of Padova, University of Padova, Padova, Italy [4]

Introduction: Rhabdomyosarcoma (RMS) is the most frequent soft tissue tumor in childhood and arises from immature mesenchymal cells committed to skeletal muscle differentiation. RMS shows extensive heterogeneity in histology, age, site of onset, clinical course and prognosis. Recently, the Anaplastic Lymphoma Kinase (ALK) tyrosine kinase receptor has emerged as a promising therapeutic target for NBL. Expression of full-length ALK receptor has been observed also in RMS, the most common childhood soft tissue sarcoma (STS). However, the involvement of ALK in RMS has not yet been comprehensively investigated.

Methods: To investigate the role of ALK and its clinical relevance in RMS, we have selected: a panel of 13 RMS cell lines, an institutional cohort of 73 FFPE pediatric RMS (28 alveolar, ARMS, and 46 embryonal, ERMS), and an external cohort of 27 FFPE pediatric RMS (6 ARMS and 17 ERMS) for validation. For these cases we investigated: 1) ALK expression by IHC; 2) genomic status of ALK by FISH, 3) ALK mutations (on 30 frozen RMS specimen available at our Institute).

Results:. IHC and FISH analysis revealed an ALK protein high or focal expression associated to copy number gains of the ALK gene in: RH30 cell line, 41% of institutional RMS (19% ERMS and 70% ARMS), and 48% of external RMS (38% of ERMS and 83% of ARMS). Overall, an ALK structural rearrangement was observed in 1 ERMS associated with an high protein expression. Furthermore, no mutations were identified by the mutational analysis of 6 exons (20-25) of ALK gene.

Conclusion: The strong association of ALK protein expression with a gene copy number gain, more frequently in ARMS, does identify a subgroup of RMS that likely could benefit from an anti-ALK targeted therapy and possibly provide a new prognostic tools for RMS stratification.

B 9
NEXT-GENERATION SEQUENCING OF COLORECTAL CANCER SAMPLES FOR IDENTIFICATION OF GENES INVOLVED IN CETUXIMAB RESISTANCE

Laura Lupini [1] - Cristian Bassi [1] - Jitka Mlcochova [2] - Petra Vychytilova-faltejskova [2] - Marta Russo [1] - Silvia Sabbioni [3] - Ondrej Slaby [2] - Massimo Negrini [1]

Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Ferrara, Italy [1] - Department of Comprehensive Cancer Care, Masaryk Memorial Cancer Institute, Brno, Czech Republic [2] - Department of Life Science and Biotechnology, University of Ferrara, Ferrara, Italy [3]

Introduction. Colorectal cancer (CRC) is one of the most frequent tumors worldwide. Cetuximab, a monoclonal antibody

that binds the extracellular domain of epidermal growth factor receptor (EGFR), is often administered to metastatic CRC harboring wild-type KRAS. However, a high percentage of tumors does not respond to the drug. Beside the well-known KRAS, some other few genes involved in development of cetuximab resistance have already been found and they include some genes of EGFR pathways. In order to improve our knowledge about the genetic mechanisms of cetuximab resistance acquisition, next generation sequencing (NGS) technologies could be of help, thanks to their capability of analyzing in a single run several genome regions in different samples.

Materials and methods. Through Ion Torrent PGM, we performed a high-throughput sequencing of a panel of 21 genes involved in CRC, in 31 wild-type-classified-KRAS colorectal cancers, that were treated with cetuximab, among which 14 samples showed no clinical response to treatment. Among all alterations found, only potentially pathogenic ones were considered. Mutations were validated by Sanger sequencing.

Results. All the segments of 21 genes of the panel were sequenced with an average coverage of 487 reads each. Overall, the most frequently mutated genes in the 31 samples were TP53 (61%) and APC (53%), in agreement with the scientific literature. In order to distinguish between Cetuximab responder and resistant samples, the most relevant mutations were found in EGFR-pathway genes (KRAS, NRAS, BRAF). In particular, the finding that 4 samples had mutation in KRAS gene demonstrated an improved accuracy of NGS technologies in predicting low frequency single nucleotide variations, if compared to other methods currently used in clinical practice.

Conclusions. This work, in addition to finding new potential markers of EGFR inhibitors sensitivity, demonstrated that next-generation sequencing technology could be of help in clinical practice, for its ability to analyze in a single reaction several cancer-related genes that can guide clinical decision and patients management.

B 10
CIRCULATING TUMOR CELLS IN METASTATIC MEDULLARY THYROID CANCER

Cinzia Marinaro [1] - Cristina Romei [2] - Fiammetta Falcone [1] - Francesca Casella [2] - Rossella Elisei [2] - Giuseppe Viglietto [1]

Experimental and Clinical Medicine Department, Magna Graecia University, Catanzaro, Italy [1] - Clinical and Experimental Medicine Department, Azienda Ospedaliero-Universitaria Pisana, PISA, Italy [2]

Introduction: Thyroid cancer is the most common malignancy of the endocrine system. Medullary thyroid cancers (MTC) represent 7% of thyroid tumors; 80% of these tumors have a sporadic origin while 20% a familiar origin. MTC's distant metastases are the main cause of mortality; survival after detection of metastases is of about 50% after one year. Interesting information about the metastatic process arise from the phenotypic and genotypic characterization of circulating tumor cells (CTCs), cancer cells of solid tumor origin found in the peripheral blood.

Materials and methods : The total number of CTCs in thyroid cancer patients was estimated by an immunomagnetic separation of peripheral blood mononuclear cells using EpCAM antibody (Cell Search Technology, Veridex); CTCs were defined as nucleated cells expressing cytokeratin and lacking CD45 expression. Single CTC sorting was obtained by the DEPArray (Silicon Biosystems), a semiautomated system that allows the isolation of rare cells based on dielectrophoretic manipulation. To identify genetic alterations responsible for the tumor phenotype, next-generation sequencing (ION Torrent platform) was performed.

Results: CTCs were detected in 19,35% of metastatic MTC patients (n=6 of 31). CTC numbers ranged between 0 and 200/7.5 ml blood and 9,6% of CTC-positive patients had $\geq$ 5 CTCs. Single cell preparations resulting from CTC-enriched samples were subjected to whole genome amplification and different technologies were evaluated to obtain the best performance and genome representativeness. The mutational status of single CTCs was assessed and the bioinformatic analysis is ongoing.

Conclusions: In order to better understand the molecular and genetic features of metastatic medullary thyroid carcinoma a schematic flowchart was adopted; starting from enriched samples through single cell mutational analysis it was possible to identify, for the first time, circulating cells with malignant phenotype in medullary thyroid cancer patients.

B 11
SCREENING FOR OF FIG-ROS1-VARIANT IN BILIARY TRACT CARCINOMAS BY NESTED PCR

Caterina Peraldo Neia (1) - Giuliana Cavalloni (2) - Antonella Balsamo (2) - Tiziana Venesio (3) - Francesca Napoli (4) - Massimo Aglietta (5) - Francesco Leone (5)

Department of Oncology, IRCCS-Candiolo, Italy, University of Turin Medical School Turin, Italy, Candiolo, Italy (1) - Fondazione del Piemonte per l'Oncologia (FPO), IRCCS-Candiolo, Italy, Fondazione del Piemonte per l'Oncologia (FPO), IRCCS-Candiolo, Italy, Candiolo, Italy (2) - IRCCS-Candiolo, Italy, Molecular Pathology Laboratory, Unit of Pathology, Candiolo, Italy (3) - Department of Oncology, IRCCS-Candiolo, Italy, University of Turin Medical School Turin, Candiolo, Italy (4) - Univerity of Turin Medical School, Fondazione del Piemonte per l'Oncologia (FPO), IRCCS-Candiolo, Italy, Univerity of Turin Medical School, Fondazione del Piemonte per l'Oncologia (FPO), Candiolo, Italy (5)

ROS1 rearrangements have been detected in a variety of tumors and they are considered as suitable targets of anticancer therapies. We developed a new, quick, specific, and sensitive Polymerase Chain Reaction (PCR) test to screen FIG-ROS1 variant and applied to a series of Italian patients with bile duct carcinoma (BTC).

Formalin-fixed, paraffin-embedded tissue derived from 65 Italian BTC patients and six cell lines were analyzed by nested PCR to investigate the prevalence of a previously reported FIG-ROS1 variant. The specificity and sensitivity of nested PCR were set up on FIG-ROS1 positive U118MG cells in reconstitution experiments with peripheral blood mononuclear cells.

We found that 6 out of 65 (9.23%) BTC patients were positive for the FIG-ROS1 fusion product, 2 out of 14 (14.28%) gallbladder carcinoma (GBC) patients, 4/25 (16%) extrahepatic

cholangiocarcinoma (ECC) patients. None of the 26 intrahepatic cholangiocarcinoma (ICC) patients presented FIG-ROS1 variant. All the cell lines were negative for this variant.

In conclusion GBC and ECC subpopulations resulted positive for FIG-ROS1 rearrangement in a case series of Italian patients. This may have clinical implication, since these patients will potentially benefit from the treatment with specific ROS1 inhibitors.

B 12
HUMAN BUT NOT MOUSE P53 LOCALIZES AT THE CENTROSOMES IN MITOSIS AND ALLOWS DIAGNOSIS OF MUTANT ATM ZYGOSITY

Andrea Prodosmo [1] - Laura Monteonofrio [1] - Claudia Contadini [1] - Giuliana Di Rocco [1] - Cecilia Nisticò [1] - Enrico Cundari [2] - Luciana Chessa [3] - Silvia Soddu [1]

Regina Elena National Cancer Institute, I.F.O., Rome, Italy [1] - I.B.P.M, C.N.R., Rome, Italy [2] - Department of Clinical and Molecular Medicine, La Sapienza, University, Rome, Italy [3]

Introduction: Centrosomes duplicate at each cell cycle and are the major organizers of the bipolar mitotic spindle. A direct role of p53 in centrosome duplication has been proposed based on studies performed in mouse cells in which p53 localizes at the centrosome in interphase both in quiescent and proliferating cells. In contrast to mouse cells, we observed that in human cells p53 does not permanently resides at the centrosome but transiently moves to centrosomes at each mitosis in a microtubule- and ATM-dependent manner. This behavior is independent of DNA damage and it is so consistent at each cell cycle to allow us to develop a simple, fast, minimally invasive, reliable, and inexpensive test that functionally determines mutant ATM zygosity.

Materials and methods : Based on the presence or absence of the human p53 mitotic centrosomal localization (p53-MCL) in cell-cycle stimulated peripheral blood mononucleated cells, we diagnose ataxia-telangiectasia (A-T) homozygotes and heterozygotes and confirmed *ATM* as a breast cancer-susceptibility gene. This human p53 behavior is independent of the type of *ATM* mutations and the amount of ATM protein expression, and is so stable within each genotype to allow a significant discrimination without the need of reference samples.

Results: We found that alterations of p53-MCL are specific for A-T since no p53-MCL alterations were found in other genetic diseases causing ataxia, showing radiosensitivity, or carrying mutations in the *ATR*, *TP53* and *BRCA1/2* genes.

Conclusions: These data open the possibility of cost-effective, early diagnosis of A-T homozygotes and large-scale screenings for heterozygotes based on a p53-specific subcellular localization

B 13
EGFR MUTATION TESTING IN PULMONARY ADENOCARCINOMA: EVALUATION OF TUMOR CELL NUMBER AND TUMOR PERCENT IN PARAFFIN SECTIONS OF 120 SMALL BIOPSIES

Stefania Scarpino [1] - Federica Pulcini [1] - Arianna Di Napoli [1] - Emanuela Pilozzi [1] - Luigi Ruco [1]

Department of Clinical and Molecular Medicine, Pathology Unit, Sant'Andrea Hospital, Sapienza University, Rome, Italy [1]

Purpose: Successful evaluation of EGFR mutational status in tissue sections of small biopsies is profoundly influenced by number and percent of tumor cells. Criteria for adequacy are actually lacking.

Experimental design: Tumor area, tumor cell number and percent tumor were measured on digitalized slides of 120 small biopsies. DNA was extracted from the whole paraffin section without microdissection and was processed for EGFR mutation analysis using a Real-Time PCR technique. In other experiments, the minimal tumor area necessary for successful EGFR mutation testing was determined by laser capture microdissection of 12 EGFR-mutated cases of lung adenocarcinoma.

Results and Conclusions: The incidence of EGFR-mutated cases was similar in 132 surgical specimens (20.5%), and in 145 small biopsies (21.4%). In both series mutated cases were more numerous in female patients. Tumor cell number and tumor percent were evaluated in digitalized slides of 120 small biopsies. It was found that 11 of the 26 EGFR-mutated cases (42%) had <20% tumor cells, that only a single case had <200 tumor cells, and that 364 tumor cells occupying a tumor area of 0.12 mm2 was the lowest tumor content still effective for detection of EGFR mutations. This finding was validated experimentally using laser capture microdissection in 12 surgical samples of EGFR-mutated adenocarcinoma. It was found that a tumor area of 0.12 mm2, containing 140±34 tumor cells, was large enough to allow detection of EGFR mutation in 11 of 12 cases. It was also demonstrated that EGFR gene amplification and/or chromosomal polysomy cause a 2- 4 fold increase in the sensitivity of the assay. Conclusions: Our findings suggest that whenever a small biopsy sample is adequate for making a histological diagnosis of adenocarcinoma, it probably contains enough tumor cells for a reliable EGFR mutation testing if a mutation-specific Real Time PCR technique is used.

B 14
WHOLE EXOME SEQUENCING REVEALED A NOVEL PALB2 MUTATION IN A MALE BREAST CANCER FAMILY

Valentina Silvestri [1] - Piera Rizzolo [1] - Anna Sara Navazio [1] - Virginia Valentini [1] - Veronica Zelli [1] - Giovanni Chillemi [2] - Marco Montagna [3] - Laura Ottini [1]

Department of Molecular Medicine, Sapienza University of Rome, Rome, Italy [1] - CASPUR, Inter-universities Consortium for Supercomputing Applications, Rome, Italy [2] - Immunology and Molecular Oncology Unit, Veneto Institute of Oncology IOV-IRCCS, Padua, Italy [3]

Introduction: Male breast cancer (MBC) is a rare and poorly characterized disease. A large percentage of men affected with breast cancer (BC) showed BC family history and personal his-

tory of other cancers, thus pointing to a relevant genetic component in MBC pathogenesis. We have showed that about 15% of Italian MBC cases can be assigned to mutations in known susceptibility genes, such as *BRCA1/2*. However, a large fraction of high-risk, *BRCA1/2*-negative MBCs remain to be assigned to specific genetic factors and deserve to be further investigated.

In this study, we investigated a high-risk *BRCA1/2*-negative MBC family by Whole Exome Sequencing, in order to identify germ-line variants that could explain an additional genetic component of MBC susceptibility.

Materials and methods: A family with 6 BC cases, including two first-degree MBC cases, was selected for the study. Three BC cases, including a MBC case, were examined. DNA was extracted from blood samples and the exome was captured with the Illumina TruSeq Exome Enrichment kit. Whole Exome Sequencing was performed with the Illumina Genome Analyzer IIx platform. Candidate germ-line mutations were identified using a bioinformatics pipeline, including BWA for sequence alignment, SAMtools for alignment filtering and Varscan for mutation calling, and validated by Sanger sequencing.

Results: A list of candidate genes was defined, giving priority to not in-frame or unknown variants in genes potentially involved in cancer pathogenesis and present in at least two of the three affected family members. A novel *PALB2* truncating mutation, c.419delA, resulting in p.K140X, was the most relevant candidate.

PALB2 is a known BC susceptibility gene, whose mutations are frequently observed in families with cases of both female and male BC. The mutation was found in the index case and her paternal uncle, diagnosed with melanoma at age 65 and MBC at age 76, but not in her maternal aunt, diagnosed with BC at age 60, indicating that the mutation came from the paternal side of the family.

Conclusions: Our results add to the accumulating evidence that *PALB2* is involved in BC risk in both sexes. Consideration may be given to clinical testing of *PALB2* for high-risk *BRCA1/2*-negative families in which there is more than one male member diagnosed with the disease.

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B 15
CHROMATIN REMODELING SWI/SNF COMPLEX ROLE IN PANCREATIC CANCER PROGRESSION

Maria Svelto [1] - Giannicola Genovese [2] - Marisa Farina [1] - Caterina Fanali [3] - Donatella Lucchetti [4] - Valerio Cufino [4] - Alessandro Sgambato [5] - Lynda Chin [6]

Institute of Pathology, Catholyc University, Rome, Italy [1] - Genomic Medicine, MD Anderson Cancer Center, Houston, United States [2] - Institute of Pathology, catholyc university, Rome, Italy [3] - Institute of Pathology, Catholyc university, Rome, Italy [4] - Institue of Pathology, Catholyc University, Rome, Italy [5] - Genomic Medicine, MD Anderson Cancer center, Houston, United States [6]

Introduction: Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive disease and little is known about the broad epigenetic changes associated with malignant tranformation. Upon acute oncogenic Kras activation, normal cells activate fail-safe mechanisms which result in cell cycle arrest and cellular senescence also described as Oncogene Induce Senescence (OIS). OIS has been extensively observed in a spectrum of pre-malignant lesion suggesting that aberrant oncogenic activity in the early, no invasive, stages of cancer progression may trigger a senescence response and that additional genetic/epigenetic events may be necessary for the development of aggressive cancer.

Materials and methods : We investigate the molecular events occurring during OIS escape in a Genetically Engineered Mouse Model (GEMM) system of PDAC progression in the context of oncogenic Kras activation (*Pdx1-Cre; Kras*$^{G12DLSL/+}$). By using a stochastic system of senescence escape we found that the escaper lines showed full oncogenic potential as assessed by subcutaneous and orthotopic transplants in nude mice and through an RT-qPCR array we showed that 1 of the top 5 down-regulated genes is member of the conserved SWI/SNF chromatin remodeling complex *Snf5*, suggesting a functional role of the complex in the establishment of a senescent cell fate. We next sought to determine the functional role of *Snf5* by RNAi mediated depletion in pancreatic ductal epithelial cells upon *Kras* activation.

Results: The stable knockdown of both genes resulted in a significant survival advantage in culture *in vitro*, clonogenic growth in soft agar and tumorigenesis *in vivo*. Moreover, we generated a conditional GEM model of PDAC by crossing *Snf5* floxed mice with the pancreatic specific knock-in allele of oncogenic Kras (*Pdx1-Cre; Kras*$^{G12DLSL/+}$*; Snf5*$^{L/L}$ and *Pdx1-Cre; Kras*$^{G12DLSL/+}$*; Snf5*$^{L/+}$) where we noticed a robust acceleration of pancreatic tumorigenesis by coupling exit from quiescence with a differentiation block and induction of an EMT (Epithelial Mesenchymal Transition) program ultimately resulting in aggressive anaplastic ductal adenocarcinomas.

Conclusions: These data support the hypothesis that alterations affecting the chromatin remodeling machinery may promote the development of senescence escape and pancreatic tumor progression.

B 16
CONSISTENCY OF EGFR AND KRAS MUTATIONAL STATUS IN NON-SMALL CELL LUNG CANCER (NSCLC) FORMALIN-FIXED AND SNAP FROZEN TISSUE SAMPLES BY NEXT GENERATION SEQUENCING (NGS)

Irene Vanni (1) - Simona Coco (1) - Anna Truini (1) - Maria Giovanna Dal Bello (1) - Angela Alama (1) - Erika Rijavec (1) - Carlo Genova (1) - Giulia Barletta (1) - Federica Biello (1) - Mauro Truini (2) - Francesco Grossi (1)

Lung Cancer Unit, IRCCS A.O.U San Martino-IST Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy (1) - Pathology and Cytohistology Division, IRCCS AOU San Martino IST, Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy (2)

Introduction: NSCLC is one of the most common cause of cancer-related death worldwide. In view of a personalized treatment, the identification of driver mutations in *EGFR* are already

included as standard care for advanced NSCLC patients, and *KRAS* represents an emerging druggable target in NSCLC therapy. NGS shows several advantages in the screening of clinically relevant mutations such as the higher sensitivity over the Sanger Sequencing (SS). We evaluated a target sequencing approach focused on somatic hotspot cancer mutations based on semiconductor sequencing. Since the majority of tumours from advanced patients derived from formalin-fixed paraffin embedded (FFPE) biopsies and the fixative process may result in DNA fragmentation and nucleotide artefacts, we also evaluated the method reliability concerning mutation type and data reproducibility comparing FFPE with the matched snap frozen (SF) sequencing results.

Materials and methods To explore a potential clinical role of NGS, we selected 2 NSCLC cell lines harbouring known *EGFR* mutations (del E746-A750 NCI-H1650;L858R/T790M NCI-H1975) and 5 patients with FFPE and matched SF tissue, previously positively tested for *EGFR* and *KRAS* mutations by SS. DNA extracted from FFPE and SF tissues underwent an accurate quality control assay for FFPE DNA based on a multiplex-PCR approach. The samples were amplified using the "AmpliSeq Colon and Lung Cancer Panel" that analyzes targeted regions of 22 genes frequently mutated in lung cancer, and sequenced by Ion Torrent PGM® (Life Technologies). Sequence data were analysed using the Variant Caller plugin for all classes of genomic alterations comparing to prior SS mutation results.

Results NGS confirmed *EGFR* and *KRAS* mutations in both cell lines and NSCLC samples showing identical results starting from FFPE and SF tissue belonging to the same patient. Only one FFPE sample disclosed two novel additional SNV compared to matched SF tissue, but with a low frequency (5%-12%). Moreover, the sequencing identified several additional gene variants in others genes (e.g. *TP53, FGFR3*) identifying both known SNPs and pathogenic mutations. Further, all samples hotspot regions reached at least 500x sequencing coverage.

Conclusions

We demonstrate the feasibility to obtain a NGS suitable DNA from a FFPE tissue performing an appropriate DNA quality control assay, and thus reaching comparable data either starting from SF and FFPE tissue.

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B 17
CHARACTERIZATION OF CIRCULATING-FREE DNA IN PLASMA SAMPLES FROM NON-SMALL CELL LUNG CANCER PATIENTS

Carla Verri [1] - Paola Ostano [2] - Lidia Sacchetto [2] - Maurizia Mello Grand [2] - Chiara Ghimenti [3] - Giovanna Chiorino [2] - Luca Roz [1] - Gabriella Sozzi [1]

Tumor Genomics Unit, Fondazione IRCCS -Istituto Nazionale dei Tumori, Milan, Italy [1] - Cancer Genomics Lab, Fondazione Edo ed Elvo Tempia, Biella, Italy [2] - Ospedale di Empoli, Ospedale di Empoli, Empoli, Italy [3]

Introduction: Plasma levels of circulating free DNA (cfDNA) are higher in NSCLC patients compared to disease free smokers and represent potential diagnostic markers for lung cancer. Several studies showed that most of cfDNA is released by normal cells whereas only a small fraction is tumor-related, prompting the hypothesis that changes in tumor microenvironment contribute to circulating DNA. At present, the biological mechanisms responsible for the release of cfDNA in plasma are not known. A molecular characterization of cfDNA circulating in plasma, besides providing clues on the origin and mechanism involved in the release of DNA in the blood, could also improve the robustness of diagnostic/prognostic tests based on a non-invasive detection of biomarkers in plasma samples.

Materials and Methods: To gain insights into differential representation of genomic regions in plasma of lung cancer patients and control subjects, we studied the genomic profile of cfDNA from 22 patients and 9 disease free controls using Agilent aCGH 2x105A Microarray platform after Whole Genome Amplification (Sigma). Amplified cfDNA was cohybridized with reference genomic DNA, using a reference+sample vs reference design. After background correction and linear intensity normalization, the green reference intensity was subtracted from the red reference+sample intensity to obtain estimate of the true signal from probes recognizing circulating fragments.

Results and Conclusions: Only 768 unique probes were consistently detectable in all plasma samples, representing 0.8% of total probes analyzed. The average number of circulating probes in control samples was 35919 (36%), in ADC was 63727 (64%) and in SCC 44353 (45%). 22 genomic regions were found to be exclusively present in plasma samples from lung cancer, including also unknown genomic regions. Interestingly, by performing analysis according to tumor histotype, an increased release of tumor-related regions was observed in plasma samples from ADC compared to SCC patients (121 and 15 positive probes, respectively) and ALK gene was among the top-ten probes identified, with the highest log(Intensity) value. Validation of the genomic regions involved, in particular those of the ALK gene, is ongoing by real time PCR in an independent set of plasma samples from patients with corresponding tumor frozen tissue also available for the cytogenetic characterization of these targets by FISH.

B 18
SEARCHING FOR ONCOGENIC DRIVERS IN THE STEPWISE PROCESS OF HEPATOCARCINOGENESIS

Patrizia Zavattari [1] - Andrea Perra [2] - Marta Anna Kovalik [2] - Maria Maddalena Angioni [2] - Silvia Menegon [3] - Annalisa Petrelli [3] - Luca Quagliata [4] - Giovanna Maria Ledda-columbano [2] - Luigi Terracciano [4] - Silvia Giordano [3] - Amedeo Columbano [2]

Department of Biomedical Sciences, University of Cagliari, Cagliari, Italy [1] - Department of Biomedical Sciences, University of Cagliari, Italy [2] - IRCC, Candiolo, Italy [3] - Institute of Pathology, University of Basel, Switzerland [4]

Introduction: Hepatocellular carcinoma (HCC) develops through a multistage process but the nature of the molecular changes associated to the different steps, particularly the very early ones, is largely unknown. Recently, we have shown that activation of the Keap1-Nrf2 pathway is a very early event, suggesting that dysregulation of this pathway may have a causal role in HCC development.

Materials and methods : The aim of the present study was to investigate by Sanger sequence analysis whether and when *Nrf2*

mutations occur in the Resistant-Hepatocyte (R-H) rat model of hepatocarcinogenesis. The effect of Nrf2 silencing in the colony forming ability and tumorigenic properties of HCC cells was also investigated *in vitro* and *in vivo*.

Results: *Nrf2* mutations are extremely frequent (18/20;90%) in early preneoplastic lesions positive for the stem/progenitor cell marker KRT-19, considered to be the precursor cell population of HCC in this model. Notably, all the mutations were missense and involved the Nrf2-Keap1 binding regions. Mutations of *Keap-1* were rare and were found only in preneoplastic lesions lacking *Nrf2* mutations (10%). KRT-19 positive HCCs also showed a high percentage of *Nrf2* mutation (> 50%), suggesting that mutation of this gene provides a selective advantage towards progression to HCC. Unlike *Nrf2*, mutations of b-catenin were found in late, but not early stages of cancer development. Silencing of *Nrf2* impaired colony forming ability of rat tumorigenic cells. Moreover, while untreated rat carcinoma cells develop HCC when injected into syngenic animals, their tumorigenic capacity was inhibited when *Nrf2* was silenced prior to injection.

Conclusions: i) The Nrf2-Keap1 pathway is strongly activated in early and late stages of rat hepatocarcinogenesis; ii) Activating mutations of *Nrf2* are most likely the cause of dysregulation of the Nrf2-Keap1 pathway; iii) These mutations are also present in very early stages of the carcinogenic process suggesting that Nrf2 might be a "driver" oncogene in HCC development; iv) *Nrf2* mutations are more frequent in a subset of aggressive preneoplastic lesions characterized for their positivity to KRT-19; v) Silencing of *Nrf2* impaired both colony forming ability as well as tumorigenic capacity of rat carcinoma cells. In view of the recent finding of *NRF2* mutations in human HCC, our results suggest that *NRF2* can be a therapeutic target for HCC.

Cancer Stem Cells

C 1
BALANCE BETWEEN EPITHELIAL AND MESENCHYMAL FEATURES REGULATES PLASTICITY AND RESPONSE TO MICROENVIRONMENTAL CUES IN LUNG CANCER CELLS

Francesca Andriani [1] - Giulia Bertolini [1] - Tiziana Caputo [1] - Federica Facchinetti [1] - Erika Baldoli [1] - Massimo Moro [1] - Roberto Caserini [1] - Gabriella Sozzi [1] - Luca Roz [1]

Fondazione IRCCS, Istituto Nazionale dei Tumori, Milan, Italy [1]

Introduction: solid cancers can be seen as abnormal organs with their own hierarchical organization where only a subpopulation of cells, defined as "cancer stem cells" (CSCs) has potential to initiate a new tumor. Signals from the microenvironment are involved in modulation of stemness properties and recent evidence related to the plasticity of cancer cells suggests that, CSCs can be generated de novo by a dynamic phenotypic switch between non-CSC and CSC populations.

Materials and methods: to investigate how signals from microenvironment could influence stemness properties, six lung cancer cell lines were treated with TGFb. FACS analysis showed an heterogeneous increase of CD133 (1 to 10 fold) and stemness-related genes, associated in general to an up regulation of mesenchymal EMT-related genes and downregulation of epithelial marker CDH1 assessed by Real Time PCR.

Results: extent of response was found to be strictly dependent on the ratio between mesenchymal (M) and epithelial (E) features estimated by the expression of SNAI2 and E-cadherin (CDH1) markers. Cells displaying both, mesenchymal and epithelial features, responded to microenvironmental stimuli more efficiently than cells with a fully mesenchymal phenotype suggesting that a hybrid state could be more conductive to plasticity. Overexpression of miR200c was used to establish whether modulation of epithelial-mesenchymal balance could affect the acquisition of stemness phenotype under microenvironmental stimuli. MiR200c induced upregulation of CDH1 without any alteration of mesenchymal markers and resulted in a shift, driving cells toward a more epithelial phenotype. Only cell lines that reached a critical threshold in the balance between E and M markers were able to generate stem-like cells in response to external signals. Moreover, to verify the importance of microenvironment in providing the right cues also for tumor progression in vivo, cell lines were co-injected with fibroblasts in mice. Fibroblasts promoted tumor growth and dissemination in vivo in particular in cells with hybrid features (E/M), while cells with mesenchymal traits resulted more cell-autonomous and not responsive to signals derived from fibroblasts.

Conclusions: together these data provide evidence that external stimuli can generate cells with stem-like features and that phenotypic state, mesenchymal (M), epithelial (E) or hybrid (M/E), is critical for the regulation of plasticity induced by the microenvironment.

C 2
IMPAIRMENT OF V-ATPASE AS A THERAPEUTIC MODALITY TARGETING CANCER STEM CELLS OF RHABDOMYOSARCOMA

Sofia Avnet [1] - Manuela Salerno [2] - Shigekuni Hosogi [3] - Nicola Baldini [2]

Orthopaedic Pathophysiology and Regenerative Medicine, Istituto Ortopedico Rizzoli, Bologna, Italy [1] - Department of Biomedical and Neuromotor Sciences, University of Bologna, Bologna, Italy [2] - Department of Molecular Cell Physiology, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kyoto, Japan, Bologna, Italy [3]

Introduction: Rhabdomyosarcoma (RMS) is a very aggressive tumor with a high incidence of relapse and metastasis. Since treatment options for RMS patients are limited, the identification of novel targets is strongly needed. According to the cancer stem cell (CSC) theory, the development of new agents that selectively block key pathways in the CSC subpopulation would be predictably successful. We have previously isolated and characterized CSC from several sarcomas, including RMS.[1] In

this work, we investigate the vacuolar proton pump (V-ATPase) activity as a candidate therapeutic target.

Materials and methods : RMS-CSC were obtained by the 3D sphere system assay, under low attachment conditions plus bFGF and EGF exposure. Stemness, chemoresistance, invasiveness, lysosome and cytosolic acidity, and V-ATPAse V0c expression of CSC were evaluated and compared to those of parental cells. Finally, we analyzed the effects of the V-ATPase V0c blocker, omeprazole, on survival, invasiveness, and drug resistance of CSC.

Results: isolated RMS-CSC showed stem-like features (high expression of NANOG and OCT3/4 genes, self-renewal and multiple differentiation ability) and higher invasive ability and chemoresistance to doxorubicin (DXR) in comparison to native cells. Interestingly, RMS-CSC were also resistant to DXR through a mechanism unrelated to the classical multidrug resistance process but dependent on a high level of lysosome acidity mediated by an overexpression of vacuolar ATPase (V-ATPase). Inhibition of lysosomal acidification by the V-ATPase inhibitor omeprazole (OME) significantly modified chemoresistance of RMS-CSC. Unexpectedly, lysosomal targeting also blocked cell growth and reduced the invasive potential of RMS CSC, even at a very low dosage (OME 10 and 50 μM, respectively).

Conclusions: Based on these observations, we propose lysosome acidity as a valuable target to overcome chemoresistance of RMS-CSC, and suggest the use of anti-V-ATPase agents in combination with standard regimens as a promising tool for the eradication of minimal residual disease or the prevention of metastatic disease.

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Reference:

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C 3

GENE EXPRESSION AND MICRORNA PROFILES OF GBM STEM-LIKE CELLS DISCRIMINATE DIFFERENTIALLY AGGRESSIVE CELL CLUSTERS

Mariachiara Buccarelli [(1)] - Michele Signore [(2)] - Liliana Morgante [(2)] - Daniele Runci [(1)] - Ramona Ilari [(2)] - Roberto Pallini [(3)] - Lucia Ricci-vitiani [(2)]

Department of Hematology, Oncology and Molecular Medicine,Istituto Superiore di Sanità, Rome, Italy, Università di Roma 'La Sapienza', Roma, Italy [(1)] - Department of Hematology, Oncology and Molecular Medicine,Istituto Superiore di Sanità, Rome, Italy, Department of Hematology, Oncology and Molecular Medicine,Istituto Superiore di Sanità, Rome, Italy, Roma, Italy [(2)] - Institute of Neurosurgery,Università Cattolica del Sacro Cuore, Roma, Italy, Institute of Neurosurgery,Università Cattolica del Sacro Cuore, Roma, Italy, Roma, Italy [(3)]

Glioblastoma multiforme (GBM) is the most common and aggressive adult brain tumor. Despite aggressive surgery, radiation and chemotherapies, the life expectancy of patients with GBM is about 14 months after diagnosis. The extremely aggressive behaviour of GBM has been ascribed to a rare fraction of self-renewing, multipotent tumor-initiating cells, called GBM stem-like cells (GSCs), responsible for tumor progression, maintenance, and recurrence. GSCs can be maintained *in vitro* using specific serum-free conditions. During the last years, we have collected GSCs from more than 50 GBM patients. These cells have been expanded and validated for their stem cell properties. The tumorigenic potential of GSCs has been assayed *in vivo* by orthotopic and heterotopic xenograft in immunodeficient mice.

Gene expression profiling (Affymetrix assay) has been performed in a large panel of GSC lines. Clustering of the 40 GSC samples using only the top 1000 most variable genes/transcript revealed two distinct clusters. A clustering of GBM SC samples into two distinct clusters has been reported in the literature. According to this previous works we also classified the two clusters as a "GSf" full stem-like phenotype (more closely related to GBM samples) and a "GSr" restricted stem-like phenotype (more closely related to GBM stem-like cell lines).

MicroRNA (miRNAs) gene profiling (Agilent assay) has also been performed on GSC lines identifying 9 miRNAs as differentially expressed between GSf and GSr groups. Some of them were previously reported to be de-regulated in malignancies, including GBM. These miRNAs are mainly involved in cell growth and survival as regulators of the EGFR signaling pathway.

C 4

RESVERATROL EFFECTS ON MIGRATION AND WNT SIGNALING PATHWAY IN CANCER STEM CELL LINES FROM GLIOBLASTOMA MULTIFORME

Chiara Cilibrasi [(1)] - Gabriele Riva [(2)] - Valentina Butta [(1)] - Leda Dalpra' [(3)] - Angela Bentivegna [(2)]

Phd in neuroscience, department of surgery and translational medicine, university of milan-bicocca, monza (mb), italy [(1)] - department of surgery and translational medicine, university of milan-bicocca, monza (mb), italy

(2) - department of surgery and translational medicine / department of clinical pathology, medical genetics laboratory, university of milan-bicocca / san gerardo hospital, monza (mb), italy (3)

Introduction Glioblastoma multiforme (GBM) is a grade IV astrocytoma and the least successfully treated solid tumors: current therapies provide a median survival of 12-15 months after diagnosis, due to the high recurrence rate. GBM is characterized by an highly infitrative nature and a remarkable intratumoral heterogeneity, mirrored by the presence of distinct subpopulations of cells with different tumorigenic capabilities: Glioma stem cells (GSCs) are believed to be the real driving force of tumor initiation, progression and relapse. Better therapeutic strategies GSC-targeted are needed.

Resveratrol (RSV) is a polyphenolic phytoalexin with pleiotropic health benefits. Many studies highlighted its antiproliferative and proapoptotic effects and its ability to reduce tumor invasion and cell migration in several types of cancers, including GBM.

RSV might represent an attractive agent for the treatment of GBM because of its minimal toxicity and blood brain barrier permeability.

Material and Methods In this study, we analyzed the effects of RSV exposure (48-72-96h) on cell motility, through Wound Healing assay, in six GSC lines isolated from GBM (GBM2, GBM7, G179, GliNS2, G144, GBM04). Moreover we evaluated the effect of RSV administration on WNT signaling pathway using RT-PCR technology on Applied Biosystems platform. The WNT signaling expression profile was performed using PCR custom arrays (Qiagen) on untreated and treated (100mM 96h RSV) cells in order to explore expression variations of 7 WNT-related genes (WNT1, FZD4, CTNNB1, EP300, CREBBP, TCF7, MYC) after RSV exposure.

Results RSV strongly reduces cellular motility in all GSC cell lines and differently modulates WNT signaling pathway in the GSC cell lines taken into account. WNT1 is upregulated in GBM04 and G144 cell lines, while FZD4 is upregulated in GBM2, GBM04 and G144 cell lines after treatment. CTNNB1 and EP300 show an unchanged transcriptional activity in most of the cell lines. CREBBP and TCF7 are upregulated in some cell lines and downregulated in others. MYC is upregulated in most of the cell lines.

The validation of these data in order to verify, at protein level, the RSV modulation of MMPs and WNT signaling pathway is in progress.

Conclusions RSV treatment could represent, in combination with other therapeutic strategies, a new approach in order to inhibit the infiltrative nature of GSCs. Moreover the study of the WNT signaling pathway could suggest new insights on GBM oncogenesis.

Keywords: GBM, Glioma stem cells, Resveratrol, WNT signaling pathway

C 5
EFFECTS OF CHEMOPREVENTIVE AGENTS ON MELANOMA STEM CELLS

Katiuscia Dallaglio (1) - Tiziana Petrachi (1) - Martina Chiappelli (1) - Adriana Albini (2)

Tranlastional Research Laboratory, IRCCS 'Tecnologie Avanzate e Modelli Assistenziali in Oncologia', Arcispedale Santa Maria Nuova, REGGIO EMILIA, Italy (1) - Department of Research and Statistics Infrastructure, IRCCS 'Tecnologie Avanzate e Modelli Assistenziali in Oncologia', Arcispedale Santa Maria Nuova, REGGIO EMILIA, Italy (2)

Introduction: Melanoma is the deadliest form of skin cancer, displaying resistance to conventional therapies. Recently discovered drugs targeting melanoma induce some degree of tumor regression, however relapse often occurs within few months. A possible mechanism explaining this phenomenon is the persistence of stem-like tumor cells, namely cancer stem cells (CSC), in treated patients. Therapies targeting the CSC compartment are warranted, however to date no currently available drug kills exclusively or preferentially melanoma stem cells. Chemoprevention is the use of natural or synthetic chemical agents suppressing or preventing the carcinogenic progression. Several chemopreventive agents have shown to be able to kill melanoma cells, however, little is known on the cellular targets of chemoprevention in melanoma.

Materials and methods: We evaluated the effect of the chemopreventive biguanides metformin and phenformin on melanoma cells using both monolayer and 3D spheroid cultures. Given that in melanoma, cells expressing high levels of aldehyde dehydrogenase (ALDH) are CSC, we isolated ALDH+ and + melanoma cells by flow cytometric cell sorting and analyzed these cells by Real Time PCR. We also tested the sensitivity of ALDH+ and - cells to 10mM metformin and 1mM phenformin by blu trypan exclusion dye.

Results: Metformin and phenformin abrogate melanoma cell viability in monolayer cell cultures and reduce sphere size and the number of viable cells/sphere at day10. Once sorted, ALDH+ melanoma cells express stem cell markers including SOX2 and CD271 and generate slightly bigger and less necrotic spheres as compared to ALDH- cells. Cell viability is higher in ALDH+ derived spheres as compared to ALDH- derived ones. To test if AMP-Activated Protein Kinase (AMPK), a molecular hub activated by metfomin, is involved in this mechanism we analysed AMPK expression in ALDH+ and – cells. AMPK levels are similar in both cell types, consistently with a similar decrease in size of spheres derived from ALDH+ and – cells after treatment with metformin and phenformin. However, the number of viable cells/sphere is markedly and significantly decreased in ALDH+ derived spheres treated with metformin/phenformin, but not in ALDH- ones.

Conclusions: Preliminary results suggest that both metformin and phenformin decrease melanoma cell viability, preferentially targeting ALDH+ melanoma cells. Further studies analysing the molecular mechanisms (including AMPK involvement) underlying metformin/phenformin- effects on melanoma CSC are necessary. Overall these data indicate a possible approach to target melanoma stem cells with cancer chemopreventive therapies.

C 6
INHIBITION OF GSK 3BETA ACTIVITY RESULTS IN MESENCHYMAL TO EPITHELIAL REVERTING TRANSITION IN PRIMARY COLON CANCER CELL CULTURES

Marina De Rosa [1] - Valeria Costabile [1] - Raffaella Accetta [1] - Paolo Delrio [2] - Daniela Rega [2] - Ugo Pace [2] - Francesca Duraturo [1] - Raffaella Liccardo [1] - Giovanni Battista Rossi [3] - Rita Genesio [4] - Lucio Nitsch [4] - Paola Izzo [1]

Department of Molecular Medicine and Medical Biotechnology and CEINGE Biotecnologie Avanzate, University of Naples "Federico II", Naples, Italy [1] - Colorectal Surgical Oncology - Abdominal Oncology Department, Istituto Nazionale per lo studio e la cura dei tumori, "Fondazione Giovanni Pascale"-IRCCS, Naples, Italy [2] - Endoscopy Unit, Istituto Nazionale per lo studio e la cura dei tumori, "Fondazione Giovanni Pascale" -IRCCS, Naples, Italy [3] - Department of Molecular Medicine and Medical Biotechnology, University of Naples "Federico II", Naples, Italy [4]

Introduction. Epithelial-to mesenchymal transition (EMT) confers stem cell-like phenotype and more motile properties to carcinoma cells. During EMT, the expression of E-cadherin decreases, resulting in loss of cell–cell adhesion and increased migration. Expression of Twist1 and other pleiotropic transcription factors, such as Snail, is known to activate EMT.

We aimed to realize a tissue bio-bank from patients affected by colorectal cancer and to establish primary cell cultures with the main purpose to study EMT.

Materials and methods. We sampled pairs of normal colorectal mucosa and its matched cancer tissues from 110 patients with sporadic and hereditary colorectal cancer, established primary cell cultures from some of these patients and validated coltures with cytogenetic and molecular biology approaches. Western blot assay, quantitative Real-Time PCR and immunofluorescence were performed to investigate the expression of several markers, such as: E-cadherin, N-cadherin, vimentin, beta-catenin, cytokeratin 20 and cytokeratin 18, twist1, Snail, CD44, ki67, sox-2, oct-4 and nanog. Moreover, cell differentiation was induced by incubation with 5% FBS and 30mM LiCl containing medium for 10 days.

Results. Our prymary colorectal cancer cells lose the expression of E-cadherin epithelial marker, which is instead expressed in cancer and normal colon mucosa of the same patient, while over-express vimentin (mesenchymal marker) Twist1, Snail (EMT markers) and Ki67. Cytokeratin 18 was expressed both in tissues and cell cultures. Expression of stem cell markers, such as CD44, Sox-2, oct-4 and nanog were also observed. Following differentiation with the GSK3b inhibitor LiCl, the cells began to express E-cadherin and, at once, twist1 expression was strongly down-regulated, suggesting a mesenchymal to epithelial reverting transition process.

Discussion. We have established primary colorectal cancer cell cultures, isolated from CRC patients, that express mesenchymal and epithelial bio-markers together with high level of EMT transcription factors. Moreover, inhibition of GSK3 beta by LiCl causes mesenchymal to epithelial reverting transition. In our opinion this approach could represent an interesting way to further investigate the clinical relevance of EMT in human colorectal cancer and the molecular basis of pharmacological resistance and metastasis.

C 7
EFFECTS OF CD133 MUTANTS ON COLON CANCER CELLS PHENOTYPE

Caterina Fanali [1] - Maddalena Corbi [2] - Maria Svelto [3] - Marisa Farina [3] - Donatella Lucchetti [3] - Valerio Cufino [3] - Alma Boninsegna [3] - Federica Calapà [3] - Achille Cittadini [3] - Alessandro Sgambato [3]

pathology institute, università cattolica del sacro cuore, roma, Italy [1] - Università Cattolica del Sacro Cuore - Istituto di Patologia Generale [2]

Background: Colorectal cancer (CRC) is the third most common cause of cancer-related death. It has been reported that a subpopulation of cells within CRC possesses the potential to initiate and sustain tumor growth. These cells are called cancer stem cells (CSCs) and present special surface markers.

The CD133 has proved a useful marker to identify and isolate the colon CSCs and although many study have demonstrated that CD133 expression has prognostic significance, the exact roles of the molecule in human tumorigenesis remain unknown.

CD133 contains a short C-terminal cytoplasmatic domain with five tyrosine residues, including a consensus tyrosine phosphorylation site. To analyze the role of C-terminal domain of CD133, the HCT116 human colon cancer cells were engineered to express an exogenous CD133 without C-terminal domain and 5 mutants of CD133 in which the tyrosine residues of C-terminal domain have been mutated in phenylalanines.

Materials and Methods: The cDNA encoding the CD133 deleted of C-terminal domain was transfected into HCT116 cells; these cells was compared with cells overexpressing the full-length CD133. Mutagenesis experiments were conducted to mutate the tyrosine residues in phenylalanine and subsequent experiments were performed to test the potential function of these aminoacid residues within the molecule.

Results: Cells overexpressing the CD133 deleted of C-terminal domain, have shown a reduction in proliferation, migration, invasiveness and tumorigenicity compared to cells overexpressing the full size CD133. Moreover cells overexpressing the CD133 wild type have shown an increased phosphorylation of Akt, which was not observed in the cells overexpressing the truncated protein.

To test if CD133 C-terminal domain could be involved in the activation of AKT pathway through the tyrosine phosphorylation, the HCT116 have been engineered to express mutants of CD133 in which the tyrosine residues have been mutated in phenylalanines.

Real time PCR have shown that some of these mutants displayed a genic expression profile more similar to cells overexpressing the CD133 deleted of C-terminal domain that to cells overexpressing the CD133 wild type .

Conclusions: Our studies show that the C-terminal domain tirosynes are involved in the activation of intracellular signaling CD133-dependent and that the activation of AKT pathway

might be involved in this signaling. Further studies are needed to assess which tyrosine are more involved in this activation.

C 8
BONE MORPHOGENETIC PROTEINS SIGNALING PROMOTES CANCER INITIATING CELLS PHENOTYPE

Chiara Garulli [1] - Cristina Kalogris [1] - Lucia Pietrella [1] - Caterina Bartolacci [1] - Cristina Andreani [1] - Valentina Gambini [1] - Martina Tilio [1] - Maria Elexpuru Zabaleta [1] - Cristina Marchini [1] - Augusto Amici [1]

Department of Bioscience and Biotechnology, University of Camerino, Camerino, Italy. [1]

INTRODUCTION Increasing evidence supports the theory that tumor growth, homeostasis, and recurrence are dependent on a small subset of cells with stem cell properties, redefined cancer initiating cells (CICs) or cancer stem cells. Bone morphogenetic proteins (BMPs) are involved in cell-fate specification during embryogenesis, in the maintenance of developmental potency in adult stem cells and may contribute to sustain CIC populations in breast carcinoma. Using the mouse A17 cell model previously related to mesenchymal cancer stem cells and displaying properties of CICs, we investigated the role of BMPs in the control of breast cancer cell plasticity.

MATERIALS AND METHODS A17 cells were treated with BMPs type-1 receptor inhibitor Dorsomorphin and with BMP4 siRNA.

MTT assay was performed. Treated versus control cells were analyzed after 96 hours (0,5μM and 2 μM) by cell cycle analysis, migration assays and 3D growth *in vitro*.

Cells extracts were collected at the same time to perform RT-PCR and Western-blot assays.

The activity of COX2 promoter, in relation to BMPs pathway was studied by promoter luciferase assay.

RESULTS: We showed that an autocrine activation of BMP4 signaling is crucial for the maintenance of mesenchymal stem cell phenotype and tumorigenic potential of A17 cells. Pharmacological inhibition of BMP signaling cascade by Dorsomorphin resulted in the acquisition of epithelial-like traits by A17 cells, including expression of Citokeratin-18 and E-cadherin, through downregulation of Snail and Slug transcriptional factors and Cyclooxygenase-2 (COX2) expression, and in the loss of their stem-features and self-renewal ability. Similiar results were obteined directly silencing BMP4.

This phenotypic switch compromised A17 cell motility, invasiveness and in vitro tumor growth..

CONCLUSION: These results reveal that BMPs are key molecules at the crossroad between stemness and cancer.

C 9
EPIGENETIC SUPPRESSION OF MIR-326 AND ITS HOST GENE ß-ARRESTIN1 REGULATES PROLIFERATION AND HEDGEHOG SIGNALLING IN NEURONAL STEM CELLS AND IN MEDULLOBLASTOMA STEM CELLS

Evelina Miele [1] - Agnese Po [2] - Danilo Cucchi [2] - Enrico De Smaele [3] - Gianluca Canettieri [2] - Laura Antonucci [2] - Antonello Mai [4] - Sergio Valente [4] - Angela Mastronuzzi [5] - Carlo Efisio Marras [6] - Franco Locatelli [5] - Isabella Screpanti [1] - Massimo Levrero [2] - Alberto Gulino [1] - Elisabetta Ferretti [3]

Department of Molecular Medicine and CLNS@Sapienza, University of Rome Sapienza / Istituto Italiano di Tecnologia, Rome, Italy [1] - Department of Molecular Medicine, University of Rome Sapienza, Rome, Italy [2] - Department Experimental Medicine, University of Rome Sapienza, Rome, Italy [3] - Department of Chemistry, University of Rome Sapienza, Rome, Italy [4] - Department of Pediatric Hematology-Oncology, Ospedale Pediatrico Bambino Gesù, Rome, Vatican City State [5] - Department of Neuroscience, Opsedale Pedaitrico Bambino Gesù, Rome, Vatican City State [6]

Introduction: Neuronal stem cells (NSC) and medulloblastoma stem cells (CSC) have been reported to share gene expression features and recent studies have highlighted the crucial role of microRNAs both in CSC/NSC and tumors signaling pathway deregulation. We have previously identified microRNAs deregulated medulloblastoma and among them we showed that miR-326 is strongly downregulated and is a repressor of the Hedgehog/Gli signalling.

Materials and methods: Human and murine NSC and CSC were derived and cultured as neurospheres. We evaluated the expression levels of miR-326 and its host gene β-arrestin1 in medulloblastoma specimens as well as in NSC and in CSC. We investigated the role of the two molecules in stem cell context. We studied the regulation of miR-326/ β-arrestin1 transcriptional unit in NSC and CSC. A pharmacological approach was utilized to modulate the expression of of miR-326/ β-arrestin1 in CSC both in vitro and in vivo.

Results: We found that miR-326 cooperates with its host gene β-arrestin1 as a tumor suppressor locus, which is lost in tumor as well as in stem cells. We showed that such locus blocks NSC and CSC proliferation by p27 activation and suppresses Hedgehog/Gli signaling at multiple levels. In detail, β-arrestin1 inhibits Hedgehog/Gli through the modulation of Gli1 K518 acetylation while the intragenic miR-326 controls Gli2 and smoothened (SMO), activatory molecules of the pathway. Then, we analyzed the potential mechanisms involved in the down regulation of this locus, finding that β-arrestin1/miR-326 locus is silenced through epigenetic mechanisms at both DNA and histone levels. Indeed, epigenetic drugs are able to reactivate the miR-326/β-arrestin1 locus and suppress CSC in vitro and in vivo**.**

Conclusions: Our study reveals a new microRNA/host gene network in NSC and CSC and proposes β-arrestin1 as tumor suppressor for medulloblastoma patients, susceptible to be re-expressed by epigenetic treatments.

C 10
BEVACIZUMAB-INDUCED INFILTRATIVE SHIFT OF GBM IS DRIVEN BY THE VASCULAR ENDOTHELIUM

Liliana Morgante [1] - Giorgio Quintino D'alessandris [2] - Daniele Runci [3] - Lucia Ricci-vitiani [1] - Stefano Giannetti [4] - Luigi Maria Larocca [5] - Tonia Cenci [5] - Roberto Pallini [2]

Istituto Superiore di Sanità, Department of Hematology, Oncology and Molecular Medicine, Roma, Italy [1] - Institute of Neurosurgery,Università Cattolica del Sacro Cuore, Rome, Italy, Institute of Neurosurgery,Università Cattolica del Sacro Cuore, Rome, Italy, Roma, Italy [2] - Istituto Superiore di Sanità Department of Hematology, Oncology and Molecular Medicine, Università di Roma "La Sapienza", Roma, Italy [3] - Institute of Human Anatomy Università Cattolica del Sacro Cuore, Rome, Institute of Human Anatomy Università Cattolica del Sacro Cuore, Rome, Roma, Italy [4] - Institute of Pathology, Università Cattolica del Sacro Cuore, Rome, Italy;, Institute of Pathology, Università Cattolica del Sacro Cuore, Rome, Italy;, Roma, Italy [5]

Glioblastoma multiforme (GBM) remains one of the most lethal form of cancer. The progression and the infiltration of GBM is supported by a robust angiogenesis, one of the most peculiar feature of GBM. Thus one of the approach in the treatment of GBM is to target the vascular endothelial growth factor (VEGF) which has a pivotal role in GBM angiogenesis. The monoclonal antibody anti VEGF (bevacizumab) is currently the only FDA-approved target therapeutic agent for recurrent GBM. Bevacizumab treatment however, may trigger a phenotypic change in GBM that acquires a gliomatosis-like growth pattern. This change is referred as "infiltrative shift". In this study we investigate the morphological aspect of the infiltrative growth induced by bevacizumab. To this end we used an intracranial xenografts model of fluorescently labeled U87MG cells and we stained the brain vessels with endothelial cell (EC) markers by immunofluorescence to understand the interaction between tumor cells and the ECs. In addition to a stronger vessel cooption we were able to identify at least three additional phenomena involving GBM cells after exposure to bevacizumab: mosaic tube formation, endothelium oriented migration and aberrant expression of EC markers. We can hypothesize that the antiangiogenic treatment induces a repertory of vascular like behaviors by tumor cells. Moreover, in our GBM clinical case of a bevacizumab-induced infiltrative shift we performed a FISH Assay with a probe for the amplified EGFR coupled with the staining for EC markers. By this approach we were able to identify that a substantial fraction of the tumor cells infiltrating the brain were closely related to EC even on the distant vessels, thus reminding to the phenomena observed in our animal model. To conclude, this study shows that the brain infiltration induced by bevacizumab is mainly driven by EC structures arguing that bevacizumab elicits a vascular behaviour by the GBM cells that mimics the stem-like phenotype.

C 11
LKB1 MUTATION AS A POTENTIAL VULNERABILITY IN KRAS MUTANT NSCLC

Massimo Moro [1] - Luca Roz [1] - Roberto Caserini [1] - Ugo Pastorino [2] - Gabriella Sozzi [1]

Dipartimento Genomica Tumorale, Fondazione IRCCS, Istituto Nazionale dei Tumori, Milano, Italy [1] - Unità di Chirurgia Toracica, Fondazione IRCCS, Istituto Nazionale dei Tumori, Milano, Italy [2]

Introduction: Non Small Cell Lung Cancer (NSCLC) is poorly chemosensitive and the use of targeted therapies is limited to about 15% of patients where a specific target lesion is observed (i.e. EGFR, ALK). Therefore, the identification of further selected subpopulations is essential. RAS is one of the most frequently mutated gene in NSCLC, accounting for 20-25% of all NSCLC cases, however personalized treatments for this driver mutation are still lacking (1). Current estimates suggest that at least 10% of all NSCLC are co-mutated both in KRAS and LKB1 (2). The liver kinase B1 (LKB1 or STK11) tumor suppressor is a master regulator of growth, metabolism and cell survival. Therapeutic approaches in KRAS-dependent NSCLC genetically engineered mouse models (GEMMs) revealed that tumors with KRAS and LKB1 mutations showed selective response to phenformin as a single agent (3).

The aim of the study was to confirm LKB1 co-vulnerability of KRAS mutated NSCLC to metabolic stress induced by compounds such as biguanides (phenformin and metformin)

Results: NSCLC cell lines with different LKB1 and KRAS status have been treated with biguanides to evaluate *in vitro* dose/response. Mutated cell lines were more sensitive to phenformin treatment (IC50: 70μM and 98μM in A549 and H460 double mutated cell lines respectively; 106μM in LT73 LKB1 mutated cell lines and 184μM in H1299 wild type cell lines). The effects of phenformin have been confirmed *in vivo*, exploiting the panel of Patient Derived Xenografts (PDXs) generated in our laboratory. Indeed, treatments of KRAS/LKB1 mutated PDXs resulted in a significant reduction of tumor growth (30 to 50% growth inhibition in phenformin treated tumors, 100mg/Kg/day, 6 weeks) whereas no effects were appreciable in tumor growth of *wild type* PDXs. Metformin is less selective towards KRAS/LKB1 mutated NSCLC, but its lower toxicity allows an increase in dosage and a treatment in combination with currently available chemoterapeutics. Interestingly, both biguanides are more active on $CD133^+$ Tumor Initiating Cells (TICs) than chemotherapeutics (20-25 fold, 2-12 fold and 0.5-3 fold enrichment of TICs after cisplatin, phenformin and metformin treatment, respectively).

Conclusions: Taken together, these preliminary data indicate a selective action of phenformin on KRAS/LKB1 mutated ADC *in vitro* and *in vivo*. Further investigation are needed to elucidate the differences observed in the effects of metformin and phenformin.

C 12
REGULATION OF STEAROYL-COA DESATURASE-1 (SCD1), A KEY FACTOR FOR PROPAGATION AND SURVIVAL OF LUNG CANCER STEM CELLS

Maria Elena Pisanu [1] - Nadia Lobello [1] - Claudia De Vitis [2] - Alessia Noto [3] - Luigi Fattore [3] - Debora Malpicci [1] - Barbara Bonacci [4] - Salvatore Raffa [3] - Stefania Morrone [5] - Giuseppe Roscilli [6] - Luigi Aurisicchio [6] - Laura Luberto [6] - Maria Rosaria Giovagnoli [7] - Salvatore Mariotta [7] - Alberto Ricci [7] - Mariafranca Panebianco [1] - Ilenia Aversa [1] - Francesco Saverio Costanzo [1] - Maria Rosaria Torrisi [3] - Gennaro Ciliberto [2] - Rita Mancini [3]

Magna Graecia University, Department of Experimental and Clinical Medicine, Catanzaro, Italy [1] - IRCCS Istituto Nazionale Tumori, Fondazione 'G. Pascale', Napoli, Italy [2] - Sapienza University, Department of Clinical and Molecular Medicine, Roma, Italy [3] - Sapienza University, Department of Surgery 'P.Valdoni', Roma, Italy [4] - Sapienza University, Department of Experimental Medicine, Roma, Italy [5] - Takis, Takis srl, Roma, Italy [6] - Sapienza University, S.Andrea Hospital, Roma, Italy [7]

Introduction According to the cancer stem cell hypothesis, cancer is sustained by a population of cells with stem cell like properties (CSCs), whose distinctive feature is their capacity of self renewal. CSC have the capacity to form 3D spheroids in appropriate conditions and show increased resistance to chemotherapy. We observed that in non small cell lung cancer (NSCLC) spheroids SCD1, enzyme involved in the conversion of saturated into monounsaturated fatty acids, is upregulated. This was confirmed both in a stable NSCLC H460 and in primary tumor cells derived from malignant pleural effusions. Importantly, SCD1 inhibition leads to lung cancer spheroid collapse and to the selective apoptosis of $ALDH^+$ cells, a marker enriched in cells with stem like properties. Therefore, the study of SCD1 and of its regulation in lung cancer may provide further insights into the understanding of the mechanisms responsible for the propagation of lung CSCs.

Methods The cell profile of ALDH was determined incubating H460 cells with ALDEFLUOR substrate BAAA in presence or in absence of ALDH inhibitor DEAB. Cells suspended in BAAA and DEAB, were used to define the ALDEFLUOR positive region. Cells that catalyzed BAAA were considered $ALDH^+$. Sphere forming assays were carried out H460 cells in presence or absence of cisplatin (CDDP) or SCD1 inhibitor MF438. RT-PCR was performed using SYBR green detection and the $\Delta\Delta$Ct method was used for relative quantification.

Results We demonstrate that $ALDH^+$ H460 cells have a much greater spheroid forming efficiency than $ALDH^-$ cells. This goes in parallel with a greater sensitivity to the disrupting effect of SCD1 inhibitors and to the combined effect of the SCD1 inhibitor MF438 and CDDP. Indeed combination studies with CDDP and MF438 show that they act synergistically to inhibit cancer spheroid propagation. We also observed that $ALDHA1^+$ cells show enriched expression of transcription factor NFY. Previous studies identified a binding site for NFY in the proximal promoter region of the SCD1 gene. Cotransfections with NFY confirmed its ability to transactivate a fusion construct between the SCD1 promoter and the luciferase gene.

Conclusions Current studies are directed to dissect the interplay between NFY and other transcription factors in the regulation of the SCD1 gene promoter and how this may affect maintenance and survival of $ALDH^+$ lung CSCs.

C 13
HEDGEHOG-GLI SIGNALING PATHWAY IN LUNG CANCER STEM CELLS AND ITS DRUG MEDIATED TARGETING

Agnese Po [1] - Marianna Silvano [1] - Danilo Cucchi [1] - Adriana Eramo [2] - Evelina Miele [3] - Valentina Salvati [2] - Giovanni Sette [2] - Ruggero De Maria [2] - Ann Zeuner [2] - Enrico De Smaele [4] - Alberto Gulino [1] - Elisabetta Ferretti [4]

Dipartimento di Medicina Molecolare, Sapienza Università di Roma, Roma, Italy [1] - Dipartimento di Ematologia, Oncologia e Medicina Molecolare, ISS, Roma, Italy [2] - CLNS@Sapienza, IIT, Roma, Italy [3] - Dipartimento di Medicina Sperimentale, Sapienza Università di Roma, Roma, Italy [4]

Introduction: The hedgehog-Gli (Hh-Gli) pathway deregulation has been implicated in human cancers of different origins and its targeting is giving promising results in preclinical and clinical studies. Lung cancer is the leading cause of cancer-related death. Pathological Hh-Gli activation has been reported in lung cancer (small-cell and non small-cell), while there are no studies investigating Hh-Gli role in lung cancer stem cells. Therefore we decided to evaluate the Hh-Gli activity in CSC derived from lung cancer (LCSC).

Materials and methods: LCSC were obtained and maintained ad described in Eramo et al, 2008. In vivo experiments: NSG mice were injected with 200.000 cells. As the tumor was macroscopic, the treatment started. Hh-Gli antagonist, GANT-61, and Cisplatin were administered IP 40 mg/kg and 3 mg/kg respectively. After 21 days mice were sacrificed and tumors excised.

Results: LCSC express Hh-Gli signaling molecules together with stemness marker such as Oct4. LCSC expressed ABCG2, a member of ABC transporters, recently correlated to chemoresistance and to Hh-Gli in CSC. We inhibited Hh-Gli signaling on NCSLC in vitro using GANT-61, either alone or in combination with cisplatin (Cis). We had a significant inhibition of cell viability and impairment of the ability to form colonies in cell treated with GANT-61. GANT-61 was also able to down-regulate the expression of the ABC transporters. In particular ABCG2 was strongly affected while it was only slightly down-regulated by Cis treatment. The combined treatment had a strong effect on ABCG2 expression. We also investigated the direct binding of Gli1 on ABCG2 regulatory region on already described binding site and on putative sites. The binding of Gli1 was strongly affected by GANT-61 treatment. We then performed in vivo experiments. We observed an important reduction of the growth of the tumor mass in the mice treated with GANT-61 and cis, respectively. The combined treatment resulted virtually in the arrest of tumor growth. Markers of Hh-Gli pathway and of stemness were down-regulated in both the single and combined treated tumors.

Conclusions: Hh-Gli pathway is active in LCSC and tumorigenic properties of LCSC depend on level of Hh-Gli activity. ABCG2 is target of Hh-Gli and is inhibited after Hh-Gli blockade in vitro and in vivo. Hh-Gli inhibition alone or in combina-

tion with conventional chemotherapy is able to block the growth of LCSC both in vitro and in vivo.

C 14
HIGH NITRIC OXIDE PRODUCTION, SECONDARY TO HIGH INDUCIBLE- NITRIC OXIDE SYNTHASE EXPRESSION, IS ESSENTIAL IN REGULATING TUMOUR INITIATING PROPERTIES OF COLON CANCER STEM CELLS.

Maria Ausiliatrice Puglisi [1] - Valentina Tesori [1] - Marianna Cappellari [2] - Maurizio Martini [3] - Mauro Biffoni [2] - Ezio Giorda [4] - Carlo Cenciarelli [5] - Rita Carsetti [4] - Giovanni Battista Gasbarrini [6] - Lucia Ricci-vitiani [2] - Giovanni Battista Pani [7] - Antonio Gasbarrini [1]

U.O.C. Internal Medicine and Gastroenterology, Gemelli Hospital, Rome, Italy [1] - Department of Hematology, Oncology and Molecular Medicine, Istituto Superiore di Sanità, Rome, Italy [2] - Division of Anatomic Pathology and Histology, Gemelli Hospital, Rome, Italy [3] - Cytofluorimetry Laboratory, Bambino Gesù Pediatric Hospital, Rome, Italy [4] - Department of Translational Pharmacology, National Research Council, Rome, Italy [5] - Medical Research Foundation, ONLUS, Bologna, Italy [6] - Department of General Pathology, Gemelli Hospital, Rome, Italy [7]

Introduction: Several studies have indicated that continuous exposure to high concentrations of Nitric Oxide (NO), produced by inducibile-NO synthase (iNOS), promote neoplastic transformation in colon cancer (CC). Recently, it has also been suggested that high NO synthesis is a distinctive feature of "cancer stem cells" (CSC), a tumor subpopulation with self-renewal capacity, that may be identified by the expression of CD133 surface marker. Aims of this study were to explore the contribution of NO in the definition of colon CSC features and evaluate potential strategies to treat CC by modulating NO production.

Material and methods: By immunohistochemistry analysis we evaluated iNOS and CD133 expression in 30 samples of human CC. Using DAF-2DA detection system, we assayed the production of intracellular NO in 5 colon CSC lines obtained from human CC tissues. By FACS sorter, we purified the NO^{high} and NO^{low} fractions from all colon CSC lines and we compared the tumorigenic potential of both cell fractions by *in vitro* and *in vivo* assays. To tested the potential antitumor effects of iNOS modulation, we treated colon CSCs with the selective iNOS inhibitor 1400W or we stably transfected these cells with two distinct iNOS-directed short-harpin RNA(shRNAs).

Results: NO^{high} CSCs display an overespression of stem cell markers and increased tumorigenic properties *in vitro* and *in vivo* than NO^{low} fractions. Interestingly, immunohistochemistry analysis confirmed that there was a significant association between iNOS and CD133 overexpression, in human CC. The blockade of endogenous NO availability using a specific iNOS inhibitor and genetic knock-down of iNOS resulted in a significant reduction of colon CSC growth and tumorigenic capacity *in vitro* and *in vivo*

Interestingly, analysis of the genes altered by iNOS-directed shRNA showed that the knockdown of iNOS expression was associated with a significant down regulation of a wide range of signaling pathways in colon CSCs, especially genes involved in stemness and tumor progression.

Conclusion: These findings have demonstrated for the first time that endogenous NO plays an important role in defining the stemness properties of colon CSCs through cross-regulation of several cellular signaling pathways. This discovery could shed light on the mechanisms by which NO induces the growth and invasiveness of CC gathering new insights on the link between inflammation and colon tumorigenesis.

C 15
MECHANISMS OF INTERACTION BETWEEN ADIPOSE TISSUE CELLS DERIVED FROM LIPOFILING PROCEDURE AND CANCER BREAST CELLS.

Erica Repaci [1] - Michela Massa [2] - Simona Gasparini [1] - Santi Pier Luigi [2] - Rodolfo Quarto [1]

DIMES, Università degli Studi di Genova, Genova, Italy [1] - Chirurgia Plastica e Ricostruttiva, Università degli Studi di Genova, Genova, Italy [2]

Introduction. The adipose tissue transplantation, thanks to the proangiogenic factors released by mesenchymal stem cells, allows to obtain a benefit in regenerative and aesthetic outcomes of breast cancer treatment. The stroma surrounding the tumor, however, can be a stimulus to the growth of any remaining cancer cells. Our study aims to identify the interactions between adipose tissue cells and human cancer cell lines.

Material and methods. Adipose tissue collected from patients during lipofilling procedures was digested with Collagenase. Derived fibroblasts were induced to differentiate in adipocyte and then co-coltured in presence of MDA-MB-231, MCF-7 e ZR-75-1 cancer cell lines. Co-coltures of undigested intact adipose tissue with the same cancer cell lines were also performed.

Results. After 48h all cancer cell lines show 1,5-2 fold increase in proliferation with respect to controls both when co-coltured in presence of induced adipocytes and with undigested adipose tissue. Moreover cancer cells displayed a tendency to proliferate surrounding cultured adipocytes.

Discussion and conclusions. Recent studies in vitro and in mouse models have shown that the environment surrounding breast cancer cells may promote their growth and progression of any remaining still active or dormant cells. Accordingly the graft of adipose tissue could potentially promote or accelerate the development of a subclinical tumor or support the locoregional recurrence. Our study aims to identify the mechanisms ignited by the transplantation of adipose tissue in a region previously bearing a tumor and the interactions that subsequently develop between cells. In our Institute, we are currently carrying out an observational study of patients which underwent adipose tissue transplantation; so far no significant increase in the risk of recurrence in transplanted patients was detected if compared to untreated patients. The oncological safety of the outcome of the transplantation procedure is still debated and further studies and consistent follow up examination are needed to clarify this aspect.

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C 16
PROGNOSTIC IMPLICATIONS OF CD133 POSITIVE CELLS IN CHEMOTHERAPY-TREATED NON-SMALL CELL LUNG CANCER

Giulia Bertolini [1] - Roberto Caserini [1] - Eva Haspinger [2] - Marco Platania [2] - Elena Roz [3] - Elena Landoni [4] - Francesco Agustoni [2] - Marina Garassino [2] - Ugo Pastorino [5] - Filippo De Braud [2] - Gabriella Sozzi [1] - Luca Roz [1]

Tumor Genomics Unit, Department of Experimental Oncology and Molecular Medicine, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy [1] - Medical Oncology Unit 1, Department of Medical Oncology, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy [2] - Pathology Unit, Oncologic Department Casa di Cura "La Maddalena", Palermo, Italy [3] - Medical Statistics, Biometry and Bioinformatics; Unit of Clinical Epidemiology and Trial Organization, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy [4] - Thoracic Surgery Unit, Department of Surgery, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy [5]

Introduction: Primary and acquired chemoresistance represents a major cause for treatment failure in non-small cell lung cancer (NSCLC). Among factors regulating chemoresistance, a prominent role has been suggested for specific subpopulations of drug resistant cells endowed with tumor initiating potential. We have previously shown that CD133 positive cells are tumor initiating cells in NSCLC and possess intrinsic resistance to conventional cytotoxic chemotherapy in experimental studies, suggesting a possible role in chemoresistance and disease relapse. Their relevance in the clinical setting however has not been fully elucidated.

Materials and Methods: To evaluate the clinical significance of CD133 positive cells detection, we collected pre-treatment lymphnode biopsies and post-treatment surgical specimens (both lymphnodes and primary tumors) from 60 patients undergoing neo-adjuvant treatment with platinum based regimens for stage IIIA-B NSCLC followed by surgical resection. Immunohistochemistry was used to evaluate CD133 content. Positivity of tumor cells was defined as membranous staining or staining of membrane and cytoplasm as assessed by three independent observers.

Results: CD133 positive cells were detected in 35% of pre-treatment samples (8/23) and 38% of primary tumors (20/52) and 32% of metastatic lymphnodes after chemotherapy (8/25). As previously observed by flow cytometry, CD133 content was variable among different tumors but generally identified a small subpopulation (mean 5% of total tumor cells, range 1-40%). When multiple lymphnode samples were available CD133 status was concordant, while no correlation was found between CD133 status in matched lymphnodes and primary tumors, possibly indicating different dynamics in primary tumor maintenance and dissemination. Interestingly CD133 positive cells were detected more frequently in tumors responding to therapy by ≥30% size reduction (14/28, 50% vs. 6/24, 25%) suggesting enrichment of chemoresistant cells after treatment. Consistently, detection CD133+ cells in treated tumors was correlated with higher risk of local recurrence (73% vs. 53%). Finally, positivity for CD133 after treatment correlated with worse overall survival (HR 2.42, 95% CI: 0.93-6.32, p=0.086).

Conclusions: These data provide indirect evidence of chemoresistance of CD133+ cells in primary human tumors. CD133 positive cells are correlated with worse prognosis in neo-adjuvant treatment of locally advanced NSCLC and could offer clinical relevant information for personalized therapies.

C 17
THE TYPE II TRANSMEMBRANE SERINE PROTEASE TMPRSS4 IS A PROMISING CANCER STEM CELL-ASSOCIATED TARGET

Roberto Ruiu [1] - Laura Conti [1] - Maddalena Arigoni [1] - Raffaele Calogero [1] - Federica Cavallo [1] - Stefania Lanzardo [1]

Molecular Biotechnology Center, Department of Molecular Biotechnology and Health Sciences, University of Torino, Torino, Italy (1)

Introduction: Cancer stem cells (CSCs) are a sub-population of stem cell-like cells endowed with self-renewal and tumor initiating ability that are thought to be responsible for relapse, metastasis and treatment failures. Standard treatments are directed against the tumor bulk and do not eliminate CSCs, which consequently are the subject of growing interest due to the important clinical implications that the development of specific anti-CSC therapies would have.

Materials and methods : To find possible CSC-associated targets, we performed a transcriptome microarray analysis to explore genes differentially expressed between an Her2/neu$^+$ breast cancer cell line (i.e. TUBO cells) and its derived CSC-enriched tumorspheres, cultured in non-adherent conditions as floating spheroids.

Results: This analysis led us to the identification, among the differentially expressed transcripts, of that coding for TMPRSS4, a type II transmembrane serine protease highly expressed on pancreatic, thyroid, colon, and other cancer tissues. TMPRSS4 was recently shown to promote tumor growth, invasion, metastasis and epithelial-mesenchymal transition (EMT), though the molecular mechanisms underlying these processes are not fully understood. Our microarray analysis showed that TMPRSS4 is >4-fold overexpressed in tumorspheres compared to TUBO cells. This increase of expression was validated by semi-quantitative PCR analysis and observed also in other murine cell lines (4T1 and TSA) and in a human triple negative breast cancer cell line (CRL2335). We therefore investigated a possible role of TMPRSS4 in breast CSC biology, and found that TMPRSS4 downregulation by siRNA or tyroserleutide (YSL, kindly provided by Dr. Wuhan, Shenzhen Kangzhe Pharmaceutical, China) in TUBO cells significantly reduced their tumorsphere forming ability, indicating CSC self-renewal impairment. TMPRSS4 has been found to regulate diverse signaling pathways, possibly through interaction with Uroplasminogen Activator Receptor (uPAR), which can induce EMT and stem cell-like properties in breast cancer cells. Interestingly

uPAR is >4-fold overexpressed in TUBO-derived tumorspheres, suggesting that TMPRSS4-uPAR interaction may have a role in CSC stemness.

Conclusions: These preliminary results lay the foundations for a promising study on the role of TMPRSS4 in breast cancer cell stemness, tumorigeneicity and metastatic ability and for the development of therapeutic strategies targeting breast CSCs.

C 18
SURGERY-INDUCED WOUND RESPONSE PROMOTES STEM-LIKE AND TUMOR-INITIATING FEATURES OF BREAST CANCER CELLS VIA STAT3 SIGNALING

Ilenia Segatto [1] - Stefania Berton [1] - Maura Sonego [1] - Samuele Massarut [1] - Tiziana Perin [1] - Gustavo Baldassarre [1] - Barbara Belletti [1]

CRO of Aviano, National Cancer Institute, Aviano, Italy [1]

Introduction: Breast cancer (BC) is the most common malignancy in women worldwide. For early BC patients local relapse represents what mostly influences disease outcome. Thus, the understanding of the mechanisms underlying this unfavorable event represents a compelling objective in BC research. Multifocality is a hallmark of most BC, however 90% of local recurrences occur at the same quadrant of the primary cancer. It is well known that inflammation is clinically linked to cancer but the mechanisms are not fully understood. Surgery itself elicits an inflammatory response, suggesting that it could represent a perturbing factor in the process of local recurrence and/or metastasis. Post-surgery wound fluids (WF) drained from BC patients are rich in cytokines and growth factors, stimulate the *in vitro* growth of BC cells and are potent activators of STAT3 transcription factor.

Material and methods: We investigated whether STAT3 signaling was functionally involved in the response of BC cells to post-surgical inflammation. *In vitro*, we characterized the phenotype of BC cell lines with impaired STAT3 activity in response to WF. *In vivo*, we analyzed the ability of STAT3-impaired cells to survive and grow in breast microenvironment. Moreover, using an *in vivo* experimental model resembling the course of human BC, we evaluated the relevance of STAT3 in recurrence formation.

Results: Our data show that WF induced the enrichment of BC cells with stem-like phenotypes, via activation of STAT3. *In vitro*, WF highly stimulated mammosphere formation and self-renewal of BC cells. The specific inhibition of STAT3 signaling was able to counteract this event, strongly impairing stem-like properties induced by WF. *In vivo*, we observed that STAT3 was critical for tumor initiation. Moreover, inhibition of STAT3 activity decreased the formation of local relapse after surgery.

Conclusions: Overall, we demonstrate that surgery-induced inflammation promotes stem-like phenotypes and tumor-initiating abilities of BC cells. Interfering with STAT3 signaling in this setting is sufficient to suppress this process. The understanding of the crosstalk between breast cancer cells and their microenvironment may open the way to successful targeting of breast tumor-initiating cells in their initial stages and be eventually curative.

C 19
PROTEIN TYROSINE PHOSPHATASE RECEPTOR TYPE ? IS AN INHIBITOR OF CRITICAL BCR/ABL DRIVEN PATHWAYS IN CHRONIC MYELOID LEUKEMIA.

Luisa Tomasello [1] - Giovannino Silvestri [2] - Marco Della Peruta [3] - Zeno Fiorini [1] - Marzia Vezzalini [1] - Claudio Sorio [1]

Department of Pathology and Diagnostics, University of Verona, Verona, Italy [1] - Marlene and Stuart Greenebaum Cancer Center, University of Maryland School of Medicine, Baltimore, United States [2] - Cancer Research UK & UCL Cancer Trials Centre, University College London, London, United Kingdom [3]

Introduction: Chronic myeloid leukemia (CML) is a type of myeloproliferative disease associated with a characteristic chromosomal translocation called Philadelphia chromosome resulting in the rearrangement of BCR and ABL1 genes. The fusion gene BCR/ABL1 encodes for a tyrosine kinase that phosphorylates and activates some proteins involved in cellular critical processes, such as Jak2, β Catenin and Lyn. In this altered molecular arrangement, Protein Tyrosine Phosphatase Receptor Type γ (PTPRG) plays a key role as tumor suppressor gene: we have previously described how the downregulation of PTPRG in CML is correlated with increased cellular proliferation, both in vitro and in vivo. Now, we are searching for new molecular interactors of PTPRG to precisely define the biochemical pathway/s intersected by this PTP.

Materials and methods : A pull-down assay has been performed using purified recombinant His-tagged PTPRG ICD of native or phosphatase-inactive (D1028A) PTPRG bound to nichel beads and K562 cell line lysates to investigate the binding between PTPRG and its putative interactors. Co-immunoprecipitaton was applied to confirm the interactions thus detected.

Results: Our preliminary data show a specific, direct interaction between PTPRG and proteins critically involved in the BCR/ABL pathway. In particular, two members of the family of non-receptor type Tyrosine protein kinases, LYN and SYK, regulatory molecules involved in signal transduction.

We next applied the Bio-Plex Suspension Array System (Bio-Rad) to evaluate the levels of phosphorylation of key signalling molecules. Reduced phosphorylation of Akt, a protein belonging to a downstream Syk pathway (PIK3/AKT pathway), correlated with a restored expression of PTPRG in K562 cells.

Conclusions: In conclusion, PTPRG acts as an antagonist of BCR/ABL1 oncogene and utilizes as substrates not only BCR/ABL but many key targets of the biochemical pathway activated by BCR/ABL1 reinforcing its role as a key tumor suppressor gene in CML and, likely, other types of neoplasia.

C 20
SILENCING OF SNAI2/SLUG GENE IN PROSTATE CANCER AND ITS ROLE IN NEUROENDOCRINE DIFFERENTIATION, METASTASIS-SUPPRESSOR AND PLURIPOTENCY GENE EXPRESSION

Maria Grazia Tupone [1] - Silvia Esposito [1] - Marco Vincenzo Russo [1] - Irma Airoldi [2] - Serena Di Meo [1] - Carlo Sorrentino [1] - Emma Di Carlo[1]

Department of Medicine and Sciences of Aging, 'G. d'Annunzio' University, Chieti, Italy [1] - Laboratory of Oncology, Istituto Giannina Gaslini, Genova, Italy [2]

Introduction: Prostate cancer (PCa)-related deaths are mostly due to metastatic diseases developed from high grade cancers often endowed with neuroendocrine differentiation (NED) areas. SNAI2/Slug is a zinc-finger transcription factor that acts as a key regulator of cell migration during both embryonic development and tumor metastatization. Its role in prostate cancer development and progression is not yet fully understood. We thus investigated SNAI2 gene expression, regulation and role in human PCa.

Materials and methods: Epithelium and stroma were microdissected from PCa specimens and then analyzed by real-time RT-PCR to detect SNAI2 gene expression levels in both compartments. Immunostainings was used to localize the expression of SNAI2 protein and that of its down-stream target molecules in prostate samples. Genomic sequencing was performed to assess the involvement of epigenetic mechanisms in the SNAI2 gene expression regulation, while knockdown of SNAI2, by specific siRNA, was performed to clarify its biological role.

Results: SNAI2 expression was considerably down-regulated in most malignant epithelia, microdissected from PCa semples, in association with gene promoter methylation, with the exception of few cancer foci and cell clusters forming: I. Chromogranin-A+ neuroendocrine differentiation (NED) areas, II. the expansion/invasion front of high-grade PCa or, III. lymph-node metastasis.

SNAI2 was methylated in 22Rv1 and LNCaP cell lines and treatment with 5-aza-2'-deoxycytidine restored its expression, but not in PC3 cells. Depletion of SNAI2 mRNA by siRNA, in the latter cell line, increased expression levels of E-Cadh and Ep-CAM, while down-regulated the expression of N-Cadh and NED markers such as Chromogranin-A, Neuron-Specific-Enolase, Nr-CAM and N-Cadh 2. The pluripotency genes SOX2, OCT4A and NOTCH1 were also down-regulated, while the metastasis-suppressor genes KISS1 and Nm23-H1 were up-regulated.

In patient's samples, the expression SNAI2 protein was related directly to that of SOX2, OCT4A and NOTCH1 and, inversely to that of Nm23-H1.

Conclusions: SNAI2 is expressed in selected PCa areas. Silencing of SNAI2, in most PCa epithelia, may turn off the expression of NED markers and pluripotency genes, while turn on that of metastasis-suppressor genes. These data, by enlightening its role in shaping differentiation and metastatic potential of PCa, candidate SNAI2 as key therapeutic target to hamper PCa progression.

C 21
PROLYL-ISOMERASE PIN1 CONTROLS NORMAL AND CANCER STEM CELLS OF THE BREAST

Alessandro Zannini [1] - Alessandra Rustighi [1] - Elena Campaner [1] - Giovanni Sorrentino [1] - Silvano Piazza [1] - Giannino Del Sal [1]

Laboratorio Nazionale cib, Dipartimento Scienze della Vita - Università degli Studi di Trieste, Trieste, Italy [1]

Introduction: Cancer stem cells (CSCs) are proposed to be responsible for chemotherapeutic treatment failure, disease recurrence and metastatic spread. For this reason they are considered core targets to eradicate the roots of malignancy. However our understanding of the biology of these cells is still limited and elucidation of the molecular mechanisms governing their state is important in order to develop effective therapeutic strategies.

Materials and methods: the role of the Pin1-Notch axis in breast cancer stem cells and chemoresistance was investigated by both *in vitro* and in *in vivo* experiments by isolation and purification of mammary epithelial cells, western blot analyses, FACS analyses, qRT-PCR, mammospheres assays, tumor growth experiments, immunoistochemical analyses, expression- and survival- analysis in metadataset breast cancer patients.

Results: We demonstrate that CSC self-renewal, chemoresistance, tumor growth and metastasis formation *in vivo* relies on the prolyl-isomerase Pin1 functions, sustaining levels and transcriptional activities of two key cell fate determinants, Notch1 and Notch4, strongly implicated in development and progression of breast tumors. These effects involve the ability of Pin1 to favor the dissociation of the intracellular activated forms of Notch1 and Notch4 from their major E3 ub-ligase Fbxw7α, preventing their proteasome degradation. The significance of these data is evidenced by the fact that i) in breast cancer patients Notch activity is frequently deregulated despite the presence of Fbw7 and this correlates with high levels of Pin1 and ii) inhibition of Pin1 elicits breast CSC exhaustion and chemosensitivity.

Conclusions: This study has two major impacts. First, our findings provide explanation to understand how, in the absence of mutations in NOTCH pathway genes, in a consistent proportion of breast cancer patients, activated Notch1 can exist in spite of the presence of its major constrain, the tumor suppressor Fbxw7α. The data presented, in fact, suggest that deregulated activity of Pin1, at least in breast cancer, where mutational events in NOTCH1 and FBXW7 are rare, could reduce the selective pressure for mutations providing an alternative mechanism for Notch oncogenic activation. Second, our findings pinpoint Pin1 as a crucial target in aggressive breast cancers, providing the rational for a therapeutic strategy based on Pin1 inhibition to hit CSCs, restore chemosensitivity and inhibit metastatic spread.

Cell Adhesion, Migration, Invasion And Metastasis

D 1
TROP-2 IS A UNIVERSAL DETERMINANT OF CANCER METASTATIZATION AND SURVIVAL

Saverio Alberti [1] - Emanuela Guerra [2] - Romina Tripaldi [2] - Rossano Lattanzio [2] - Giovanna Vacca [2] - Romina Zappacosta [2] - Marco Trerotola [2] - Pasquale Simeone [2] - Valeria Relli [2] - Robert De Lange [3] - Ulrich H. Weidle [3] - Rossana La Sorda [2] - Patrizia Querzoli [4] - Massimo Pedriali [4] - Enzo Bianchini [5] - Domenico Angelucci [6] - Giuseppe Pizzicannella [7] - Xiao-feng Sung [8] - Carla Di Loreto [9] - Carlo Alberto Beltrami [9] - Laura Antolini [10] - Mauro Piantelli [2]

Unit of Cancer Pathology, Center of Excellence for Research on Aging, Foundation University "G. D' Annunzio", Chieti, Italy & Department of Neuroscience and Imaging, University 'G. d'Annunzio', Via Colle dell' Ara, 66100 Chieti Scalo, Italy [1] - Unit of Cancer Pathology, CeSI, Foundation University 'G. d'Annunzio', Via Colle dell' Ara, 66100 Chieti Scalo, Italy [2] - Roche Diagnostics GmbH, Pharma Research, Nonnenwald 2, D-82372 Penzberg, Germany [3] - Institute of Pathology, University of Ferrara, Via Luigi Borsari 46, 44121 Ferrara, Italy [4] - Department of Pathology, Rovigo Hospital, Viale Tre Martiri 140, 45100 Rovigo, Italy [5] - eDepartment of Pathology, Ortona Hospital, Contrada S.Liberata 1, 66026 Ortona, Italy [6] - Department of Pathology, Lanciano Hospital, Via per Fossacesia 1, 66034 Lanciano, Italy [7] - Division of Oncology, Department of Clinical and Experimental Medicine, Faculty of Health Sciences, County Council of Östergötland, University of Linköping, S-581 85 Linköping, Sweden [8] - Department of Pathology, University of Udine, Via Palladio 8, 33100 Udine, Italy [9] - Biostatistics Center, Department of Clinical Medicine, Prevention and Biotechnology, University of Milano-Bicocca, 20900 Monza, Italy [10]

Introduction: Metastases are the main cause of cancer death. Hundreds of proteins/genes have been linked to the metastatic phenotype in different experimental systems. Yet, no unique markers of cancer aggressiveness and metastatic potential have been identified.

Materials and methods : Gene-expression profiling, metastatic models transcriptome meta-analysis, RT-PCR and IHC analysis of training and validation patient case-series of breast, uterus, stomach, colon and ovary metastatic cancers were utilized to define global and *TROP2* expression patterns. Lentiviral vector-driven down-regulation versus overexpression of Trop-2 and target downstream effectors were utilized to define the Trop-2 mechanics of metastatic induction. E-cadherin, ß-catenin and Trop-2 expression patterns were challenged against a breast cancer case series to define prognostic relevance and survival.

Results: Gene-expression profiling of murine, rat and human metastatic cells identified *TROP2* as the only globally-shared upregulated gene. Metastatic upregulation of Trop-2 was validated in breast, uterus, colon, stomach and ovary cancer patients. Trop-2 was then shown to induce aggressive metastatization through loss of cell-cell adhesion and increased cell migration and survival. We discovered that Trop-2 binds to E-cadherin and inactivates it, by disanchoring it from the cytoskeleton. Functional blockade of E-cadherin then triggers β-catenin activation, nuclear translocation and downstream transcription. The Trop-2/E-cadherin/β-catenin module was recapitulated in tumor patients, with unprecedented impact on metastatic survival of aggressive triple-negative breast cancers.

Conclusions: Our findings candidate *TROP2* as a universal driver of metastatic spreading. The paradigm of a global, Trop-2-driven pro-metastatic program paves the way for novel clinical approaches to patient diagnostics, prognostics and cure.

D 2
EFFECTS OF NOREPINEPHRINE ON MIGRATION AND INVASION OF DU145 CELLS IN XENOGRAFT MOUSE MODEL OF PROSTATE CANCER.

Antonio Barbieri [1] - Sabrina Bimonte [1] - Giuseppe Palma [1] - Antonio Luciano [1] - Domenica Rea [1] - Aldo Giudice [2] - Giosuè Scognamillo [3] - Elvira La Mantia [3] - Renato Franco [3] - Paola Stiuso [4] - Michele Caraglia [4] - Claudio Arra [1]

Animal Facility Unit, National Institute of Tumours of Naples "Pascale" Naples, Italy, Hospital, Naples, Italy [1] - National Institute of Tumours of Naples "Pascale" Naples, Italy, Hospital, Naples, Italy [2] - Pathology Unit, National Institute of Tumours of Naples "Pascale", Naples, Italy, Hospital, Naples, Italy [3] - Department of Biochemistry, Biophysics and General Pathology, Second University of Naples, Naples, Italy, University, Naples, Italy [4]

Metastasis causes most of the deaths for cancer and this process still represents an enigmatic aspect of the disease. It is note that catecholamines, such as norepinephrine, are released by the sympathetic nervous system in chronic stress conditions. Since it has also been demonstrated that norepinephrine stimulates the motility of breast and colon cells through β-adrenergic receptor, we decided to examine its possible role in the development and metastasis formation, by *in vitro* and *in vivo* studies. We demonstrate that treatments with norepinephrine (β2 - adrenoreceptor agonist) in mice injected with human prostate cancer cells DU145 increase the metastatic potential of these cells. Our date showed that the treatment of mice with norepinephrine leads to a significant increase of the migratory activity of cancer cells in a concentration-dependent manner and that this process is blocked by propanolol (β-adrenergic receptors). Mice treated with norepinephrine, displayed an increased number of metastatic foci of DU145 cells in inguinal lymph nodes and also showed an increased expression of MMP2 and MMP9 in tumor samples compared to controls.

All together these data show that β2-AR plays an important role in the formation of metastasis in prostate cancer and suggest that the treatment with antagonist propranolol, could represents an interesting tool to control metastasis formation in cells over-expressing β2AR.

Keywords: Stress, Norepinephrine, cell migration, cell invasion, metastases, β-Adrenergic receptors.

D 3
PD-L1 EXPRESSION IDENTIFIES A SUBPOPULATION OF MELANOMA CELLS CHARACTERIZED BY ENHANCED INVASIVENESS AND AGGRESSIVENESS

Davide Brusa [1] - Daniela Massi [2] - Barbara Merelli [3] - Michela Ciano [1] - Valentina Audrito [4] - Sara Serra [4] - Gianna Baroni [2] - Romina Nassini [5] - Daiana Minocci [5] - Laura Cattaneo [6] - Alessandra Carobbio [7] - Silvia Deaglio [4] - Mario Mandalà [3]

Human Genetics Foundation (HuGeF), University of Turin, Turin, Italy [1] - Division of Pathological Anatomy, Department of Surgery and Translational Medicine, Univerity of Florence, Florence, Italy [2] - Unit of Medical Oncology, Department of Oncology and Hematology, Papa Giovanni XXIII Hospital, Bergamo, Italy [3] - Department of Medical Sciences, University of Turin, Turin, Italy [4] - Unit of Clinical Pharmacology and Oncology, Department of Health Science, University of Florence, Florence, Italy [5] - Division of Pathological Anatomy, Papa Giovanni XXIII Hospital, Bergamo, Italy [6] - Research Foundation, Papa Giovanni XXIII Hospital, Bergamo, Italy [7]

Introduction: Melanoma is one of the most aggressive malignant tumors, with a high mortality in the metastatic setting. The discovery of somatic mutations in the BRAF oncogene in some patients led to the introduction of BRAF inhibitors in the clinical management. Clinical responses to BRAFi are dramatic, but they may be short lived. For this reason, there is intense investigation into alternative therapeutic strategies, including novel immunomodulatory agents such as anti-PD-1 and anti-PD-L1 antibodies. PD-L1 is a transmembrane molecule that can be expressed by melanoma cells, where it mediates immunosuppression by interacting with the PD-1 receptor on T cells. The clinical and biological significance of PD-L1 expression in metastatic melanoma remains to be fully uncovered.

The aim of this work is to understand the prognostic role and functional significance of PD-L1 expression in metastatic melanoma.

Materials and methods: PD-L1 expression was analyzed by immunohistochemistry using different antibodies in primary tumors and paired metastases from 83 melanoma patients. PD-L1$^+$ and PD-L1$^-$ subsets of the A375 cell line were stabilized *in vitro* by cell sorting and compared using gene expression profiling and functional assays studying growth, migration and invasion. Results were confirmed *in vivo* using xenograft models.

Results: PD-L1 membrane positivity was detected in 24/83 (19%) of patients. By multivariate analysis, PD-L1 membrane expression (95% CI 1.48-4.67, P< 0.001), age >57 years [CI 95% 1.20-3.71, P< 0.009] and stage M1c (95% CI 1.01-3.38, P< 0.045) were independent predictors of poor prognosis. Furthermore, PD-L1 expression defined a subset of the A375 cell line characterized by a specific genetic profile. PD-L1$^+$ A375 cells showed a highly invasive phenotype and enhanced ability to grow *in vivo* in immunocompromised mouse models.

Conclusions: PD-L1 may be proposed as a novel prognostic marker in metastatic melanoma, associated to a more aggressive disease and a worse clinical prognosis. Furthermore, it defines a distinct morpho-phenotypic subset of the disease with a specific genetic signature and a different biological behavior with a more activated status in terms of invasiveness and enhanced ability to grow in vitro and in vivo. Future studies will tell whether PD-L1 expression is also a marker of resistance to selected therapies and whether it may be successfully exploited alone or in combination as a target for specific subsets of melanoma patients.

D 4
AN ANGIOPOIETIN-LIKE PROTEIN 2 AUTOCRINE SIGNALING PROMOTES EMT DURING PANCREATIC DUCTAL CARCINOGENESIS

Carmine Carbone [1] - Geny Piro [2] - Matteo Fassan [3] - Anna Tamburrino [1] - Maria Mihaela Mina [2] - Marco Zanotto [1] - Paul J Chiao [4] - Claudio Bassi [5] - Aldo Scarpa [3] - Giampaolo Tortora [2] - Davide Melisi [1]

Digestive Molecular Clinical Oncology Research Unit, Università degli studi di Verona, Verona, Italy [1] - Laboratory of Oncology and Molecular Therapy, Università degli Studi di Verona, Verona, Italy [2] - ARC-Net Research Centre and Department of Pathology and Diagnostics, Università degli studi di Verona, Verona, Italy [3] - Department of Molecular and Cellular Oncology, MD Anderson Cancer Center, Houston, United States [4] - Department of Surgery, Università degli studi di Verona, Verona, Italy [5]

Introduction: The identification of the earliest molecular events responsible for the metastatic dissemination of pancreatic cancer remains critical for early detection, prevention, and treatment interventions. In this study, we hypothesized that an autocrine signaling between Angptl2 and its receptor LilrB2 might be responsible for the epithelial-to-mesenchymal transition (EMT) and, in turn, the early metastatic behavior of cells in pancreatic preneoplastic lesions.

Materials and methods: We used an in vitro experimental cell transformation model system consisting in the sequential and stable expression of activated K-Ras, HER2, and p16/p14 shRNA sequences in the human pancreatic ductal epithelial (HPDE) cell line to study the role of Angptl2 on the features of EMT in the different steps of the pancreatic ductal carcinogenesis. The expression of Angptl2 has been analyzed in a series of 62 different human Pancreatic Intraepithelial Neoplasia (PanIN) and 15 pancreatic cancer lesions.

Results: We demonstrated that the sequential activation of K-Ras, expression of Her2 and silencing of p16/p14 are sufficient to progressively and significantly reduce the expression of the epithelial marker E-cadherin and to induce migratory properties in HPDE cells. In this multistep model, we measured the progressively increased secretion of Angptl2, and the overexpression of LilrB2. Exogenous Angptl2 significantly increased proliferation rate and migratory properties of LilrB2-expressing HPDE/K-Ras cells, and HPDE/K-Ras/Her2/p16p14shRNA cell lines. Conversely, silencing the expression of ANGPTL2 reverted EMT and reduced migration in HPDE/K-Ras, and HPDE/K-Ras/Her2/p16p14shRNA cell lines. An increasingly significant overexpression of Angptl2 was observed in human PanIN and neoplastic lesions if compared with normal pancreatic parenchyma.

Conclusions: These findings showed that the autocrine signaling of Angptl2 and its receptor LilrB2 plays key roles in sustaining EMT and the early metastatic behavior of cells in pancreatic preneoplastic lesions supporting the potential role of Angptl2 for early detection, metastasis prevention, and treatment in pancreatic cancer.

D 5
ENDOTHELIAL PODOSOME ROSETTES REGULATE VASCULAR BRANCHING IN TUMOR ANGIOGENESIS

Giulia Chiaverina [1] - Giorgio Seano [2] - Paolo Armando Gagliardi [1] - Laura Di Blasio [1] - Alberto Puliafito [1] - Roberto Sessa [3] - Claire Bouvard [4] - Guido Tarone [5] - Dominique Helley [6] - Lydia Sorokin [7] - Guido Serini [1] - Federico Bussolino [1] - Luca Primo [1]

Institute for Cancer Research and Treatment at Candiolo, Turin, Italy, Department of Oncology, University of Torino, Turin, Italy, Candiolo, Italy [1] - Harvard Medical School, Massachusetts General Hospital, Boston, MA, USA, Harvard Medical School, Massachusetts General Hospital, Boston, United States [2] - Cardiovascular Research Institute, University of California, San Francisco, CA, USA, University of California, San Francisco, CA, USA, San Francisco, United States [3] - UMR-S 765, Université Paris Descartes, Sorbonne Paris Cité, Paris, France, The Scripps Research Institute, La Jolla, CA, USA., Paris, France [4] - Department of Molecular Biotechnology and Health Sciences, University of Torino, Molecular Biotechnology Center, Turin, Italy, University of Torino, Turin, Italy, Turin, Italy [5] - UMR-S 765, Université Paris Descartes, Sorbonne Paris Cité, Paris, France, Université Paris Descartes, Sorbonne Paris Cité, Paris, France, Paris, France [6] - Institute of Physiological Chemistry and Pathobiochemistry, Muenster University, Muenster, Germany, Muenster University, Muenster, Germany, Muenster, Italy [7]

Introduction: The mechanism by which angiogenic endothelial cells break the physical barrier of vascular basement membrane and consequently sprout, forming new vessels in mature tissues, is unclear. Here we show how the angiogenic endothelium is characterized by the presence of podosome rosettes. Podosomes and invadopodia, collectively called invadosomes, are specialized cell-matrix contacts with an inherent ability to degrade ECM components, thus they are considered key structures of cells that are able to cross anatomical boundaries.

Materials and methods : We studied the formation of podosome rosettes in HUVECs, in mouse aortic ring (mAR) assay and in genetic mouse models.

Results: We found that VEGF-A stimulation induces the formation of endothelial podosome rosettes by up-regulating integrin α6β1; in contrast, the binding of α6β1 integrin to vascular basement membrane laminin impairs the formation of podosome rosettes by restricting α6β1 integrin to focal adhesions and hampering its translocation to podosomes. By analyzing genetic mouse models of cancer and by exploiting a function blocking antibody, we demonstrate how the interaction between α6β1 integrin and laminin controls tumor blood vessel branching by influencing the dynamics of podosome rosettes.

Conclusions: We provide the first evidence that endothelial podosome rosettes are critical regulators of sprouting angiogenesis and promote tumor blood vessels branching. The vascular normalizing effects of podosome rosette inhibition thus open new avenues to study their role in tumor angiogenesis and blood vessel branching in general.

D 6
PROSTAGLANDIN E2 TRANS-ACTIVATES THE COLONY-STIMULATING FACTOR-1 (CSF-1) RECEPTOR AND SYNERGIZES WITH CSF-1 IN THE INDUCTION OF MACROPHAGE CHEMOTAXIS VIA THE MITOGEN-ACTIVATED PROTEIN KINASE ERK1/2

Graziana Digiacomo [1] - Marina Ziche [2] - Persio Dello Sbarba [1] - Sandra Donnini [2] - Elisabetta Rovida [1]

Università, Università degli studi di Firenze, Firenze, Italy [1] - Università, Università degli Studi di Siena, Siena, Italy [2]

Introduction: Prostaglandin E2 (PGE2) is a key mediator of immunity, inflammation and cancer. Besides the existence of four G-protein-coupled E-prostanoid receptors (EP1-4), the complexity of PGE2 signaling is further increased by the existence of a cross-talk between EP receptors and tyrosine kinase receptors (TKR) such as Fibroblast Growth Factor Receptor 1 (FGFR1) or Epidermal Growth Factor Receptor (EGFR). The Colony-Stimulating Factor-1 Receptor (CSF-1R) is a TKR that sustains the survival, proliferation, and motility of monocytes/macrophages, which are essential components of innate immunity and cancer development.

Materials and Methods: The experiments were performed in two murine macrophage cell lines, BAC-1.2F5 and RAW264.7, that physiologically express high amounts of CSF-1R protein. BAC-1.2F5 cells were incubated in the absence of CSF-1 for 18 h, whereas RAW264.7 cells in the absence of FBS for 24 h, respectively, before being stimulated with the appropriate stimulus. Cells were then lysed and protein lysates subjected to SDS-PAGE and immunoblotting with the indicated antibodies. To determine whether CSF-1R is necessary for PGE2-induced ERK1/2 activation and for the macrophage chemotaxis, we inhibited CSF-1R genetically (by specific siRNA) or pharmacologically (by a CSF-1R specific drug, GW2580). Migration assay was performed with modified Boyden chambers.

Results: In either BAC-1.2F5 or RAW264.7 1 μM PGE2 induced rapid CSF-1R phosphorylation, wich was dependent on Src family kinases activation. Indeed, SFK pharmacologic inhibition prevented PGE-induced CSF-1R hosphorylation. Genetic inhibition of CSF-1R reduced PGE2-elicited ERK1/2 phosphorylation and macrophage chemotaxis, indicating that CSF-1R plays a role in PGE2-mediated immuno-regulation. Furthermore, at low concentrations PGE2 synergized with CSF-1 in inducing macrophage chemotaxis, as well as ERK1/2 phosphorylation. Accordingly, ERK1/2 inhibition completely blocked chemotaxis induced by the combination CSF-1/PGE2.

Conclusions: Our results indicate that PGE2 trans-activates CSF-1R and synergizes with CSF-1R –dependent signaling at the level of ERK1/2 in promoting macrophage chemotaxis. This synergistic interaction is likely to play an important role in the initiation and progression of inflammatory diseases as well as cancer.

D 7
MELANOMA-DERIVED EXOSOMES AND MIR-222 PROMETASTATIC FUNCTIONS.

Alessandra De Feo [1] - Carolina Coscia [1] - Maria Bellenghi [1] - Maria Cristina Errico [1] - Nadia Felli [1] - Gianfranco Mattia [1] - Alessandra Carè* [1] - Federica Felicetti* [1]

Istituto Superiore Sanità, Dipartimento Ematologia, Oncologia e Medicina Molecolare, Rome, Italy [1]

Introduction: Our previous results indicated that miR-221&222 and miR-126&126* take part in a complex balance able to induce or inhibit melanoma progression. In particular, miR-221&222 are key factors for melanoma development and dissemination, whereas miR-126&126* play tumor suppression functions. Growing evidence is showing that miRNAs are not strictly cellular, but are secreted through the release of small vesicles, called "exosomes", and therefore present in extracellular compartments, including blood and other body fluids, and in cell culture media. Tumor exosomes contain miRNAs as well as intact and functional mRNAs and proteins that can alter the cellular environment to favor tumor growth. Increasing our understanding of exosomes and extracellular miRNAs should clarify this new communication network.

Materials and methods: Exosomes were isolated by ultracentrifugation or Exoquick-TC methods. The expression of miRNAs, mRNAs and/or proteins in exosomes was evaluated by Real Time PCR or Western Blot analysis. Biological assays were performed according to standard procedures.

Results: Our data show that miR-222 is carried by exosomes secreted from melanoma and that its expression show a direct correlation with melanoma malignancy. In addition a significant level of miR-222 seems to represent a key factor in increasing exosome release, suggesting that this miRNA may drive traffic of microvesicles within tissues and tumor mass. We performed a series of biological assays looking for the capability of miR-222-containing exosomes (exo-miR-222) to convey the same effects obtained by directly overexpressing miR-222 in melanoma cells. Interestingly, in exomiR-222 fused melanoma cell line we observed a significant induction of the invasion and chemotactic capacities compared to exo-control cells. In order to gain insight in the molecular mechanisms underlying this exo-based increased malignancy, we investigated (by TaqMan Array Plate) genes differentially expressed in exo-miR-222 and exo-control. Some tumor promoting genes, like VEGF, FGF2, MGAT5, PNN and FXYD5 were upregulated in exosomes secreted by miR-222 overexpressing cell line.

Conclusion: These data support the conclusion that at least a fraction of miR-222 is secreted by exosomes. In turn, miR-222 was identified as a component required for exosomal secretion in melanoma, further confirming the role played by this miRNA in the genetic changes underlying tumor progression.

D 8
NOTCH3 DEREGULATES CXCR4 EXPRESSION IN IMMATURE THYMOCYTES

Francesca Ferrandino [1] - Giovanni Bernardini [2] - Ambra Ciuffetta [2] - Antonello Campanese [2] - Paola Grazioli [2] - Diana Bellavia [2] - Alberto Gulino [2] - Isabella Screpanti [2] - Maria Pia Felli [3]

Department of Experimental Medicine, "Sapienza" University of Roma, Roma, Italy [1] - Department of Molecular Medicine, "Sapienza" University, Viale Regina Elena 291, 00161 Roma [2] - Department of Experimental Medicine, "Sapienza" University, Viale Regina Elena 324, 00161 Roma [3]

Constitutive activation of Notch signaling is one of the major cause of acute T cell lymphoblastic-leukemia (T-ALL) in humans and mice. The oncogenic function of Notch3 in T-ALL was demonstrated by a murine model of our laboratory, characterized by enforced expression of the Notch3 active form (N3-IC) in immature thymocytes (N3-ICtg). Deregulated proliferation and maturation at the preT/T transition phase and constitutive activation of preTCR were observed in N3-ICtg mice .

T cell differentiation depends on multiple signals furnished by the stroma, which elaborates cytokines, chemokines and ligands and sustains thymocyte maturative process within the thymic micro-environment. Cooperative signaling among the preTCR, CXCR4 and Notch are required at β selection for the continued differentiation from Double Negative (DN) to Double Positive (DP) T cells. The stromal cell derived factor SDF-1(CXCL12) and its receptor CXCR4 promote survival of DN thymocytes and regulate the migration during the DN/DP transition. The CXCR4/SDF-1 axis has been suggested to play a role in the pathogenesis of T-ALL.

FACS experiments demonstrated decreased percentages of CXCR4 positive cells in DN-gated thymocytes of N3-ICtg mice with respect to wt, not attributable to any reduction of DN cell numbers in N3-ICtg mice. Furthemore, mRNA from selected DN thymocytes evidenced a reduced transcription of CXCR4 gene in N3-ICtg with respect to wt mice. Thus it may suggest that Notch3 disrupts early event in preT-cell progressive maturation accompanied by altered migration through the thymus, as further supported by reduced EpCAM expression, a homotypic adhesion molecule known to play a role in thymocytes/T cell interactions with epithelial cells. On the other hand, CXCR4 expression per cell was increased in DP-gated cells of N3-ICtg with respect wt mice. We hypothesize that Notch3 deregulation may lead to aberrant positioning of immature DN thymocytes and may modulate DP cells egress from thymus, in early steps of T-ALL development.

D 9
VAV1 AS A POSITIVE PROGNOSTIC BIOMARKER IN BREAST TUMORS: A ROLE IN MODULATING THE EFFICIENCY OF THE METASTATIC PROCESS

Silvia Grassilli [1] - Federica Brugnoli [1] - Rossano Lattanzio [1] - Marco Marchisio [1] - Ervin Nika [1] - Silvano Capitani [1] - Valeria Bertagnolo [1]

Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Ferrara, Italy [1]

Introduction: Vav1 is one of the signaling proteins normally restricted to hematopoietic cells that results ectopically expressed in solid tumors, including breast cancer. Contrarily to other solid tumors, in which Vav1 positively correlates with malignancy, the *Vav1* transcript in breast cancers seems to be higher in tumors from patients who remained disease free than in patients who developed recurrence. This work was aimed to establish the significance of Vav1 expression in breast tumor cells, in terms of tumor progression measured as risk of recurrence in patients with invasive breast cancer without lymph node involvement.

Materials and methods: Vav1 expression was evaluated by immunohistochemical analysis on TMAs containing T1-T2, N0, M0 breast cancer sections and correlated with clinicopathological features of tumors and follow-up of breast tumor patients. Breast cancer-derived cell lines with different morphology, immunoprofile and invasive properties were used to investigate the in vitro and in vivo Vav1-related metastatic potential.

Results: Vav1 is expressed in almost all the breast cancers investigated and shows a peculiar localization inside the nucleus of tumor cells. High amounts of nuclear Vav1 are positively correlated with low incidence of relapse, regardless phenotype and molecular subtype of breast neoplasia. In particular, Kaplan-Meier plots showed an elevated risk of distant metastasis in patients with low Vav1 expression compared to patients with high Vav1 expression in their tumors, that persisted after adjusting for other prognostic factors in multivariate analysis. Experiments performed with breast tumor-derived cells indicated that Vav1 negatively modulates their *in vitro* invasiveness and their *in vivo* metastatic efficiency, possibly by affecting the expression of genes involved in tumor invasion and/or metastasis of breast tumors.

Conclusions: Our findings indicate that Vav1 is a positive prognostic factor in early-stage breast tumors, regardless cancer subtypes, as it reduces the ability of tumor cells to form metastasis. Since the high heterogeneity of breast tumors makes difficult to predict the evolution of early breast neoplasias, the evaluation of nuclear Vav1 levels may help in the characterization and management of early breast cancer patients. In addition, Vav1 may serve as a target for new therapies aimed to prevent breast cancer progression.

D 10
THE ROLE OF THE INTEGRIN-LINKED KINASE (ILK) IN THE NHE1-ß1 INTEGRIN DEPENDENT REGULATION OF INVADOPODIA FUNCTION IN BREAST AND PROSTATE CANCER CELLS

Maria Raffaella Greco [1] - Stefania Forciniti [1] - Loredana Moro [2] - Katrine Zeeberg [1] - Valeria Casavola [1] - Stephan Joel Reshkin [1] - Rosa Angela Cardone [1]

Department of Bioscience, Biotechnology and Biopharmaceutics, University, Bari, Italy [1] - Institute of Biomembranes and Bioenergetics (IBBE), CNR,, University, Bari, - [2]

Tumor invasion requires changes in cell adhesion, motility and proteolytic remodeling of the extracellular matrix (ECM). It is now well established that tumor invasive ability is driven by the activated beta1 (β1)-integrin , whose cytoplasmic tail is linked to integrin linked kinase (ILK), and proteases in actin rich plasma membrane structures called invadopodia. The invadopodia play a central role in cancer cell dissemination through the directed proteolysis of the ECM. The acidic tumor microenvironment assumes a fundamental importance. In recent years, the predominant regulator of tumor intracellular/extracellular pH (pHi/pHe) has been shown to be the Na+/H+ exchanger, NHE1. It is localized at invadopodia and its activity has a double function in driving both β1-integrin stimulated invadopodia formation and their proteolytic activity. ß1 integrin is known to activate multiple signaling cascades involved in the regulation of motility and invasion but its role in the regulation of the formation and activity of invadopodia, remains unknown.

Here we demonstrate that in the invadopodia of breast and prostate cancer cells there is a signal axis "β1 integrin-ILK- NHE1" in which the signal mediated by activation of integrin interacts with the invasive signal mediated by NHE1.

The protein expression studies conducted on both cell lines show that NHE1 coimmunoprecipitates

with β1 integrin and ILK in invadopodia. Digestion and migration assays demonstrate that this events are mediated (i) by NHE1 because the treatment with cariporide, that blocks the activity of the exchanger, reduces the entity of the two processes and (ii) they are mediated also by β1 integrin because the stimulation of its activity increases the entity of the two processes. Interestingly, ILK has an important role in invasion because its inhibition reduces both migration and the degradation of ECM. Moreover experiments with Proximity Ligation Assay (in Situ PLA) demonstrate that ILK associates with T567-phosphoEzrin. These data suggest that ECM-mediated stimulation of invadopodia occurs through the formation of a protein-protein complex formed by NHE1-β1 integrin-ILK-NHERF1 and p-Ezrin that serves as a linker between ILK and NHE1. This increased understanding of the molecular interactions within the invadopodia should permit the design of small molecule inhibitors to be tested in the future for preventing invadopodia mediated-invasion.

D 11
SEMA6A AND MICAL-1 ARE POTENTIAL THERAPEUTIC TARGETS IN BRAFV600E HUMAN MELANOMAS

Rossella Loria [1] - Giulia Bon [1] - Valentina Perotti [2] - Enzo Gallo [3] - Ilaria Bersani [2] - Paola Baldassari [2] - Manuela Porru [4] - Carlo Leonetti [4] - Selene Di Carlo [1] - Paolo Visca [3] - Andrea Anichini [2] - Roberta Mortarini [2] - Rita Falcioni [1]

Regina Elena National Cancer Institute, Molecular Oncogenesis Laboratory, Dept. of Experimental Oncology, Rome, Italy [1] - IRCCS National Cancer Institute , Milano, Human Tumors Immunobiology Unit, Dept. of Experimental Oncology and Molecular Medicine, Milan, Italy [2] - Regina Elena National Cancer Institute, Pathology Laboratory, Rome, Italy [3] - Regina Elena National Cancer Institute, Chemotherapy Laboratory,Dept. of Experimental Oncology, Rome, Italy [4]

BRAFV600E- and MEK-specific inhibitors have shown significant impact on progression-free and overall survival in advanced melanoma, but primary and secondary resistance mechanisms restrict the efficacy of these target-specific drugs, suggesting that additional therapeutic targets need to be identified, first of all in the highly frequent BRAF-mutant tumors. To this end, we used whole genome microarray analysis to identify differentially expressed genes in a set of neoplastic clones, isolated from a single melanoma metastasis, and characterized by mutually exclusive expression of BRAFV600E or NRASQ61R. By this approach we identified two genes, SEMA6A and Mical-1 belonging to the semaphorin-plexin signaling pathway and highly expressed, at mRNA and protein levels, in BRAF-mutant neoplastic clones. Real-time PCR, Western blot analysis and immunohistochemistry confirmed the preferential expression of SEMA6A and Mical-1 in BRAF-mutant neoplastic cells from melanoma clones, primary and metastatic cell lines and tissue sections from melanoma lesions. SEMA6A depletion, by specific RNA-interference experiments, led to cytoskeletal remodeling, loss of stress fibers, generation of actin-rich protrusions, and cell death, while SEMA6A overexpression, in NRAS mutant clones, promoted *in vitro* invasion. Mical-1 depletion, by siRNA, in BRAF mutant melanomas, did not alter the actin cytoskeleton organization, but caused a strong NDR phosphorylation and NDR-dependent apoptosis. Overall, these results demonstrate that SEMA6A and Mical-1 are two potential therapeutic targets with independent pro-survival function in BRAFV600E mutant melanomas.

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D 12
SCD5 ?9 STEAROYL-COA-DESATURASE BLOCKS SPARC SECRETION AND, IN TURN, REDUCES METASTASES IN MELANOMA

Maria Bellenghi [1] - Alessandra De Feo [1] - Federica Felicetti [1] - Lisabianca Bottero [1] - Sabina Sangaletti [2] - Francesca Pedini [1] - Marina Petrini [1] - Rossella Puglisi [1] - Cinzia Gesumundo [1] - Massimo Denaro [1] - Luca Pasquini [1] - Claudio Tripodo [3] - Mario Paolo Colombo [2] - Alessandra Carè [1] - Gianfranco Mattia [1]

Istituto Superiore di Sanità, Reserach Istitute, Rome, Italy [1] - Istituto Nazionale Tumori, IRCCS, Milan, Italy [2] - Unità di Immunologia, Dipartimento di scienze della salute, Università di Palermo, Palermo, Italy [3]

Introduction Alterated fatty acids composition of plasma membranes significantly contributes to local tumor growth and metastatic dissemination. Saturated fatty acids (SFA), as palmitic and stearic, are desaturated by Δ9 stearoyl-coA-desaturase enzymes (SCD1 and SCD5) to produce the monounsaturated (MUFA) palmitoleic and oleic fatty acids, according to a correct SFA/MUFA ratio. Here we have analyzed the expression and function of SCD5 in melanoma and its involvement in regulating the secretion of SPARC, a matricellular protein significantly associated with metastatic dissemination.

Materials and methods Expression studies were performed by western blot, real time PCR and immunofluorescence and all the biological assays performed according to standard procedures. Different pH culture conditions were utilized to evaluate cell secretion. For *in vivo* studies, xenografted nude mice were utilized to evaluate SCD5 functional role on tumor growth and metastatic potential.

Results Our data show that SCD5 decreases during melanoma progression and that its enforced expression in A375M metastatic melanoma blocks SPARC release leading to a significant intracellular accumulation paralleled by collagen retention. *In vivo,* A375M-SCD5 cells injected into athymic nude mice produced significant less metastases in the lung than A375M counterpart confirming that SCD5 hampers the malignant phenotype. Accordingly, IHC analyses revealed a partial mesenchymal-epithelial transition according to restored E-cadherin expression and β-catenin membrane relocalization. Following the notion of acidic microenvironment enhancing SPARC activity and proper ECM remodeling toward dissemination, we cultured A375M-SCD5 cells in low pH vs buffered conditions (pH 6.0 vs 7.4). Interestingly, acidic culture conditions restored SPARC secretion in SCD5 expressing cells, inducing cysteine cathepsin release.

Conclusion These results indicate the requirement of SCD5 down-modulation for tumor malignancy and extracellular matrix component degradation. Accordingly, in A375M-SCD5 cells, SPARC retention is associated with the recovery of some epithelial characteristics. As these data do not clarify the specific role of SCD5 in downgrading melanoma malignancy, further studies are ongoing to evaluate a possible direct effect of SCD5 on pH modulation or indirect responses caused by changes of membrane composition. Funded by AIRC to AC (IG13247)

D 13
CD157-FIBRONECTIN INTERACTION AT THE CROSS-ROAD BETWEEN INFLAMMATION AND CANCER PROGRESSION

Simona Morone [1] - Stefania Augeri [2] - Massimiliano Cuccioloni [3] - Matteo Mozzicafreddo [3] - Mauro Angeletti [3] - Nicola Lo Buono [1] - Alice Giacomino [2] - Francesca Martinetto [2] - Erika Ortolan [2] - Ada Funaro [1]

Department of Medical Sciences, University ot Torino, Torino, Italy [1] - Department of Medical Sciences, University of Torino, Torino, Italy [2] - School of Biosciences and Veterinary Medicine, University of Camerino, Camerino, Italy [3]

Introduction: CD157/BST1 is a glycoprotein belonging to the NADase/ADP-ribosyl cyclase gene family involved in the control of leukocyte adhesion to extracellular matrix (ECM) proteins, migration and diapedesis. CD157 is also expressed in epithelial ovarian cancer where it enhances tumor aggressiveness by promoting mesenchymal differentiation and is an independent prognostic factor of poor survival. However, the molecular interactions underpinning the role of CD157 in these processes remain obscure. The biological functions of CD157 and its partnership with specific integrins led us to assume the existence of a direct interaction between CD157 and an unknown component of the ECM.

Materials and methods: The interaction between CD157 and ECM was studied using i) an immunoenzymatic assay based on the binding of recombinant human soluble CD157 (rh-sCD157) to microtiter plates coated with ECM proteins, and ii) Surface Plasmon Resonance analysis allowing real-time monitoring of binding kinetics. The biological significance of the CD157-ECM interaction was studied in both native and engineered Met-5A human pleural mesothelial cells.

Results: We demonstrated that CD157 binds fibronectin with high affinity within its heparin-binding domains (HBD) 1 and 2. The CD157-HBD binding is mediated by the protein core and it is stabilized by the glycosidic chains. Moreover, we found that CD157 binds to other ECM proteins containing HBDs, such as fibrinogen, laminin and collagen I, but not to vitronectin or to the polysaccharide components of ECM (such as heparin and hyaluronan). The CD157-ECM interaction occurs with living cells, where it elicits CD157-mediated cell responses. Indeed, i) CD157 expressed by Met-5A cells bound fibronectin and its HBDs; ii) anti-CD157 antibodies significantly reduced cell adhesion to selected ECM proteins, and iii) knockdown of CD157 expression in Met-5A cells changed their morphology and cytoskeleton organization, reduced cell spreading and remarkably decreased the fibronectin-mediated phosphorylation of FAK, SRC and Akt tyrosine kinases. These morphological and functional changes resulted in impaired cell adhesion.

Conclusions: These findings indicate that the CD157-ECM interaction drives many of the biological effects exerted by CD157 in different physiological (*e.g.*, leukocyte trafficking) and pathological contexts (*e.g*, inflammatory diseases and cancer), and make CD157 an attractive therapeutic target in inflammation and cancer.

D 14
GLYPICAN-5 EXERTS CELL (AND CANCER) TYPE-DEPENDENT ANTITHETIC EFFECTS ON TUMOUR GROWTH AND SPREADING AND IS AN INDISPENSABLE TUMOUR MICROENVIRONMENTAL COMPONENT

Silvia Rossi [1] - Carlotta Alias [1] - Elena Garusi [1] - Enrica Balza [2] - Anna Rubartelli [2] - Ottavia Barbieri [3] - Simonetta Astigiano [3] - Pier Andrea Nicolosi [4] - Roberto Perris [1]

COMT– Centre for Molecular and Translational Oncology, Dept. of Life Sciences, University of Parma, Parma, Italy [1] - Cell Biology Unit, University of Genova, IRCCS AOU San Martino-IST National Cancer Research Institute, Genova, Italy [2] - Embryogenesis and Tumorigenesis in Animal Models Unit, University of Genoa, IRCCS AOU San Martino-IST National Cancer Research Institute, Genova, Italy [3] - S.O.C. for Experimental Oncology 2, The National Tumour Institute Aviano, CRO-IRCCS, Aviano, Pordenone, Italy [4]

Cell surface proteoglycans are critically involved in several aspects of cancer biology, starting from tumour onset to uncontrolled growth and metastasis formation. While data has been accrued concerning the role of NG2/CSPG4 and syndecans, very little is known about how glypicans (GPCs) may influence tumorigenesis, either when presented on the cancer themselves or when accumulated in the tumour microenvironment and metastatic niches. The current idea is that several GPCs, including GPC5 (one of the 6 GPCs known in human), may act as a tumorigenesis promoter in rhabdomyosarcomas, whereas it appear as a putative tumour suppressor in lung carcinoma leaving its significance in NSCLC controversial. We have therefore specifically addressed the role of GPC5 in mesenchymal derived tumours and the growth environment of these tumours. Forced expression of GPC5 in sarcoma cell lines produces an oncosuppressing function as it significantly impedes growth of cells in vitro and interferes with primary tumour formation and growth in vivo. Growth reduction in vivo is accompanied by a concomitant reduction in intra-lesional vessel density. Highly aggressive melanoma cells implanted into $GPC5^{-/-}$ mice also show strongly retarded growth and impaired mediastinal lymph-nodal infiltration and distant metastases, yielding significantly prolonged survival. Intriguingly, opposite results were obtained when GPC5 was overexpressed in melanoma cells. In fact, GPC5 overexpressing A375 cells showed an enhanced capability to growth and proliferate in different serum concentration and in response to FGF-2, HGF, IGF and Wnt-1; enhanced capability to adhere to collagen type I and fibronectin and to migrate in scratch assays in response to conditioned media and IGF-1 and Wnt1; increased ability to evade from Matrigel drops, to form more and larger colonies in anchorage-independent conditions; and the propensity to form bigger tumour spheroids on Poly-HEMA substrates when compared to non-transduced cells. The results highlight cell type-dependent antithetic roles GPC5 in tumorigenesis and DNA microarray-based global gene profiling and comprehensive ~800-spot antibody array-based phospho-proteomic screens of tumour masses formed by $GPC5^{+}$ and $GPC5^{-}$ cells have been exploited to dissect the gene expression and signal transduction pathways underlying these antipodal effects, Meanwhile, parallel ongoing studies are being pursued to define whether other GPCs share the same features.

D 15
CADHERIN 6 AND THYROID CANCER: BEYOND A STRUCTURAL FUNCTION

Valentina Sancisi [1] - Greta Gandolfi [1] - Moira Ragazzi [2] - Ione Tamagnini [2] - Simonetta Piana [2] - Alessia Ciarrocchi [1]

Laboratory of Translational Research, Arcispedale Santa Maria Nuova - IRCCS, Reggio Emilia, Italy [1] - Pathology Unit, Arcispedale Santa Maria Nuova - IRCCS, Reggio Emilia, Italy [2]

Introduction: Modifications in adhesion molecules profile may change the way tumor cells interact with the surrounding microenvironment. The Cadherin switch that takes place during epithelial-mesenchymal transition (EMT) contributes to loosening the rigid organization of epithelial tissues and to enhance motility and invasiveness of tumor cells. We recently discovered that Cadherin-6 (CDH6) is a hallmark of aggressive cells in thyroid cancer and that in vivo its expression marks specifically invasive cells at the edge of tumor invasion.

Materials and methods: We analyzed the expression of CDH6 and a panel of EMT markers by qPCR in cancer and normal thyroid cell lines with or without TGF-β treatment and in a series of human thyroid carcinomas. CDH6 expression and localization in thyroid carcinomas was evaluated also by immunihistochemistry. For functional assays we down-regulated CDH6 by siRNA approach and we performed proliferation assays, migration assays and analysis of cytoskeleton structure by immunofluorescence.

Results: Using a combination of in vitro and in vivo approaches we demonstrated that CDH6 is a late mesenchymal marker and that its expression is controlled by TGF-β during EMT in thyroid cancer. Furthermore, we demonstrated that the transcription factor Runx2 is required for CDH6 expression in thyroid cancer cells. Knockdown of CDH6 by siRNA determined a profound reorganization of the cytoskeleton architecture and of the cell-cell interactions. These morphological alterations are accompanied by reduction in cell migration. Surprisingly, besides the structural effect, the siRNA mediated knockdown of CDH6 determined also a profound reduction in cell proliferation suggesting the possibility that this protein play a function in supporting cellular viability and growth.

Using a yeast-two hybrid screening approach we searched for CDH6 new interactors in a cDNA expression library from human thyroid cancer samples. Intriguingly, proteins involved in authophagy, vescicle trafficking and mitochondrial metabolism were found to interact with CDH6 in thyroid cancer samples indicating that the role of this cadherin in supporting thyroid cancer progression is not limited to a mere structural function.

Conclusions: Overall, these observations provide novel information on the involvement of CDH6 in leading thyroid tumors aggressiveness and suggest its possible role in different pathways at the interface between cell sustenance and microenvironment interaction.

D 16
STRUCTURAL-FUNCTIONAL DIVERSITIES OF NOVEL NG2/CSPG4 PROTEOGLYCAN ISOFORMS DICTATE THE BEHAVIOUR OF CANCER CELLS AND THEIR PUTATIVE THERAPEUTIC TARGETING

Elisa Tamburini [1] - Alice Dallatomasina [2] - Pier Andrea Nicolosi [3] - Elisabetta Lombardi [3] - Maria Teresa Mucignat [3] - Serena Galati [2] - Carlotta Alias [4] - Domenica Mangieri [5] - Roberto Perris [6]

COMT- Centre for Molecular and Translational Oncology, University of Parma, PARMA, Italy [1] - COMT- Centre for Molecular and Translational Oncology/ Department of Life Sciences, University of Parma, PARMA, Italy [2] - Division for Experimental Oncology 2, CRO National Cancer Institute, Aviano, Pordenone, Italy [3] - Department of Life Sciences, University of Parma, PARMA, Italy [4] - Section for Anatomic Pathology, Parma University Hospital, PARMA, Italy [5] - COMT- Centre for Molecular and Translational Oncology/ Department of Life Sciences/CRO National Cancer Institute, University of Parma, PARMA, Italy [6]

NG2/CSPG4 is a proteoglycan (PG) believed to dictate a number of cellular functions to represent a poor prognostic factor and to be a therapeutic target in many tumour types. Its post-translational processing lends support to the hypothesis that diverse isoforms of the PG may be expressed by different cancer cells and that such structural variations may be associated with a functional diversity.

We have generated a panel of monoclonal antibodies against the NG2 ectodomain to create distributional cancer maps of NG2 isoforms in situ and in circulating cancer cells and to correlate these distribution patterns with structural functional traits of the isoforms to find unique immune-targets. We observe a variation in the distribution of NG2 variants in different tumour types and within the same tumour entity accompanied by a diverse distribution of the isoforms in neovascular structures associated with tumour lesions and healthy foetal and adult tissues. The same isoform variation was noted to be reproduced in soft-tissue sarcoma, melanoma and breast carcinoma cell lines used as model cancer cells, while combined glycomic and proteomic analyses on immunoprecipitated NG2 molecules allowed us to delineate the structural features of the detected isoforms. The same mass spectrometric approaches were also employed to identify extracellular binding partners of NG2 and several such could be identified. The significance of these molecular interactions is currently under investigation. In vitro flow assays, vascular transmigration assays and CAM assays entailing intravital microscopy indicate a definite role of NG2 in intra-extravasation phenomena and support its potential in promoting specific steps of the metastatic cascade. To further establish whether solitary NG2 expressing subsets of tumour lesions would correspond to the more aggressive cancer cell populations of the lesion we sorted $NG2^+$ and $NG2^-$ cells, compared their in vitro and in vivo tumorigenic behaviour and established the gene expression patterns through DNA microarray. The PG-expressing subset was found to be consistently more aggressive and to exhibit a significantly diverse gene expression pattern and a differential phosphorylation of key intracellular signalling components. The data suggest that different NG2 isoforms may exert diverse functions and that antibodies specifically recognizing these isoforms may be exploited to better tailor immune-directed, anti-NG2 therapeutic strategies.

D 17
CROSS-TALK BETWEEN BONE MARROW-DERIVED MESENCHYMAL STEM CELLS AND TUMOR CELLS: ROLE OF CXCR4 AND AQP1 IN OSTEOSARCOMA AND HEPATOCARCINOMA PROGRESSION

Antonella Zannetti [1] - Alessandra Pelagalli [2] - Anna Nardelli [1] - Enrico Lucarelli [3] - Stefania Scala [4] - Bruno Alfano [1] - Arturo Brunetti [2] - Marco Salvatore [2]

Istituto di Biostrutture e Bioimmagini, CNR, Napoli, Italy [1] - Dipartimento di Scienze Biomediche Avanzate, Università degli Studi di Napoli "Federico II", Napoli, Italy [2] - Laboratorio di Patologia Ortopedica e Rigenerazione Tissutale Osteoarticolare, Istituto Ortopedico Rizzoli, Bologna, Italy [3] - Istituto Nazionale per lo Studio e la Cura dei Tumori "Fondazione Giovanni Pascale", IRCCS, Napoli, Italy [4]

Introduction: Increasing evidences suggest that bone marrow-derived mesenchymal stem cells (BM-MSCs) are recruited into the stroma of developing tumors where they contribute to progression by enhancing tumor growth and metastasis, or by inducing anticancer-drug resistance. Although many experimental evidences exist supporting the therapeutic potential of MSCs, the mechanism of homing and recruitment of MSCs into tumors and their potential role in malignant tissue progression is still not well understood. The aim of this study was to elucidate the role of BM-MSCs to promote tumor cell proliferation and invasion. Therefore, we analyzed whether chemokine receptor type 4 (CXCR4) and water channel molecule, aquaporin 1 (AQP1), both known to play a key role in cancer metastases, could affect MSCs mediated osteosarcoma and hepacarcinoma progression.

Materials and methods: BM-MSCs were grown in medium with 1% fetal bovine serum to obtain conditioned medium (MSCs-CM). CXCR4 and AQP1 protein level was evaluated in human hepatocarcinoma (SNU-398) and osteosarcoma (U2OS) cells exposed to MSCs-CM. Proliferation of tumor cells co-cultured with MSCs or cultured in presence of MSCs-CM was evaluated by MTT assay. Tumor migration and invasion were assayed in 24-well transwell chambers pre-coated with collagen/fibronectin or matrigel, respectively. Tumor cells were added to the upper chamber and incubated in presence of MSCs-CM or MSCs (lower chamber) used as chemoattractants. Tumor cells pre-treated with a CXCR4 antagonist (AMD3100) or AQP1 antibody were also analyzed for proliferation, migration and invasion. Furthermore, involvement of Akt and/or Erk signalling pathways in tumor progression MSCs-mediated was examined.

Results: Our results showed that CXCR4 and AQP1 expression is increased in human hepatocarcinoma and osteosarcoma cells in presence of MSCs secreted factors. Conditioned medium from MSCs promoted proliferation, migration and invasion of tumor cells, whereas inhibition of CXCR4 and AQP1 significantly down-regulated these effects. Furthermore, SNU-398 and U2OS cells showed an enhancement of p-Akt and p-Erk levels when they were cultured in presence of MSCs-CM.

Conclusions: In conclusion these findings suggest that BM-MSCs can promote the proliferation and invasion of osteosarcoma and hepatocarcinoma cells through CXCR4 and AQP1 overexpression. In addition, we found that MSCs may contribute to tumor progression by PI3K/Akt pathway and/or Ras/Erk cascade.

Cell Cycle Regulation

E 1
INVESTIGATING THE ROLE OF ERß IN MELANOMA GROWTH AND ITS INTERACTION WITH THE TUMOR EPIGENOME

Monica Marzagalli [1] - Lavinia Casati [1] - Marina Montagnani Marelli [1] - Roberta Moretti [1] - Patrizia Limonta [1]

Department of Pharmacological and Biomolecular Sciences, Università degli Studi di Milano, Milan, Italy [1]

Introduction: Cutaneous melanoma remains a mostly incurable disease. The identification of novel molecular pathways involved in melanoma progression might help increase the therapeutic options for this pathology. Clinical observations indicate that estrogen receptor-b (ERb) is expressed in melanoma tissues and its expression decreases with tumor progression, suggesting its anticancer activity. Moreover, epigenetic alterations were proposed to play a key role in melanocyte malignant transformation. Preliminary data from this laboratory indicate that ERb (but not ERa) is expressed in human melanoma cells (BLM, WM115).

These experiments were performed to clarify the effects of ERb activation on melanoma cell growth and on the melanoma epigenome.

Materials and methods: Cell count by hemocytometer; immunofluorescence assay (ERb translocation); gene reporter assay (ERb transcriptional activity); digestion of genomic DNA by sensitive DNA methylation restriction enzymes followed by CG-sites PCR and colorimetric assay (global DNA methylation); fluorescent Western blot (histone acetylation and methylation). Receptor activation was achieved by means of specific agonists: E2, DPN, KB1, KB2, KB4.

Results: In BLM cells, we found that: 1) ERb agonists significantly inhibit cell proliferation, and this effect is completely abrogated by the ER antagonist ICI 182,780 (similar results were reported in WM115 cells); 2) ERb activation triggers its translocation from the cytoplasm into the nucleus and its transcriptional activity, confirming the classical mechanism of action of steroid receptors; 3) global DNA is hypomethylated with respect to normal melanocytes; 4) the acetylation level of histone H4 (H4K16Ac) and the methylation level of histone H3 (H3K4me3) are significantly higher than the corresponding histone modifications in normal melanocytes; 5) ERb activation increases global DNA methylation as well as the levels of H4K16Ac and of H3K4me3.

Conclusions: in human melanoma cells ERb is associated with a significant antitumor effect and its activation affects the melanoma epigenome, suggesting that its activity might be mediated by an epigenetic modification of the expression of oncogenes/tumor suppressor genes. These data support the notion that ERb might be considered as a molecular target for novel therapeutic strategies in melanoma. *(Supported by Fondazione Banca del Monte di Lombardia, and by PRIN 2010-2011; KB compounds were provided by Karo Bio AB)*

E 2
INHIBITION OF PHOSPHATIDYLCHOLINE-SPECIFIC PHOSPHOLIPASE C AS A NEW STRATEGY TO PREVENT THE PROLIFERATION OF TRIPLE-NEGATIVE BREAST CANCER CELLS

Paola Pisterzi [1] - Laura Mercurio [1] - Maria Josè Caramujo [1] - Alessandro Ricci [1] - Egidio Iorio [1] - Franca Podo [1] - Serena Cecchetti [1]

Section of Molecular and Cellular Imaging, Department of Cell Biology and Neurosciences, Istituto Superiore di Sanità, Rome, Italy [1]

Introduction: Accruing evidence supports functional implications of choline metabolism on cell signaling and the possible role of phosphatidylcholine (PtdCho)-cycle as target for anticancer treatment (*Podo F et al, NMR Biomed 2011; Glunde K et al, Nat Rev Cancer 2011*). An abnormal choline phospholipid metabolism is emerging as a hallmark that is associated with oncogenesis and tumour progression and we contributed in this field identifying an upmodulation of PC-specific phospholipase C (PC-PLC) in breast and ovarian cancer (*Iorio E et al, Cancer Res 2010; Paris L et al, Breast Cancer Res 2010; Abalsamo L et al, Breast Cancer Res 2012*). Purpose of this study was to investigate whether the pharmacological inhibition of PC-PLC could be used as a potential antitumor strategy against triple-negative breast cancer (TNBC) cells by affecting both EGFR signaling and cell cycle progression.

Materials and methods: EGFR receptor and the direct interaction of PC-PLC with this receptor were investigated in two TNBC cell lines (MDA-MB-231 and MDA-MB-468) compared with the HER2-overexpressing (SKBr3) and the non tumoral MCF10A cells, using flow cytometry, confocal laser scanning microscopy and immunoprecipitation techniques. SDS-page, western blotting and flow cytometry analyses were utilized to investigate the effects of PC-PLC inhibition on the EGFR-mediated signaling cascade (activation/phosphorylation status of the receptor and key proteins such as AKT and ERK1/2) and on changes induced in the expression of molecules involved in the progression through the cell cycle.

Results: PC-PLC was more highly expressed in the TNBC than in HER2-overexpressing SKBr3 and in non tumoral (MCF-10A) cells. Pharmacological inhibition of the PC-PLC enzymatic activity resulted in a reduction of the signaling cascade mediated by the EGFR receptor and was associated with cell growth arrest in G0/G1 phase, in absence of apoptosis. Moreover, following exposure to the inhibitor only the tumor cell lines showed significant changes in the expression of molecules regulating proliferation and cell cycle progression, such as down-regulation of cyclin D1, cyclin E, and Rb protein, while p21 was up-regulated.

Conclusions: These results highlighted the role of the PC-PLC enzyme in the proliferation of triple-negative breast cancer cells, suggesting that PC-PLC inhibition could represent a powerful strategy to control malignant transformation and tumor progression of this tumor cells.

E 3
PLAGL1 GENE TRANSCRIPTION DURING HEPATOCELLULAR CARCINOMA CELL PROLIFERATION

Ana Florencia Vega Benedetti [1] - Roberta Vanni [2] - Paola Caria [2] - Patrizia Zavattari [2] - Luis Antonio Parada [1]

Instituto de Patología Experimental, Universidad Nacional de Salta, Salta, Argentina [1] - Dipartment of Biomedical Sciences, University of Cagliari, Cagliari, Italy [2]

Introduction: *PLAGL1* gene encodes a homonym Zinc-finger protein that regulates cell cycle arrest and apoptosis. Regulation occurs through convergent mechanisms including the induction of the expression of $p21^{Cip1}$ and PPARγ and the interaction with p53. Deletion of chromosome 6q is a common finding in hepatocellular carcinoma (HCC). The important tumorigenic event associated with this abnormality has been the loss of the tumor suppressor gene *IGFIIR* (6q25.3). However we speculate that *PLAGL1* (6q24.1) may also have an important role in HCC development. In fact, abnormal promoter methylation of this gene is a frequent event in ovary and breast tumors. We studied the trancriptional level of *PLAGL1* in the context of proliferation of hepatoma cells.

Materials and methods: Human HCC cell lines HepG2, Huh7, PLC and SkHep1 were plated onto 75 cm^2 flasks for proliferation assays, and cultured in D-MEMF12 media supplented with 10% FBS. Normal fibroblasts were used as control. Cell count and Real Time PCR (RT-PCR) for *PLAGL1* and *PPIA* (reference gene) was performed at 48h, 72h and 96h during cell proliferation. The transcription level of *PLAGL1* and *PPIA* was quantified using the $2^{-\Delta\Delta Ct}$ method. The statistical analysis were performed by 1 way ANOVA.

Results: We determined that the cancer cell lines have higher proliferation rates, and significant lower level of *PLAGL1* mRNA than fibroblasts. Significant differences were found in the transcription level of *PLAGL1* in fibroblast between the different time points. Whereas the tumor cell lines did not exhibit changes in the transcription level of *PLAGL1* during the experimental period, except for SkHep1 at 96h. Comparing the level among tumor cell lines, significant differences were found, being SkHep1 cells those with the lowest expression.

Conclusions: We conclude that the low transcription level of *PLAGL1* could be associated with abnormal proliferation of HCC cells. Further experiments will be performed on HCCs mouse model and human samples.

Cell Metabolism

F 1
EXTRACELLULAR NICOTINAMIDE PHOSPHORIBOSYLTRANSFERASE (NAMPT) SHAPES THE CLL MICROENVIRONMENT PROMOTING MACROPHAGE M2 POLARIZATION VIA A NON-ENZYMATIC MECHANISM

Valentina Audrito [1] - Sara Serra [1] - Davide Brusa [1] - Francesca Mazzola [2] - Francesca Arruga [1] - Tiziana Vaisitti [1] - Davide Rossi [3] - Marta Coscia [4] - Gianluca Gaidano [3] - Cynthia Wolberger [5] - Nadia Raffaelli [2] - Silvia Deaglio [1]

Dept. of Medical Sciences, University of Turin & Human Genetics Foundation, Torino, Italy [1] - Dept of Agricultural, Food and Environmental Sciences, Università Politecnica delle Marche, Ancona, Italy [2] - Division of Hematology, Department of Translational Medicine, "Amedeo Avogadro" University of Eastern Piedmont, Novara, Italy [3] - Division of Hematology, AO Città della Salute e della Scienza, Torino, Italy [4] - Dept. of Biophysics and Biophysical Chemistry,, Johns Hopkins University School of Medicine, Baltimore, - [5]

The cancer-associated microenvironment provides malignant cells with a cocktail of signals that increase survival, reprogram their metabolism and help escape from the action of the immune system. Nicotinamide phosphoribosyltransferase (iNAMPT) is the rate-limiting enzyme in NAD biosynthesis. An extracellular form of this protein (eNAMPT) exerts cytokine/adipokine-like actions. Both iNAMPT and eNAMPT levels are increased in tumors, suggesting that this molecule is at the crossroad between metabolism and inflammation. Here we show that NAMPT mRNA, as well as intracellular and plasma protein levels are significantly up-regulated in chronic lymphocytic leukemia (CLL) patients compared to healthy donors. eNAMPT, secreted by activated CLL lymphocytes in the extracellular milieu, polarizes resting monocytes towards macrophages with an M2 phenotype. These cells express high levels of CD163, CD206 and indoleamine 2,3-dioxygenase (IDO) and secrete immunosuppressive cytokines, such as IL-10, CCL18 and IL-6. Furthermore, NAMPT-primed M2 macrophages reduce antigen-driven T cell proliferation and support Treg expansion. Once exposed to eNAMPT, M2 macrophages activate a signaling pathway characterized by ERK1/2 phosphorylation and by the activation of STAT3 and NF-kB. These effects are independent of the enzymatic activity, as inferred by the use of an enzymatically-deficient NAMPT mutant. Overall, these results reveal that eNAMPT is a critical element in the induction of an immunosuppressive and tumor-promoting microenvironment in CLL, in a way apparently independent of its enzymatic activities.

F 2
FGF/FGFR SIGNALING SUSTAINS CELL SURVIVAL AND CELL METABOLISM THROUGH THE ACTIVATION OF PI3K/AKT AND MAPK PATHWAYS IN SQUAMOUS CELL CARCINOMA OF THE LUNG.

Cristina Caffarra [1] - Claudia Fumarola [1] - Roberta Alfieri [1] - Marcello Tiseo [2] - Andrea Ardizzoni [2] - Pier Giorgio Petronini [1]

Department of Clinical and Experimental Medicine, University of Parma, Parma, Italy [1] - Division of Medical Oncology, University Hospital of Parma, Parma, Italy [2]

Background: Fibroblast Growth Factors (FGFs) signaling is a complex pathway which controls a variety of biological functions, including cell proliferation, survival, differentiation and migration. Moreover, it has been demonstrated that FGFR1 influenced cell metabolism through the direct phosphorylation of the glycolitic enzyme pyruvate kinase M2 (PKM2). It is known that the FGFR signaling pathway is aberrantly activated in at least a subset of NSCLCs, especially of squamous hystotype (SQCLC) where FGFR is often over-expressed or amplified. In this study we evaluated the role of FGFR signaling in cell survival and cell metabolism, both in normoxic and hypoxic conditions, in a panel of SQCLC cell lines using Dovitinib, a multi-target RTK inhibitor that is able to target FGFR1-2-3.

Results: Dovitinib reduced cell proliferation, in association with the induction of apoptosis, in a dose dependent-manner, in particular in H1703 and H520 cells showing FGFR1 amplification. In these cells we observed a down-regulation of PI3K/AKT/mTOR and MAPK pathways, due to the inhibitory action of the drug on FGFR activation. In addition, we demonstrated that dovitinib impaired glucose metabolism by down-regulating the glucose uptake and the expression of GLUT-1 and HIF-1 both in normoxic and hypoxic conditions. Moreover, we found out that the growth-inhibitory effects of dovitinib were enhanced under hypoxia.

Conclusions: Our results indicate the importance of FGFR-1 signaling pathway in sustaining cell survival and energy metabolism in SQCLC cell lines showing overexpression of FGFR1. This study suggests that inhibition of FGFR signalling may represent a valuable therapeutic strategy for treatment of SQCLC with specific alterations of FGFR.

F 3
METABOLOMIC SIGNATURES OF ALTERED PHOSPHATYDILCHOLINE METABOLISM IN BREAST CANCER CELLS

Maria José Caramujo [1] - Serena Cecchetti [1] - Paola Pisterzi [1] - Franca Podo [1] - Egidio Iorio [1]

Istituto Superiore di Sanità, Department of Cell Biology and Neurosciences, Rome, Italy [1]

Introduction: Altered glucose and phosphatydilcholine (PtdCho) metabolism are metabolic hallmarks of various cancer phenotypes [Galluzzi et al. Nat Rev Drug Discov 2013; Glunde et al. Nat Rev 2011]. High level of intracellular lactate results from the up-regulation of glycolitic pathway. Both biosynthetic

and catabolic pathways of PtdCho contribute to the increased phosphocholine (PCho) levels in cancer cells involving phosphorylation of choline (Cho) in *de novo* PtdCho biosynthesis, as well as by activation of PtdCho−specific phospholipase C (plc) [Podo et al. NMR Biomed 2011].

We assessed the metabolic signature in breast cancer (BC) cells and their non-tumoural counterpart, and investigated the effect of plc inhibition on intracellular metabolism and cell growth to explore plc as a possible target for antitumor treatments.

Materials and methods: High-resolution NMR experiments were performed (9.4 T, Bruker Avance) on aqueous cell extracts of BC cell lines MCF-7 and MDA-MB-231 and their non-tumoural MCF-10A cell line [Iorio et al., Cancer Res 2010]. Activity and expression of plc were assessed by biochemical assay and western blot. D609 (inhibitor of plc) was added (150 μM) after 24 hours of culture of plated cells (20000 cells/cm^2). Evaluation of necrotic, apoptotic and living cells were performed by flow cytometry after stained with Propidium Iodide (Lugini et al., J Immunol 2012).

Results: Tumoural cells lines presented significantly higher content of PCho, ATP, ADP, succinate, acetate and lactate than MCF-10A. The activity of plc was higher in tumoural cells lines than in MCF-10A. The exposure to D609 decreased the difference in these metabolites, with the exception of lactate, between the BC cells and MCF-10A. D609 inhibited cell proliferation during the 48h exposure in all cells lines, and also induced apoptosis up to 20% in BC cells under our experimental conditions.

Conclusions: Tumoural cells lines show signs of both higher PtdCho metabolism and glycolysis than MCF-10A. The exposure to D609 elicits decrease in the PCho content in tumoural cells but not its suppression, thus pointing to biosynthetic pathway contribution to high PCho content of cells; the higher glycolitic metabolism of tumoural cells is mantained. Evidence of abnormal PtdCho metabolisms appears to have implications for BC biology and is a potential avenue to the development of non-invasive clinical tools for BC diagnosis and treatment follow-up.

F 4
EPITHELIAL OVARIAN CANCER: AFFECTING TUMOR AGGRESSIVENESS AND DRUG SENSITIVITY BY TARGETING ABERRANT METABOLISM.

Anna Granata [1] - Roberta Nicoletti [1] - Balaji Krishnamachary [2] - Zaver Bhujwalla [2] - Franca Podo [3] - Silvana Canevari [4] - Egidio Iorio [3] - Marina Bagnoli [1] - Delia Mezzanzanica [1]

Unit of Molecular Therapies, Department of Experimental Oncology and Molecular Medicine, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy [1] - Dept. Radiology, The Johns Hopkins University School of Medicine, Baltimore MD, United States [2] - Department of Cell Biology and Neurosciences, Istituto Superiore di Sanità, Rome, Italy [3] - Functional Genomics Unit, Department of Experimental Oncology and Molecular Medicine, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy;, Milano, Italy [4]

Epithelial ovarian cancer (EOC) remains a highly lethal malignancy due to late diagnosis and early relapse associated with development of chemoresistance. EOC possesses an altered MRS-choline profile, characterized by increased phosphocholine (PCho) content to which mainly contribute over-expression and activation of Choline kinase-alpha (ChoK-alpha). Aberrant metabolism has been proposed as a novel cancer hallmark. Aim of the study is the evaluation of the biological relevance of increased ChoK expression and activity focusing the attention on its possible druggability in EOC. We recently showed that transient CHKA silencing induced: a significant reduction in PCho accumulation as assessed by MRS analysis; inhibition of in vitro cell growth; down modulation of genes related to inflammation and EOC aggressiveness; a significantly reduced cell motility and invasion and a significant increase of sensitivity to platinum, paclitaxel and doxorubicin even in a drug-resistant context. Since the dynamics of biological effects related to CHKA silencing could not possibly be detectable by transient RNAi, we stably downregulated ChoKα mRNA expression. Lentiviral vector, expressing GFP and specific CHKA shRNA, was used to transduce two EOC cell lines, INTOV11 and SKOV3. In both transduced cell lines, we confirmed the data obtained with the transient downmodulation, as reduction of PCho content, inhibition of in vitro cell growth and motilicy. Furthermore, a significant decrease of in vivo tumor growth (sub-cutaneous injection) was evident in both sh-CHKA cellular models as compared to their controls. The global metabolic profiles of sh-CHKA and control EOC cell lines, performed with the Metabolon technology platform, revealed in both cellular models an alteration of glutathione metabolism, commonly characterized by a decrease of reduced glutathione (GSH) content in shCHKA cells as compaired to their controls. Interestingly, sh-CHKA showed an increased sensitivity to drug treatment. These observations suggest that CHKA knocking down may change antioxidant cellular defense thus increasing drug sensitivity. Assessments of the correlation between knocking down CHKA and changes in antioxidant defense is currently ongoing.

F 5
ON THE ROLE OF NEUROFIBROMIN IN MITOCHONDRIAL BIOENERGETIC REGULATION

Ionica Masgras [1] - Giulia Guzzo [1] - Matteo Curtarello [2] - Stefano Indraccolo [2] - Alberto Gambalunga [1] - Federica Chiara [1] - Reuven Stein [3] - Paolo Bernardi [1] - Andrea Rasola [1]

University of Padova, University of Padova, Padova, Italy [1] - Istituto Oncologico Veneto-IRCCS, Istituto Oncologico Veneto-IRCCS, Padova, Italy [2] - Tel Aviv University, Tel Aviv University, Ramat Aviv, Israel [3]

Introduction: NF1 is a genetic disease characterized by an elevated propensity to develop a variety of tumours, mainly complex neoplasms called neurofibromas composed by transformed Schwann cells, mast cells and fibroblasts. The molecular determinants of the neoplastic progression occurring in NF1 cells are only partially characterized. Neurofibromin, the protein encoded by the *NF1* gene, is a negative regulator of the signalling axes controlled by Ras, which are also involved in the metabolic rewiring of tumour cells. We have investigated whether the neurofibromin loss of function that characterizes NF1 tumour cells prompts metabolic changes.

Materials and methods: We have studied the bioenergetic properties of mouse embryonic fibroblasts (MEFs) obtained

from mice in which the *Nf1* gene is ablated: we measured the mitochondrial Oxygen Consumption Rate (OCR) and the activity and the assembly of the respiratory chain complexes. Moreover, we tested the tumorigenic capability of cells both *in vitro* and *in vivo*.

Results: We found that the absence of neurofibromin confers tumorigenic properties to MEFs both *in vitro* and *in vivo*, and inhibition of the MEK/ERK pathway abrogates their tumorigenic potential. Compared to wild-type MEFs, $Nf1^{-/-}$ cells have a decreased OCR and lower Complex II (succinate dehydrogenase) and Complex I (NADH dehydrogenase) activities, the latter being paralleled by a decrease in the expression level of Complex I subunits. Inhibition of ERK signalling increases the expression of Complex I subunits resulting in its augmented assembly and activity.

Conclusions: Altogether these data indicate that *Nf1* deletion causes up-regulation of Ras/ERK pathway, which is responsible for MEFs tumorigenicity and for a metabolic switch toward respiratory inhibition. We hypothesize that the absence of neurofibromin, and the ensuing induction of Ras/ERK signalling, is upstream to the regulation of mitochondrial bioenergetics, and that the metabolic rewiring prompted by Ras/ERK activation can contribute to the transformed phenotype that we observe in $Nf1^{-/-}$ MEFs.

F 6
METABOLIC AND MOTILE REPROGRAMMING OF ER POSITIVE BREAST CANCER CELLS FOLLOWING LONG-TERM ESTROGEN DEPRIVATION

Andrea Morandi [1] - Marina Bacci [1] - Lesley-ann Martin [2] - Maria Letizia Taddei [1] - Elisa Giannoni [1] - Paola Chiarugi [1]

Dipartimento Scienze Biomediche Sperimentali e Cliniche, Università degli Studi di Firenze, Firenze, Italy [1] - Breakthrough Breast Cancer Research Centre, Institute of Cancer Research, London, United Kingdom [2]

Introduction: Approximately 2/3 of the breast tumors are positive for estrogen receptor-α (ER) expression and aromatase inhibitors (AI) have become the first-line treatment for postmenopausal women with ER+ breast cancers. However, *de novo* or acquired AI resistance still limits their benefit for many patients. Metabolic reprogramming is now considered a hallmark of cancer and a fundamental aspect of cell transformation. To elucidate whether metabolic reprogramming is linked to AI resistance we analyzed the metabolic profile of parental and AI resistant cells and their behavior when glycolysis or OXPHOS are impaired. Ultimately, motility and aggressiveness were evaluated in both cell types to correlate metabolic adaptation to cell plasticity.

Material and method: Long-term estrogen deprived MCF7 (MCF7-LTED) and MCF7 overexpressing aromatase (MCF7-2A) were used as *in vitro* models of AI resistance and sensitivity, respectively. Data mining of publicly available gene expression database was used to support the *in vitro* experiments.

Results : Androgen treated MCF7-2A cells were subject to different concentration of the AI, letrozole. The glycolysis inhibition and the cell viability inhibition induced by letrozole positively correlate, suggesting a role for letrozole in impairing glycolysis. Furthermore, metabolic comparison of LTED-MCF7 cells versus parental MCF7 cells showed an important increase in glycolysis dependence of the AI-resistant cells. When both cell lines were exposed to either the glycolysis inhibitor 2DG or to Metformin, inhibitor of mitochondrial respiratory chain complex 1, only the parental MCF7 cells showed a decrease in cell viability. MCF7-LTED cells were able to switch to either OXPHOS or glycolysis when 2DG or metformin were used, respectively: only when used in combination MCF7-LTED cell survival was impaired suggesting a high metabolic plasticity of MCF7-LTED. Importantly, MCF7-LTED cells are more motile than parental MCF7 and once MCF7-LTED cells were subjected to 2DG treatment, they further increase their motile ability. Finally, datasets analysis of patients that were treated with AI in a neo-adjuvant setting revealed an enhanced glycolytic phenotype in patients that did not respond to AI treatment.

Conclusion: Our data suggest that the metabolic plasticity is intimately linked to cancer aggressiveness. Thus, metabolic reprogramming could be a potential therapeutic target if used in combination with current therapeutic regimes.

F 7
METABOLIC CONTROL OF YAP AND TAZ BY THE MEVALONATE PATHWAY

Giovanni Sorrentino [1] - Naomi Ruggeri [1] - Valeria Specchia [2] - Michelangelo Cordenonsi [3] - Miguel Mano [4] - Sirio Dupont [3] - Andrea Manfrin [3] - Eleonora Ingallina [1] - Roberta Sommaggio [5] - Silvano Piazza [1] - Antonio Rosato [5] - Stefano Piccolo [3] - Giannino Del Sal [1]

Laboratorio Nazionale CIB, Università degli studi di Trieste, Trieste, Italy [1] - 3DiSTeBA, University of Salento, Lecce, Italy [2] - Department of Molecular Medicine, University of Padua School of Medicine, Padova, Italy [3] - International Centre for Genetic Engineering and Biotechnology, ICGEB, Trieste, Italy [4] - Dipartimento di Scienze Oncologiche e Chirurgiche, Università degli Studi di Padova, Padova, Italy [5]

The YAP and TAZ mediators of the Hippo pathway (hereafter called YAP/TAZ) promote tissue proliferation and organ growth. However, how their biological properties intersect with cellular metabolism remains unexplained. Here, we show that YAP/TAZ activity is controlled by the SREBP/mevalonate pathway. Inhibition of the rate-limiting enzyme of this pathway (HMG-CoA reductase) by statins opposes YAP/TAZ nuclear localization and transcriptional responses. Mechanistically, the geranylgeranyl pyrophosphate produced by the mevalonate cascade is required for activation of Rho GTPases that, in turn, activate YAP/TAZ by inhibiting their phosphorylation and promoting their nuclear accumulation. The mevalonate–YAP/TAZ axis is required for proliferation and self-renewal of breast cancer cells. In Drosophila melanogaster, inhibition of mevalonate biosynthesis and geranylgeranylation blunts the eye overgrowth induced by Yorkie, the YAP/TAZ orthologue. In tumour cells, YAP/TAZ activation is promoted by increased levels of mevalonic acid produced by SREBP transcriptional activity, which is induced by its oncogenic cofactor mutant p53. These findings reveal an additional layer of YAP/TAZ regulation by metabolic cues.

Control Of Gene Expression And Epigenetics

G 1

ZEB1 AND ABERRANT DNA METHYLATION COOPERATIVELY SUSTAIN MESENCHYMAL FEATURES AND STEM PHENOTYPE IN TRIPLE NEGATIVE BREAST CANCER

Giulia Brisotto [1] - Silvia Borgna [2] - Valentina Damiano [1] - Alessandra Di Gennaro [1] - Michela Armellin [1] - Roberta Maestro [1] - Manuela Santarosa [1]

CRO Aviano National Cancer Institute, Experimental Oncology, 1, Aviano (PN), Italy [1] - CRO Aviano National Cancer Institute, Experimental Oncology, 2, Aviano, (PN), Italy [2]

Introduction: During tumorigenesis, cancer cells may undergo a dedifferentiation program that leads to an increase in cell plasticity as a result of the re-expression of mesenchymal and stem-like features. The transcription factor ZEB1 is a key regulator of epithelial-to-mesenchymal transition (EMT) and of stem-like features and it is involved in a negative feedback loop with miR-200 family members, including miR-200c. The expression of miR-200c is repressed by DNA methylation and is inversely correlated with tumorigenicity and invasiveness in several human cancers. Then, we examined the role of epigenetic mechanisms in cooperation with ZEB1 in the maintenance of the EMT and stemness properties.

Materials and methods: To this aim, we investigated the mesenchymal and stem-like phenotypes in ZEB1-silenced MDAMB231 and MDAMB157 cell lines, whose profiles reflect the more aggressive breast cancers. Furthermore, we evaluated the expression level and the promoter methylation of the miR-200c in the cell lines silenced for ZEB1 and in a series of triple negative breast cancers.

Results: ZEB1 downregulation in breast cancer cells caused the reduction of stem population and increased the percentages of cells positive for the CD24 and EpCAM antigenes, typical markers of luminal breast cancer cells. The depletion of ZEB1 in the breast cancer cell lines partially reversed the mesenchymal-to-epithelial phenotype and restored the expression of E-cadherin, an important marker of the epithelial cells. In MDAMB231 the miR-200c promoter was partially methylated and CpG methylation decreased after ZEB1 silencing paralleling the re-expression of miR-200c. On the contrary, in the cell line MDAMB157, the CpG dinucleotides were fully methylated and the silencing of ZEB1 neither increase miR-200c expression level nor reduced the methylation status of its promoter. The treatment of both cell lines with a demethylating agent (5-aza-2'-deoxycytidine) increased the expression of E-cadherin and miR200c. Finally, we show an inverse correlation between the expression level of miR-200c and its promoter methylation in a series of 50 triple negative breast cancers.

Conclusion: Our findings indicate that both ZEB1 and aberrant DNA methylation are involved in the maintenance of the mesenchymal phenotypes in the breast cancers and that their cooperation suppress the miR-200c expression. Therefore, this epigenetic mechanism may represent a target therapy in breast cancer.

G 2

REGULATION OF IL-8 GENE EXPRESSION IN GLIOMAS BY MICRORNA

Enrica Fabbri [1] - Eleonora Brognara [1] - Giulia Montagner [1] - Claudio Ghimenton [2] - Albino Eccher [2] - Cinzia Cantù [2] - Susanna Khalil [2] - Valentino Bezzerri [2] - Nicoletta Bianchi [1] - Lisa Provezza [2] - Alessia Finotti [1] - Maria Cristina Dechecchi [2] - Monica Borgatti [1] - Marco Chilosi [2] - Giulio Cabrini [2] - Roberto Gambari [1]

Dept of Life Sciences and Biotechnology, University of Ferrara, Ferrara, Italy [1] - Dept of Pathology and Diagnostics, University Hospital of Verona, Verona, Italy [2]

Introduction: Interleukin-8 (IL-8, or CXCL8) is now known to be a major promoter of angiogenesis and invasiveness in human gliomas, where it is expressed and secreted at high levels. Among the different control levels of IL-8 gene expression in gliomagenesis, several have been studied and well characterized, such as hypoxia/anoxia stimulation, response to Fas ligation, death receptor activation, cytosolic Ca^{2+} transients, TNF-alpha, IL-1, and other cytokines and various cellular stresses. In addition, the expression of the IL-8 gene might be under the control of epigenetic mechanisms(s), such as those regulated by microRNAs. We found that bacterial challenge, which is known to strongly activate IL-8 gene transcription in epithelial cells, is downregulated by miR-93, which acts as a potent feedback mechanism (Fabbri, 2014). This is of peculiar interest for cancerogenesis since miR-93 has been found involved in the down-regulation of expression of VEGF which cooperates, together with IL-8, in glioma angiogenesis.

Materials and methods: Expression levels of IL-8 and VEGF genes and of miR-93, have been investigated in High Grade and Low Grade Gliomas (HGG and LGG) samples and in U251 human glioma cells. Pre-miR- and anti-miR-93 were transfected in U251 cells to check modulation of candidate target genes (IL-8 and other cytokines relevant to the glioma microenvironment).

Results: Both VEGF and IL-8 mRNAs were higly expressed in LGGs (20-200 folds) and HGGs (20-300 folds) in respect to reference RNA from healthy brains. VEGF and IL-8 mRNAs expression correlated directly, whereas MiR-93 correlated inversely with both target genes transcripts in glioma specimens. In silico analyses evidenced consensus sequences for the interaction of miR-93 in the 3'-UTR regions of IL-8 and VEGF genes. Transfection of U251 glioma cells with pre-miR-93 down-modulated both VEGF and IL-8 genes expression, whereas anti-miR-93 resulted in a consistent up-modulation. Analysis of a series of 27 candidate cytokines relevant to microenvironment released by U251 glioma cells upon transfection of pre- and anti-miR-93 suggest that intracellular modulation of miR-93 affects the expression not only of VEGF and IL-8 but also of MCP-1 and PDGF, which are also known to be involved in glioma angiogenesis.

Conclusions: Our results suggest that microRNAs, including miR-93, might be proposed as a relevant post-transcriptional regulators of angiogenesis in human gliomas.

G 3
HISTONE ACETYLTRANSFERASES INHIBITION BY CPTH2 IN 786-O CANCER CELLS ALTERS MRNAS EXPRESSION AND LEADS TO DECREASED GROWTH, MIGRATION AND INVASION ABILITY.

Elisa Cocco [1] - Claudia Canzonetta [2] - Stefania Scarpino [1] - Arianna Di Napoli [1] - Patrizia Filetici [3] - Antonella Stoppacciaro [1]

Department of Clinical and Molecular Medicine, ''Sapienza'' University, Sant'Andrea Hospital, Rome, Italy [1] - Institute Pasteur-Fondazione Cenci Bolognetti, Department of Biology and Biotechnologies C. Darwin, ''Sapienza'' University, Rome, Italy [2] - Institute of Molecular Biology and Pathology, CNR, "Sapienza" University, Rome, Italy [3]

Introduction: Renal cell carcinoma (RCC) is the ninth most common malignancy in Europe, >80 % are classified as clear cell RCC (ccRCC). The toxicity and substantial ineffectiveness of the current therapies, call for new therapeutic strategies. Histones deregulation, by histone acetyltransferases (HAT), has been linked to cancer. Inhibition of these enzymes could have a great potential in anticancer therapy. In this study, we evaluate the anti tumor effects of the HAT chemical inhibitor Cyclopentylidene-[4-(4-chlorophenyl)thiazol-2-yl)hydrazone (CPTH2) on the clear cell renal carcinoma cell line 786-O.

Materials and methods : 786-O cells were treated with CPTH2 (100μM) at increasing times. Cell viability was assessed by trypan blue dye exclusion; cell adhesion, migration and invasion,by scratch assays and boyden chamber assay. Acetylation levels and protein changes were evaluated by western blotting while gene expression changes by real time PCR. .

Results: CPTH2 diminished the acetylation level of histone H3 at the specific lysine 18, which is tightly involved in cell cycle regulation and proliferation. 90% H3-AcK18 decrease was obtained after 48h. Treatment of the 786-O cell line with CPTH2 also induced morphology changes, partial proliferation inhibition (38%, p=0,017), a significant reduction of matrigel adhesion (42%) and invasion (48%, p=0,02), and impaired cells migratory capacity (43%, p=0,000014). At the expression level, we found no changes in p300, AKT and HIF-1α mRNAs. TGF-β2 was instead significatively downregulated (37% ± 17% p=0,008), while CD44 (hyaluronic acid receptor) and Integrines α5, α6, β1, β3 mRNAs were upregulated (50%). Since CPTH2 was shown to preferentially inhibit p300 in vitro we interfered p300 by siRNA transfection. p300 silencing did not result in a significant decrease in cell proliferation or expression of TGF-β2, AKT and HIF-1α.

Conclusions: CPTH2 inhibits HAT activity determining a reduced level of histone H3K18 acetylation. CPTH2 inhibits cell migration and invasion while cell adhesion only diminishes on matrigel. CPTH2 treatment resulted in downregulation of TGF-β2 and upregulation of CD44 and Integrins (α5, α6, β1, β3) mRNA levels. Furthermore, in the 786-O cell line system silencing of p300 is not sufficient to induce the same level of functional variation induced by CPTH2 treatment, thus indicating HAT redundancy in 786-O.

G 4
MIRNAS INVOLVEMENT IN TRANSFORMING GROWTH FACTOR-BETA SIGNALING PATHWAY REGULATION IN FAMILIAL AND SPORADIC BREAST CANCER

Katia Danza [1] - Simona De Summa [1] - Rosamaria Pinto [1] - Brunella Pilato [1] - Rosanna Lacalamita [1] - Ondina Popescu [1] - Massimo Carella [2] - Orazio Palumbo [2] - Stefania Tommasi [1]

IRCCS Istituto Tumori "Giovanni Paolo II – Bari, Hospital, Bari, Italy [1] - IRCCS Casa Sollievo della Sofferenza, hospital, San Giovanni Rotondo, Italy [2]

Introduction: Transforming growth factor-beta (TGF-β) plays a bidirectional role during cancer progression through both tumor-suppressive and tumor-promoting functions. The involvement of BRCA1 in TGF-β signaling during oxidative stress responses has been demonstrated even if few data are available on TGF-β-epigenetic regulation in familial and sporadic breast tumors. Since a relevant impact of TGF-β deregulation was reported in breast cancer progression, our aim was to investigate whether a different miRNA-mediated TGF-β signaling occurs in familial and sporadic breast cancer.

Materials and methods: MiRNA expression profiling was performed on 43 familial (22 BRCA-related and 21 BRCAX) and 28 sporadic breast cancer samples through Affymetrix GeneChip miRNA Arrays. meV software for statistical analysis was used. DIANA miRPath v2.0 web-based computational tool and miRWalk database were utilized for biological interpretation of miRNA profiling data and for the identification of miRNAs target genes, respectively. Subsequently, real time PCR was performed on an independent set of 19 familial (8 BRCA-related breast cancers and 11 BRCAX tumor) and 15 sporadic breast tumors for the validation analysis.

Results: Breast cancer samples with a family history showed 28 deregulated miRNAs compared to sporadic breast tumors. Of these, 19 miRNAs target 36 genes involved in TGF-β signaling pathway. Our validation analysis, in which 4 out of 19 deregulated miRNAs were selected, highlighted miR-1184 and miR-943 trend expression similar to what observed in microarray analysis. Furthermore, in order to confirm the epigenetic-mediated TGF-β signaling pathway regulation, we explored the expression of mir-92a-1* that has TGF-β1 as validated target gene. Interestingly, as well as observed in the training set, lower expression of mir-92a-1* was reported in sporadic compared to familial breast tumors. On the contrary, higher levels of TGF-β1 were observed in breast cancer without a family history.

Conclusions: Our results highlighted epigenetic-mediated TGF-β signaling regulation in breast tumor. Because of the TGFβ dual functions in tumor, particular attention has to be paid to mir-92a-1* role in deregulating TGFβ signaling with respect to breast cancer family history.

G 5
PROMOTER METHYLATION OF TUMOUR SUPPRESSOR GENES IN PRE-NEOPLASTIC LESIONS: POTENTIAL MARKER OF DISEASE RECURRENCE

Giulia De Maio [1] - Claudia Rengucci [1] - Andrea Casadei Gardini [1] - Mattia Zucca [1] - Emanuela Scarpi [1] - Chiara Zingaretti [2] - Giovanni Foschi [1] - Maria Maddalena Tumedei [1] - Chiara Molinari [1] - Luca Saragoni [3] - Maurizio Puccetti [4] - Dino Amadori [1] - Wainer Zoli [1] - Daniele Calistri [1]

Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS), Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) Srl, Meldola (FC), Italy [1] - Foundation, INGM National Institute of Molecular Genetics, Milan, Italy [2] - Pathology Unit, Morgagni-Pierantoni Hospital, Hospital, Forlì (FC), Italy [3] - Pathology Unit, Santa Maria delle Croci Hospital, Hospital, Ravenna, Italy [4]

Abstract

Introduction: Specific epigenetic alterations of tumour suppressor genes have been associated with colorectal cancer (CRC) transformation and carcinogenesis in early stages of the disease. However, very little information is available concerning the role of methylation profile of specific tumour genes in CRC development. Moreover, it is not known whether the methylation profile of pre-neoplastic lesions can be used as prognostic biomarker for colorectal lesions recurrence. In the present study, we analyze retrospectively whether specific epigenetic alterations detected in pre-neoplastic colorectal lesions can predict disease recurrence.

Materials and methods : Pre-neoplastic colorectal lesions were endoscopically identified and surgically removed from 78 patients undergoing follow up monitoring over 5 years. Formalin fixed paraffin-embedded adenoma samples were collected and classified as low or high-risk of recurrence according to the National Comprehensive Cancer Network guidelines. Methylation profile was assessed by methylation-specific multiplex ligation probe assay (MS-MLPA). 24 different tumour suppressor genes promoters were analyzed simultaneously.

Results: High levels of methylation of several genes were found to be associated with disease recurrence. Specifically, *MLH1*, *ATM* and *FHIT* promoters were found to be significantly hypermethylated in recurring adenomas. Moreover, *TP73* and *BRCA1* showed a trend of hypermethylation with borderline significance, albeit no statistically different. CpG island methylation phenotype analysis of *MLH1*, *ATM* and *FHIT* confirmed that they could be used to accurately identify patients at a higher risk of recurrence. It is worth noticing that all these five genes were not among those most frequently methylated in our case series, suggesting that the risk of recurrence is related to specific molecular characteristics.

Conclusions: Our data suggest that a classification based on the epigenetic profile of specific genes could be successfully used to predict adenoma recurrence in patients with adenoma. Future studies in larger cohort of patients will be carried out in order to confirm these preliminary data.

G 6
LINE1 HYPOMETHYLATION ASSOCIATES WITH POOR PROGNOSIS IN HEAD AND NECK SQUAMOUS CELL CARCINOMA PATIENTS

Elisabetta Fratta [1] - Luigi Barzan [2] - Carlo Furlan [3] - Giovanni Franchin [3] - Renato Talamini [4] - Francesca Colizzi [1] - Emanuela Vaccher [5] - Sandro Sulfaro [6] - Riccardo Dolcetti [1] - Luca Sigalotti [1]

Cancer Bioimmunotherapy Unit, Centro di Riferimento Oncologico, Aviano, Italia [1] - Division of Otolaryngology, General Hospital "S. Maria degli Angeli", Pordenone, Italia [2] - Division of Radiotherapy, Centro di Riferimento Oncologico, Aviano, Italia [3] - Unit of Epidemiology and Biostatistics, Centro di Riferimento Oncologico, Aviano, Italia [4] - Division of Medical Oncology A, Centro di Riferimento Oncologico, Aviano, Italia [5] - Division of Pathology, General Hospital "S. Maria degli Angeli", Pordenone, Italia [6]

Introduction. Head and neck cancer is the sixth most common type of cancer worldwide, with approximately 600.000 new cases and 350.000 deaths every year. From a histopathological perspective, more than 90% of head and neck cancer is squamous cell carcinoma (HNSCC), which is a very aggressive neoplasm originating from the epithelial cells lining of upper aerodigestive tract. At present, no established molecular marker is available to predict the clinical course of the disease in HNSCC patients. In this setting, molecular prognostic markers in HNSCC are urgently required for improving patient management. Hypomethylation of the *Long Interspersed Nucleotide Element 1* (*LINE1*) repetitive elements, that is widely accepted as a surrogate of overall genomic DNA methylation content, was found to be associated with a poor prognosis in several cancers. At present, no studies have investigated the influence of *LINE1* methylation levels on HNSCC prognosis.

Materials and methods. The prognostic relevance of the methylation of *LINE1* repetitive sequences was evaluated by real-time Quantitative Methylation-Specific PCR (qMSP) in FFPE tissues obtained from 57 HNSCC patients. The impact of *LINE1* methylation on overall survival (OS) was assessed using Cox regression and Kaplan-Meier analysis.

Results. *LINE1* hypomethylation associated with shorter OS by Cox regression (Hazard Ratio = 3.52; 95% Confidence Interval: 1.7-7.3; P = 0.001). Median OS for hypomethylated patients was 19.7 months and was not reached in hypermethylated ones. The 5 year OS for patients with hypomethylated vs. hypermethylated *LINE1* were 14% and 63%, respectively. Multivariate analyses confirmed *LINE1* methylation as an independent prognostic marker in HNSCC.

Conclusions. The reported association of *LINE-1* hypomethylation with shorter survival in HNSCC identifies it as a potential prognostic biomarker worth further validation in a prospective study.

G 7
KEAP1/NRF2 AXIS MOLECULAR PROFILE IN SMALL CELL LUNG CANCER CELL LINES

Annamaria La Torre [1] - Angelo Sparaneo [1] - Orazio Palumbo [1] - Clelia Tiziana Storlazzi [2] - Teresa Balsamo [1] - Domenico Trombetta [1] - Fabio Pellegrini [1] - Raffaela Barbano [1] - Barbara Pasculli [1] - Paolo Graziano [1]

- Montse Sanchez-cespedes [3] - Maria Teresa Landi [4] - Paola Parrella [1] - Vito Michele Fazio [1] - Lucia Anna Muscarella [1]

Casa Sollievo della Sofferenza, IRCCS, San Giovanni Rotondo (FG), Italy [1] - Department of Biology, University of Bari, Bari, Italy [2] - Bellvitge Biomedical Research Insititute-IDIBELL, Research Centre, Barcelona, Spain [3] - National Cancer Institute, National Institute of Health, Bethesda (MD), United States [4]

Introduction. The Keap1/Nrf2 pathway is a master regulator of antioxidants and cellular stress responses implicated in resistance of tumour cells against chemotherapeutic drugs. The link between molecular alterations of this pathway in Non Small Cell Lung Cancer (NSCLC) is well studied and appears to depend on several main factors including the existence of activating mutations in *NFE2LE* gene and/or loss of function mutations and methylation in the *KEAP1* gene. At present, the data concerning the mechanism of alteration of Nrf2-Keap1 pathway in Small Cell Lung Cancer (SCLC) instead are almost incomplete and correlation analysis with therapeutic strategies targeting the molecular dysfunction of this pathway is ongoing. Here we present a comprehensive molecular alteration profile of the main partners of the Nrf2/Keap1 axis in SCLC cell lines.

Materials and methods. A total of 12 SCLC cell lines were scanned to produce a molecular profile by integrating data from SNP-Array analysis, immunofluorescence, mutation screening by direct sequencing, methylation by QMSP and expression analyses by RT-qPCR and western blotting.

Results. Our analyses confirm the global deregulation of Keap1/Nrf2 pathway in SCLC cell lines, showing an hypermethylation of the CpGs located into the P1 promoter region of the *KEAP1* in 42% (5/12) of the cell lines, and a chromosomal amplification involving the NFE2LE gene locus (2q31.1) in two cell lines. Only one just described point mutation in the kelch-repeat 2 was observed in one of the cell line analyzed.

Conclusions. Our gene-alteration profile of SCLC cell lines provides new insights into the mechanism of deregulation of Nrf2-Keap1 detoxification pathway in this group of high grade neuroendocrine lung cancers, suggesting an alternative genetic and epigenetic deregulation in this molecular axis for SCLCs. Analyses on tumour tissues are ongoing to confirm these observations. Moreover, the provided full molecular data from cell lines will be useful for in vitro functional studies aimed to establish new combined therapeutic strategies in targeted cancer treatments of this aggressive lung tumour histotype.

G 8
MGMT PROMOTER METHYLATION AND GLIOBLASTOMA: A COMPARISON OF ANALYTICAL METHODS AND OF TUMOUR SPECIMENS

Laura Lattanzio [1] - Marzia Borgognone [1] - Cristina Mocellini [1] - Fabrizio Giordano [1] - Ermanno Favata [1] - Gaetano Fasano [1] - Martino Monteverde [1] - Daniela Vivenza [1] - Federica Tonissi [1] - Annalisa Ghiglia [1] - Claudio Bernucci [1] - Marco Merlanp [1] - Cristiana Lo Nigro [1]

A.O.Santa Croce e Carle, Ospedale UNiversitario Santa Croce e Carle, Cuneo, Italia [1]

Introduction Hypermethylation of O^6-methylguanine DNA methyltransferase (MGMT) gene promoter is a predictive biomarker of response to temozolomide (TMZ) treatment and of favourable outcome in terms of overall survival (OS) and progression-free survival (PFS) in glioblastoma (GBM) patients. Nevertheless, MGMT methylation status hasn't been currently introduced in routinely clinical practice as the choice of the ideal technique and tissue sample specimen is still controversial. The aim of this study is to compare two analytical methods, methylation-specific polymerase chain reaction (MSP) and pyrosequencing (PSQ), and their use on two different tissue type samples, snap-frozen and formalin-fixed paraffin-embedded (FFPE) tissues.

Material and Methods Frozen and FFPE tissue samples were collected from 46 GBM patients treated with TMZ-containing chemo-radiotherapy protocols. Genomic DNA was extracted using standard protocols and subjected to bisulfite conversion using commercial kits. Modified DNA was amplified by MSP through nested PCR with a first external set of primers and a second PCR with specific primers for methylated/unmethylated DNA defined by Esteller et al (1999). The results were qualitatively interpreted on agarose gel. For PSQ analysis, we used a set of primers covering the same region covered by MSP primers and products were pyrosequenced using PyroMark ID System (Biotage). The average of the % methylation of 9 CpG sites was used to compare PSQ and MSP results. Kaplan-Meier curves were used to estimate patients' survival.

Results We obtained methylation data from all frozen tissues (of which 56.5% methylation-positive), while no results were obtained by 5 FFPE samples. Comparing both techniques and both tissue specimens 28% of samples showed discordant results (13/46). The highest concordance in methylation was found on frozen tissues (88.5%, 23/26 samples) and using PSQ (76.7%, 23/30 samples). Moreover, we confirmed that OS and PFS for patients carrying methylation on MGMT promoter were longer than for patients with unmethylated promoter.

Conclusions We considered MSP a limited technique on FFPE tissues; on the contrary our data indicated PSQ as the most suitable method to stratify methylated/unmethylated patients as it allows reaching quantitative results with high sensitivity and specificity. Furthermore, frozen tissues resulted the best specimen to be used due to the low DNA degradation and homogeneity in methylation throughout the tumour.

G 9
MIR-221 AND SLUG INTERPLAY IN HUMAN MESENCHYMAL STEM CELLS AND BREAST CANCER CELLS.

Marco Angelozzi [1] - Andrea Lolli [1] - Elisabetta Lambertini [1] - Federica Vezzali [1] - Martina Di Ciano [1] - Letizia Penolazzi [1] - Roberta Piva [1]

Dept. of Biomedical and Specialty Surgical Sciences, University of Ferrara, Ferrara, Italy [1]

Introduction: Regulatory interplay between specific transcription factors (TFs) and microRNAs governs the epithelial to mesenchymal transition (EMT), a crucial step in embryonic development, tissue repair, and cancer metastasis. This phenomenon involves epithelial cells that, in response to various signals, undergo morphological changes and cell-cell adhesion loss. EMT study has led to define a core regulatory system, driven by E-box binding TFs, SLUG and ZEB, and two families of microRNAs, miR-34 and miR-200. This regulatory unit, acting as "a motor of cellular plasticity", is coupled to other key cellular features such as stemness, cell-cycle arrest, apoptosis, senescence and chemotherapy resistance. In all these situations SLUG and ZEB variable levels drive cell behavior and affect cell-fate decision in a way only in part known. Better understanding of input signals that modulate expression of these TFs can help to address questions linked to cancer progression as well as normal programs of embryonic development and tissue regeneration. With this in mind we investigated the role of SLUG in determining the behavior of human mesenchymal stem cells (hMSCs) guided towards a specific phenotype with an approach based on silencing of SLUG and miR-221, both negative chondroregulators, in absence of TGFb differentiating agent. The relationship between SLUG and miR-221 under this new perspective was compared with that found in MDA-MB-231 breast cancer cells (BCs).

Materials and methods: BCs and hMSCs were treated with siRNA against Slug or antagomiR-221, without adding TGFb. We evaluated at RNA and protein level the expression of genes involved in supporting breast cancer phenotype and chondrogenesis.

Results: After TGFb exposure, hMSCs keep appreciable levels of SLUG which is however contrary to chondrogenesis. Blocking SLUG or miR-221 in hMSCs without TGFb is able to induce chondrogenic differentiation; like in BCs, siSlug strongly decreased miR-221, but SLUG expression was not affected by miR-221 content. Rescue experiment in BCs with pre-miR-221 didn't balance siSlug effects, suggesting that SLUG silencing protects cells from metastatic stimuli as miR-221 overexpression.

Conclusions: We demonstrated a functional link between SLUG and miR-221 both in BCs and in hMSCs; this is relevant to improve the knowledge on EMT. In addition, TGFb controversial outcomes in hMSCs may be relevant to set up new tools in the field of regenerative medicine and stem cancer cells.

G 10
GENE-SPECIFIC METHYLATION PROFILES IN HORMONALLY TREATED AND UNTREATED PROSTATE CANCER CASES

Piera Rizzolo [1] - Veronica Zelli [1] - Valentina Silvestri [1] - Anna Sara Navazio [1] - Virginia Valentini [1] - Mario Falchetti [1] - Raffaella Santi [2] - Gabriella Nesi [2] - Laura Ottini [1]

Department of Molecular Medicine, Sapienza University of Rome, Rome, Italy [1] - Division of Pathological Anatomy, University of Florence, Florence, Italy [2]

Introduction: DNA promoter hypermethylation is a frequent epigenetic event in prostate cancer (PCa) and many genes have been found as aberrantly methylated in PCa. Radical prostatectomy (RP) represents an effective option for PCa treatment. In selected cases, surgery may follow neoadjuvant androgen deprivation (AD) therapy. Castration-resistant PCa remains hormonally driven despite castrate levels of circulating androgens and portends a dismal outcome. The molecular mechanisms involved in castration-resistant PCa progression are still poorly understood. The aim of this study was to characterize DNA methylation profiles of PCa in a series of surgically resected samples, in order to identify possible differences related to AD therapy.

Materials and Methods: Using the candidate-gene approach, we performed promoter methylation analysis of a panel of genes involved in hormonal (*AR*, *ESR1*, *ESR2*) and tumor progression pathways (*RASSF1*, *APC*, *CD44*, *CDH1*, *BCL2*). A series of 48 PCa cases were retrospectively collected, 25 patients had surgery alone (untreated) while 23 received AD for 3 months before surgery (treated). Clinicopathologic data, including age, histology, Gleason score, stage and margin status, were recorded. Biomolecular analysis was performed by pyrosequencing and methylation levels were assessed by calculating the average of methylation for each gene. To determine microvessel density (MVD), specimens were immunostained for CD31 and LYVE-1. Kruskal-Wallis test was used for statistical purposes.

Results: Aberrant promoter methylation of the 8 genes analyzed was found in all the 48 PCa cases. Significant differences in methylation levels between treated and untreated tumors emerged for *CD44* ($p=0.015$). A significant correlation between *CDH1* methylation and positive surgical margins ($p=0.03$) was also noted. Regarding MVD, methylation of both *BCL2* and *CD44* was associated with LYVE-1 overexpression ($p=0.05$ and $p=0.01$, respectively), while *RASSF1* methylation was associated with CD31 overexpression ($p=0.03$).

Conclusion: Overall, our results showed that the methylation profiles of the genes investigated do not significantly vary in relation to hormonal therapy. Contrariwise, we observed that high methylation levels in genes involved in tumor progression were significantly correlated with tumor characteristics suggestive of a more aggressive phenotype.

G 11
IDENTIFICATION OF OPTIMAL REFERENCE GENES FOR GENE EXPRESSION NORMALIZATION IN A WIDE COHORT OF ENDOMETRIOID ENDOMETRIAL CARCINOMA TISSUES

Chiara Romani [1] - Paola Todeschini [1] - Stefano Calza [2] - Renata Tassi [1] - Laura Zanotti [1] - Elisabetta Bandiera [1] - Enrico Sartori [1] - Sergio Pecorelli [1] - Antonella Ravaggi [1] - Alessandro Santin [3] - Eliana Bignotti [1]

"Angelo Nocivelli" Institut of Molecular Medicine, Division of Gynecologic Oncology, University of Brescia, Brescia, Italy [1] - Department of Molecular and Translational Medicine, University of Brescia, Brescia, Italy [2] - Department of Obstetric, Gynecology and Reproductive Sciences, Yale University School of Medicine, New Haven, CT, United States [3]

Accurate normalization is a primary component of a reliable gene expression analysis based on qRT-PCR technique. While the use of one or more reference genes as internal controls is commonly accepted as the most appropriate normalization strategy, many qPCR-based published studies still contain data poorly normalized and reference genes arbitrarily chosen irrespective of the particular tissue and the specific experimental design. To date, no validated reference genes have been identified for endometrial cancer tissues. In this study, 10 normalization genes (GAPDH, B2M, ACTB, POLR2A, UBC, PPIA, HPRT1, GUSB, TBP, H3F3A) belonging to different functional and abundance classes in various tissues and used in different studies, were analyzed to determine their applicability. In total, 100 endometrioid endometrial cancer samples, which were carefully balanced according to their tumor grade, and 29 normal endometrial tissues were examined using SYBR Green Real-Time RT-PCR. The expression stability of candidate reference genes was determined and compared by means of geNorm and NormFinder softwares. Both algorithms were in agreement in identifying GAPDH, H3F3A, PPIA, and HPRT1 as the most stably expressed genes, only differing in their ranking order. Statistical analysis performed on the expression levels of all candidate genes confirm HPRT1 and PPIA as the most stably expressed in the study groups regardless of sample type. As the stable expression of HPRT1 and PPIA between normal and tumor endometrial samples fulfill the basic requirement of a reference gene to be used for normalization purposes, HPRT1 expression demonstrated significant differences between samples from low-grade and high-grade tumors. In conclusion, our results recommend the use of PPIA as a single reference gene to be considered for improved reliability of normalization in gene expression studies involving endometrioid endometrial tumor samples at different tumor degrees.

G 12
GENETIC AND PHARMACOLOGIC INHIBITION OF THE ENHANCER OF ZESTE HOMOLOG 2 (EZH2) HAMPERS RHABDOMYOSARCOMA TUMORIGENESIS

Laura Adesso [1] - Elena Carcarino [2] - Maria De Salvo [1] - Giorgia Bracaglia [1] - Pier Paolo Leoncini [1] - Sergio Valente [3] - Victor E. Marquez [4] - Gianni Bisogno [5] - Andrea Ferrari [6] - Pier Lorenzo Puri [2] - Antonello Mai [3] - Franco Locatelli [1] - Daniela Palacios [2] - Rossella Rota [1]

Dept. of Oncohematology, Ospedale Pediatrico Bambino Gesù, IRCCS, Research Institute, Roma, Italy [1] - Dulbecco Telethon Institute at EBRI/Santa Lucia, Fondazione Santa Lucia, Roma, Italy [2] - Istituto Pasteur - Fondazione Cenci Bolognetti, Dipartimento di Chimica e Tecnologie del Farmaco, Sapienza Università di Roma, Roma, Italy [3] - Chemical Biology Laboratory, Frederick National Laboratory for Cancer Research, CCR, National Cancer Institute, NIH, Frederick, MD, United States [4] - Oncohematology Unit, Department of Pediatrics, University of Padova, Padova, Italy [5] - Pediatric Oncology Unit, Fondazione IRCCS Istituto Nazionale Tumori, Milano, Italy [6]

Introduction: The Polycomb group (PcG) protein Enhancer of Zeste Homolog 2 (EZH2) is the catalytic subunit of the Polycomb Repressive Complex 2 (PRC2). It trimethylates lysine 27 on histone H3 (H3K27me3) repressing the transcription of differentiation genes. EZH2 prevents the anticipated differentiation of skeletal muscle progenitors and is down-regulated during the course of differentiation by promyogenic microRNAs. In rhabdomyosarcoma (RMS), a pediatric soft-tissue sarcoma of myogenic origin, EZH2 is abnormally over-expressed. Therefore, we investigated the impact of EZH2 inhibition in the RMS preclinical setting.

Material and methods: We used oligo siRNAs and shRNAs delivery and looked at the differentiation and proliferation responses. We verified the displacement of EZH2 from the promoters/enhancers of target genes by Chromatin immunoprecipitation (ChIP) and the concomitant reduction of H3K27me3. We also evaluated the modulation of selected microRNAs. Finally, we determined the effect of pharmacologic inhibition of EZH2 with DZNep, which induces protein degradation, and a catalytic inhibitor MC1945 *in vitro* and *in vivo*.

Results: EZH2 genetic silencing in RMS cell lines of the embryonal subtype (eRMS) inhibited cell proliferation and was associated with the up-regulation of $p21^{Cip1}$, Myogenin and Muscle Creatin Kinase (MCK). eRMS cells depleted of EZH2 formed multinucleated structures *in vitro* reminiscent of myofibers. ChIP assay on eRMS cell lines showed the displacement of EZH2 from the regulatory regions of these genes. Interestingly, conversely to what happened in eRMS cells, EZH2 silencing in the PAX3-FOXO1-positive alveolar RMS cell lines (aRMS) reduced Myogenin, MyoD and BCL2 expression and up-regulated FBXO32 resulting in tumor cell apoptosis. Simultaneous silencing or over-expression of FBXO32 confirmed its major role in the apoptotic response. Loss of EZH2 and H3K27me3 enrichment was detected on FBXO32 promoter by ChIP assay. A set of selected microRNAs previously shown to be directly or indirectly related to EZH2 were up-regulated in both RMS subtypes, such as miR-29b/c, miR-206, miR-101 and miR-214. *In vivo* treatments with the two inhibitors, DZNep and MC1945, mirrored the effects of EZH2 silencing inhibiting tumor xenograft growth.

Conclusions: Results from our studies suggest EZH2 inhibition as a promising adjuvant therapeutic approach against both RMS subtypes.

Dna Damage And Molecular Responses To Damage

H 1
THE G-QUADRUPLEX LIGAND EMICORON HAS ANTITUMORAL ACTIVITY AGAINST ORTHOTOPIC AND PATIENT-DERIVED HUMAN COLON CANCER XENOGRAFTS

Simona Artuso [1] - Manuela Porru [1] - Armandodoriano Bianco [2] - Marco Franceschin [3] - Maria Grazia Diodoro [1] - Daniela Passeri [4] - Augusto Orlandi [4] - Annamaria Biroccio [1] - Carlo Leonetti [1]

Regina Elena National Cancer Institute, Hospital, Rome, Italy [1] - University Sapienza, university, rome, Israel [2] - University Sapienza, rome, rome, Italy [3] - Tor Vergata university, university, rome, Italy [4]

Introduction: The evidence for the formation of G-quadruplex (G4) structures in the genome of mammalian cells strenghtened the importance to develop small molecules to target G4 for pharmacological interventation. We recently designed a molecule, namely EMICORON, having one piperidinyl group bound to the perylene bay area, sufficient to guarantee a good selectivity, and an extended aromatic core able to increase the stacking interactions with the ending tetrad of the G4. This compound rapidly triggered a strong DNA damage at telomeres and this effect was associated with the delocalization of telomeric protein POT1. Notably, EMICORON was highly active in inhibiting transformed and tumor cell proliferation, while normal fibroblasts expressing telomerase were unaffected by the treatment.

Based on these results, our aim was to evaluate the antitumoral effect of this novel G4 ligand on experimental models of human colon cancer for translational purpose.

Materials and methods : Experiments were performed to identify the maximum tolerated dose (MTD) and to analyze the toxicological profile of the compound given orally in immunosuppressed mice. Then the efficacy of EMICORON was studied on human colon xenografts established as orthotopic rectal cancer or in a disseminated neoplasia by intrasplenically injection of bioluminescent HT29 tumor cells. Moreover, experiments were done on patient-derived xenografts (PDXs) by s.c. injection of fresh tumor tissue from patients with pathologically confirmed colon cancer. Finally, immunohistochemical analysis was performed on tumor tissue to identify biological determinants of EMICORON activity.

Results: EMICORON given at the MTD was well tolerable in mice as no body weight loss or toxic deaths were observed. Moreover, major organs including bone tissue did not display microscopic difference compared to the mice treated with the vehicle. EMICORON reduced the colonization of HT29 cells at lymphnodes, intestine, stomach and especially liver. Interestingly, EMICORON was markedly active in inhibiting of more than 50% the growth of PDXs derived from a patient unresponsive to chemotherapy. Finally, we showed that inhibition of angiogenesis and proliferation were key determinant of EMICORON antitumoral activity.

Conclusions: Our results identify EMICORON as a new G4 with a promising antitumor activity on relevant experimental models of human colon cancer and warrant for further studies of EMICORON-based combination treatments.

H 2
UNCAPPED TELOMERES AS DETERMINANT FOR CELL SENSITIVITY TO G-QUADRUPLEX LIGANDS.

Chiara Cingolani [1] - Erica Salvati [1] - Angela Rizzo [1] - Sara Iachettini [1] - Pasquale Zizza [1] - Carmen D'angelo [1] - Manuela Porru [2] - Simona Artuso [1] - Carlo Leonetti [1] - Annamaria Biroccio [1]

Regina Elena National Cancer Institute, IFO- Istituti Fisioterapici Ospitalieri, Rome, Italy [1] - Regina Elena National Cancer Institute, IFO- Istituti Fisioterapici Ospitalieri, Rome, - [2]

Over the past decade, many chemical classes of G-quadruplex (G4) ligands have been described for their ability to target and damage telomeres, leading to detrimental effects on tumor cells.

The apparent selectivity of G4 ligands towards transformed cells, led us to ascertain the presence of differences in telomere structures and functions in normal vs transformed cells in a cell model system of genetically controlled transformation. Among the different comparative evaluations, the analysis of the telomere status, in interphasic and metaphasic nuclei, revealed a progressive increase in the number of telomere dysfunction (identified as TIFs) from normal to transformed cells. The majority of TIFs in interphasic nuclei exhibited the presence of gH2AX but not of 53BP1 foci, indicating that these telomere damages were not-processed by the DNA damage repair pathways. In agreement with this, gH2AX foci were detectable also in metaphase telomeres (meta-TIFs) in transformed cells and the presence of a basal telomere dysfunction correlated with the drug-induced telomere damage and cell death. The functional relevance of this observation was directly assessed by showing that fibroblasts with artificially uncapped telomeres, obtained by depleting components of the telomeric complex, became sensitive to G4 stabilization. On the other hand, telomeres of normal cells were not completely resistant to G4 stabilization, resulting damaged upon exposure to higher dosage of G4 ligands as well, and leading to cell death. Anyway the different degree of sensitivity offers a therapeutic window for a new class of molecules with anti-tumor features.

H 3
BRCANESS: A PHENOTYPE WHICH CROSSES BRCA-RELATED AND SPORADIC TRIPLE NEGATIVE BREAST TUMORS

Simona De Summa [1] - Rosamaria Pinto [1] - Katia Danza [1] - Brunella Pilato [1] - Daniela Petriella [1] - Giovanni Simone [1] - Massimo Carella [2] - Orazio Palumbo [2] - Stefania Tommasi [1]

IRCCS Istituto Tumori "Giovanni Paolo II, hospital, Bari, Italy [1] - IRCCS Casa Sollievo della Sofferenza – San Giovanni Rotondo (FG), hospital, San Giovanni Rotondo, Italy [2]

Introduction: Triple negative breast cancer (TNBC) is an heterogeneous disease. Recently, it has been demonstrated that TNBCs included different six subgroups based on gene expression profiling, characterized by peculiar ontologies. "Driver" pathways for each subgroups are different and, in particular, only basal-like tumors showed higher expression of genes involved in cell proliferation and response to DNA damage. Studies on basal-like TN cell lines regarding sensitivity to PARP inhibition demonstrated that BRCA1-mutated cell lines were more sensitive than BRCA2-mutated ones. In the recent years many studies are focusing on the identification of the so-called BRCAness phenotype, indicating with this term sporadic tumors displaying a phenotype similar to BRCA-related breast tumors. For this reason, we performed a miRNA expression profiling aiming to find epigenetic alterations, which could be able to discriminate BRCAness phenotype.

Materials and methods: 26 sporadic breast tumors were analyzed through Multiplex Ligation-dependent Probe Amplification (MLPA) assay, which contains 38 probes for BRCA1/2 genes and for chromosomal regions that are suggested to be clinically relevant in prediction for BRCA1-association. We found out 5 BRCAness cases (2 TNBC and 3 NTNBC), which underwent miRNA expression profiling through Affymetrix GeneChip miRNA Arrays together with 5 BRCA1-mutated and 6 sporadic TNBCs. meVsoftware was used to perform SAM and hierarchical clustering. Pathways enrichment analysis was performed considering validated targets for each miRNAs, obtained from Tarbase, miRtarbase and miRecords. The resulting gene lists was submitted to DAVID 6.7 tool in order to identify the targeted pathways.

Results: Comparison of BRCA1-mutated TN and BRCAness cases with sporadic TNBCs highlighted 10 deregulated miRNAs (FDR=0). Hierarchical clustering separated triple positive BRCAness cases from triple negative ones, the latter grouping with sporadic TNBCs. Moreover, BRCA1-related TNBCs are also separated in the same two clusters. Pathways enrichment analysis underlined deregulation of DNA recombination, cell proliferation, cell matrix adhesion and MAPKK cascade.

Conclusions: In conclusion, these preliminary results seemed to indicate that BRCAness phenotype could be stratified accordingly to immunophenotype and miRNA disregulation. Moreover, the separation of BRCA1-related TNBCs highlight that their molecular background is not homogeneous and such a result could explain the different sensitiveness to PARP inhibition and could improve target therapies.

H 4
THE ROLE OF DNA REPAIR IN PLATINUM RESPONSE IN RECENTLY ESTABLISHED PATIENT-DERIVED OVARIAN CARCINOMA XENOGRAFTS.

Federica Guffanti [1] - Francesca Ricci [1] - Patrizia Perego [2] - Robert Fruscio [2] - Sonia Magni [2] - Tommaso Grassi [2] - Massimo Broggini [1] - Giovanna Damia [1]

Istituto di Ricerche Farmacologiche Mario Negri - IRCCS, Istituto di Ricerche Farmacologiche Mario Negri - IRCCS, Milano, Italy [1] - Azienda ospedaliera San Gerardo, Azienda ospedaliera San Gerardo, Monza, Italy [2]

Introduction: Epithelial ovarian cancer (EOC) is the most lethal gynecological malignancy and is an heterogeneous disease. Front line therapy consists in cytoreductive surgery and platinum based chemotherapy. Despite more than 70% of patients are initially platinum-sensitive, most of them will relapse and die for chemo-resistant disease. One of the mechanisms associated to platinum resistance involves the cellular DNA repair capacity. This study was designed to elucidate the role of DNA repair in the resistance to cisplatin (DDP) in a panel of patient-derived ovarian carcinoma xenografts.

Materials and methods : Six xenografts were recently established in our laboratory directly from patient tumor samples. For chemotherapeutic trials, tumor fragments were subcutaneously implanted in nude mice and when tumor masses reached 200 mg, mice were randomized to receive or not DDP once a week for 3 weeks at the dose of 5mg/kg. A second DDP cycle was given to the re-growing tumors. The expression of DNA repair genes involved in Nucleotide Excision Repair (NER), Homologous Recombination (HR) and Fanconi Anemia (FA), were investigated by Real Time PCR in untreated and DDP-treated xenografts.

Results: Xenografts were classified as "Responder" (R) or "Non-Responder" (NR) on the basis of their response to DDP by standard criteria (tumor growth inhibition and T/C*100 - mean treated tumor weight/mean control untreated tumor weight *100). All of them were less sensitive to the second cycle of DDP, mimicking what happens in a clinical setting. The mRNA expression of DNA repair genes studied was similar in "R" and "NR" xenografts and seemed not to be modulated by the *in vivo* DDP treatment.

Conclusions: These data are preliminary and need to be corroborate in other xenografts. Tests able to mirror the *ex vivo* functional DNA repair capacity of the xenografts are under study in our laboratory and might better correlate with DDP tumor responsiveness.

H 5
RECIPROCAL REGULATION OF DBC1 AND CHK2 FUNCTIONS IN THE DNA DAMAGE RESPONSE

Martina Magni [1] - Vincenzo Ruscica [1] - Giacomo Buscemi [2] - Simona Aliprandi [2] - Enrico Fontanella [1] - Domenico Delia [1] - Laura Zannini [1]

Department of Experimental Oncology and Molecular Medicine, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy [1] - Department of Biosciences, University of Milan, Milan, Italy [2]

Introduction: human DBC1 (Deleted in Breast Cancer 1; KIAA1967; CCAR2) is a nuclear protein with controversial effects on cancer cells and with important roles in the regulation of apoptosis, transcription and histones modifications. Previously we reported that, in response to DNA damage, ATM and ATR phosphorylate DBC1 on T454, promoting the inhibition of the NAD^+ dependent deacetylase SIRT1, thus inducing p53 acetylation and apoptosis.

Materials and methods: these studies were performed in the human osteosarcoma U2OS cell line; in order to monitor SIRT1 activity, p53 acetylation was analyzed by western blot while protein-protein interactions were studied by co-immunoprecipitation. We then generated a U2OS-DBC1$^{-/-}$ cell line using the CRISPR/Cas9 system for genome engineering, in order to study the effects of DBC1 on the activity of the checkpoint kinase Chk2.

Results: we deepen characterized the molecular mechanism by which DBC1 inhibits SIRT1 and found that, beside ATM/ATR, the DBC1-dependent inhibition of SIRT1 requires also the checkpoint kinase Chk2 and the 11S proteasome activator REGγ. Specifically, in response to etoposide Chk2 phosphorylates REGγ promoting DBC1-REGγ interaction, thus increasing DBC1-SIRT1 binding and SIRT1 inhibition. In addition we report that, upon DNA damage, the catalytic activity of Chk2 is modulated by DBC1, with a final impact on DNA double strand breaks repair. Indeed, in response to etoposide, DBC1 favors Chk2 homodimerization and activation, which leads to KAP1 phosphorylation and DNA repair promotion.

Conclusions: we reveal a new physical and functional crosstalk among DBC1, SIRT1, Chk2 and REGγ, giving novel insights on their role in the DNA damage response.

Drug Resistance

I 1
CDK6 AND PLATINUM DRUG RESISTANCE IN EPITHELIAL OVARIAN CANCER

Alessandra Dall'acqua [1] - Monica Schiappacassi [1] - Maura Sonego [1] - Ilenia Pellizzari [1] - Gustavo Baldassarre [1]

CRO of Aviano, National Cancer Institute, Aviano, Italy [1]

Introduction: Epithelial Ovarian Cancers (EOCs) consist in a heterogeneous group of tumors. Late diagnosis and drug-resistant recurrences make EOCs the most aggressive among the gynecological malignancies. The central role played by CDKs (Cyclin-Dependent Kinase) in several cellular mechanisms, such as control of cell cycle progression, DNA repair and transcription and the association of their activity in different apoptotic pathways, make them attractive targets to overcome drug-resistance in EOC.

Methods: Using the RNA interference technology (targeting 23 members of the CDKs family) we performed a functional genomic screening and identified CDK6 as the CDK most significantly involved in platinum sensitivity. Interestingly, CDK6 silencing did not affect cell survival by itself but profoundly increased platinum-induced cell death.

Results: The effect of CDK6 silencing on the sensitivity to both carboplatinum (CBDCA) and cisplatin (CDDP) was first confirmed in a larger panel of EOC cell lines (MDAH 2774, OVCAR-8 and SKOV-3). Experiments performed *in vitro* with CDK6 mutants (dominant negative and constitutively active) showed that CDK6 kinase activity is necessary to prevent platinum-induced death. Based on these results, we tested the efficacy of an orally active CDK4/CDK6 inhibitor, PD0332991, in controlling EOC cell growth and platinum sensitivity. *In vitro* and *in vivo* experiments demonstrated that both CDK6 silencing and PD0332991 reduced tumor growth when combined to CBDCA treatment.

To understand the molecular mechanism whereby CDK6 regulated platinum-induced cell death in EOC we focused on specific CDK6 phosphorylation targets and demonstrated that FOXO3a is a relevant downstream target of CDK6. Biochemical analyses confirmed that, upon platinum exposure, CDK6 binds and phosphorylates FOXO3a. Moreover, our experiments also indicated that CDK6/FOXO3a axis is necessary to regulate the DNA damage response through the regulation of ATR/CHK1 pathway.

Conclusions: We demonstrated that in EOC platinum sensitivity could be increased by specific CDK6 inhibition. Since PD0332991 is currently tested in cancer patients as single agent or in combination with chemotherapy our results are of primary translational relevance. We are currently analyzing the expression of both CDK6 and FOXO3a in primary EOC to verify if they can be used as biomarkers to select those patients that may benefit from a combination therapy based on platinum and CDK6 inhibition

I 2
TARGETED RESEQUENCING APPROACH TO INVESTIGATE THE MUTATIONAL LANDSCAPE ASSOCIATED TO PLATINUM RESISTANCE IN EOC

Mariastina Di Marino [1] - Luca Beltrame [1] - Lara Paracchini [1] - Robert Fruscio [2] - Vittoria Fotia [3] - Alberto Zambelli [3] - Elena Ronchetti [3] - Tommaso Grassi [2] - Luca Clivio [1] - Enrica Calura [4] - Chiara Romualdi [4] - Maurizio D'incalci [1] - Sergio Marchini [1]

Istituto di Ricerche Farmacologiche "Mario Negri", IRCCS, Milan, Italy [1] - San Gerardo Hospital, Hospital, Monza, Italy [2] - S. Maugeri, IRCCS, Pavia, Italy [3] - Università degli studi di Padova, University, Padova, Italy [4]

Introduction Despite initial response to first line platinum-based chemotherapy, more than 80% of high grade serous ovarian cancer (HGS-EOC) patients relapse and develop resistance. The molecular and genetic features involved in drug resistance are still unknown. By gene expression profile in a cohort of 23 patients from which matched biopsies were taken at primary surgery (PS-O) when tumor was sensitive to chemotherapy and at time of relapse (SCR) when the tumor was resistant, we identified a key role of transcription modulation in tumor resistance related to the EMT pathway activation (Marchini *et al*, 2013). To go further in detail on the mechanisms leading to tumor relapse and resistance, we investigated by next generation sequencing the genomic profile of selected genes known to be involved in pathways affecting EMT and tumor response to platinum-based chemotherapy.

Methods A cohort of 33 patients affected by HGS-EOC, for whom snap frozen tumor biopsies were available at PS-O and SCR was selected from "Pandora", a tumor tissue collection of EOC. 23 out of 33 cases were those previously profiled for gene expression analysis (Marchini *et al*, 2013). DNA libraries enriched in a selected panel of 65 genes encompassing key players of signal transduction, EMT regulation, extracellular matrix interaction, cell cycle and DNA repair were generated using TruSeq Custom Amplicon kit and sequenced on MiSeq (Illumina). Data were analyzed according to established techniques using a high performance cluster computing platform (Cloud4CARE project).

Results Analysis identified a total of 13.582 mutations (96.6% SNPs and 3.4% InDels), of which 7.529 affecting PS-O and 6.015 SCR. A selection of these mutations have been independently validated for SNPs detection assay by ddPCR (Droplet Digital PCR, Bio-Rad) and PyroMark (Qiagen). With a 500X coverage, we observed BRCA1, BRCA2 and TP53 mutated in the majority of cases.

Conclusions Our preliminary results on a selection of genes involved in key processes of tumor growth and drug resistance, show an overall concordance of somatic mutations between PS-O and SCR tumor biopsies. Despite data need to be further validated, they suggest that genetic events leading to drug resistance are already present at PS-O and outgrowth under drug selective pressure, rather than being acquired *de novo* at SCR.

I 3
KEY ROLE OF GLUTATHIONE IN NEUROBLASTOMA MULTI-DRUG RESISTANCE

Renata Colla [1] - Chiara De Ciucis [1] - Barbara Marengo [1] - Roberta Ricciarelli [1] - Stefania Pizzimenti [2] - Umberto M. Marinari [1] - Maria A. Pronzato [1] - Cinzia Domenicotti [1]

Department of Experimental Medicine, University of Genoa, Genoa, Italy [1] - Department of Clinical Biological Sciences, University of Turin, Turin, Italy [2]

Introduction: Neuroblastoma (NB) is the second most common solid tumour of early childhood and it is characterised by biological and clinical heterogeneity. In fact, while low-risk NB may spontaneously regress, high-risk NB, despite it usually responding to initial chemotherapy, has a poor prognosis and many patients undergo relapses and form metastases. Therapy failure is generally caused by acquired chemoresistance of a subset of residual cells that have adapted to drugs, showing simultaneous resistance to different cytotoxic agents and increased malignant properties. Many tumours show an increase of antioxidants, such as glutathione (GSH), a key factor in the resistance to pro-oxidant drugs. Our aim is to characterise a human NB cell line resistant to etoposide, a standard chemotherapy agent, and to identify the mechanism of the resistance.

Methods: Selection of an etoposide-resistant cell line was performed treating HTLA-230, a high-risk NB cell line, for 6 months with increasing doses of etoposide (up to 1.25 μM, the dose comparable to that clinically used). Subsequently, etoposide-resistant cells were treated with higher concentrations (from 10 to 100 μM) of etoposide, doxorubicin (a classically-used chemiotherapeutic drug), buthionine sulfoximine (BSO, a GSH depleting agent) and H_2O_2 (oxidative agent). Under all conditions of treatment, cell viability was evaluated by MTT analysis and tumorigenicity was tested by a clonogenic assay. GSH/GSSG and reactive oxygen species (ROS) levels were determined by fluorescence analysis.

Results: Etoposide-resistant NB cells (Eto-R) showed a higher resistance than parental cells, not only to etoposide, but also to doxorubicin, BSO and H_2O_2. Moreover, the Eto-R were more tumourigenic and displayed an higher amount of GSH and lower ROS levels than parental cells.

Conclusion: Collectively, our results suggest that the selected etoposide-resistant NB cell line develops a Multi Drug Resistance (MDR) against different agents which are able to increase intracellular ROS production. We believe that the MDR phenotype of Eto-R cells is due to their high content of GSH that might play a crucial role in neuroblastoma chemoresistance (*Grants from CARIGE Foundation, 2013*).

I 4
CHEMOSENSITIZATION AND RADIOSENSITIZATION EFFECTS OF GLIOBLASTOMA CELLS BY THE HISTONE DEACETYLASE INHIBITOR GIVINOSTAT (ITF2357) IN GLIOBLASTOMA CELL MODELS.

Giovanni Luca Gravina [1] - Flavio Leoni [2] - Elisabetta Benedetti [3] - Simona Delle Monache [1] - Andrea Mancini [1] - Ernesto Di Cesare [1] - An-

na Maria Cimini [3] - Giuliana Porro [2] - Elisabetta Galbiati [2] - Paolo Mascagni [2] - Claudio Festuccia [1]

Department of Applied Clinical Sciences and Biotechnology, University of L'Aquila, L'Aquila, Italy [1] - Research Center Italfarmaco, Italfarmaco, Cinisello Balsamo, Milano, Italy [2] - Department of Health Sciences, University of L'Aquila, L'Aquila, Italy [3]

Background and objectives. Despite aggressive treatment with radiation therapy (RT) and concurrent adjuvant temozolomide (TMZ), glioblastoma multiform (GBM) still has a dismal prognosis. We aimed at identifying strategies to improve the therapeutic outcome of combined radiotherapy and TMZ in GBM including the use of the HDAC inhibitor Givinostat.

Methods and Materials: A set of ten glioma cell lines were used. Colony formation, DNA damage repair, mode of cell death, invasion, migration and vasculogenic potential as well as protein expression were determined. U87MG and T98G cells were also tested in vivo.

Results: Treatment of GBM cells with Givinostat caused a dose-dependent acetylation of H3 and H4 histones and reduced cell proliferation in a time (24-72 h) and dose-dependent manner in a set of 8 glioblastoma cell lines. IC50 values ranged between 0.75 and 2.5 mM with both G0/G1 and G2/M cell cycle arrest and sensible reduction of the S phase. Since clinical serum doses Givinostat range between 80 and 160 nM, we analyzed the *in vitro* effects of concentrations of the drug between 25 and 200 nM. Doses of 100 and 200 nM have significant anti-cancer activity when chronically administered (5-10 days) being able to reduce cell proliferation with a major G0/G1 cell cycle arrest but low levels of apoptosis.. These concentrations of Givinostat induced: (i) b-galactosidase activity, suggesting the induction of a senescent phenotype; (ii) Glial fibrillar actin (GFAP) expression associated with reduced nuclear levels of b-catenin and redistribution in the plasma membrane, suggesting the induction of a more differentiated phenotype. In addition, Givinostat significantly enhanced TMZ and RT efficacy both in TMZ sensitive/MGMT negative and TMZ resistant/MGMT positive cells. *In vivo*, in a subcutaneous xenograft model, the time to progression of RT, TMZ or RT plus TMZ treatments was significantly increased after administration of Givinostat. The mechanism of enhanced radio- and TMZ-sensitization by Givinostat was multifactorial, involving impaired DNA damage repair, induction of autophagy and/or apoptosis, reduced tumor stem cell re-growth, increased differentiation and reversion of EMT as well as reduced angiogenesis associated to brain microendothelial cell death. Taken together our findings show that the histone deacetylase inhibitor, Givinostat, represents a promising strategy to prime glioblastoma cells for chemotherapy and radio-induced cell death.

I 5

ACTIVATION OF THE ERBB3-AKT AXIS PROMOTES MELANOMA CELL SURVIVAL AND PROLIFERATION IN RESPONSE TO RAF/MEK INHIBITION

Debora Malpicci [1] - Luigi Fattore [2] - Emanuele Marra [3] - Giuseppe Roscilli [3] - Maria Elena Pisanu [1] - Alessia Noto [2] - Claudia De Vitis [4] - Luigi Aurisicchio [3] - Rita Mancini [2] - Paolo Antonio Ascierto [4] - Gennaro Ciliberto [4]

Department of Experimental and Clinic Medicine, "Magna Graecia" University of Catanzaro, Catanzaro, Italy [1] - Department of Clinic and Molecular Medicine, "Sapienza" University of Rome, Rome, Italy [2] - Takis, Takis srl, Rome, Italy [3] - National Institute for the Study and Cure of Cancer "Fondazione G.Pascale", National Institute for the Study and Cure of Cancer "Fondazione G.Pascale", Naples, Italy [4]

Introduction:The treatment of advanced melanomas bearing activating mutations in the BRAF oncogene with BRAF kinase inhibitors is limited by the occurrence of resistance. Reactivation of MEK-signaling is the principal mechanism of resistance. Thus MEK-inhibitors are being clinically developed in combination with BRAF inhibitors, but also in this case resistance inevitably occurs, thus limiting long term disease control. It has been recently suggested that ErbB3, a member of the EGFR receptor family, may facilitate the establishment of drug resistance. Our laboratory aims at a better understanding of the mechanisms leading to ErbB3 activation and how its inhibition by monoclonal antibodies may affect the development of long term resistance.

Materials and Methods: We tested a large panel of melanoma cell lines: LOX IMVI, M14, A375, SK-Mel5, SK-Mel28, MALME 3-M, M262, M263, M229, M397 (BRAF V600E), MST-L (BRAF V600R) and WM266, WM115 (BRAF V600D). Western blot analysis was performed on total protein extracts from untreated and BRAFi and MEKi treated cells, using anti-ErbB3, anti-AKT and anti-ERK 1/2 antibodies. The growth inhibitory effects of multiple combinations of BRAF and MEK inhibitors and/or anti-ErbB3 mAbs were evaluated by colony formation assays. Mouse xenograft studies were carried out with M14 cells injected s.c. Drug treatments began when tumors reached a mean volume of $100mm^3$ and tumor growth was measured by caliper.

Results: We show that ErbB3 is the main RTK rapidly hyperphosphorylated, in response to BRAF or MEK inhibition in melanoma cell lines harboring a variety of V600BRAF mutations. This results in a strong activation of phospho-AKT. ErbB3 activation can be fully abrogated by two distinct anti-ErbB3 monoclonal antibodies, A3 and A4. These two mAbs individually or, better, in combination strongly enhance the ability of different BRAF/MEK inhibitors to silence the oncogenic MAPK and AKT pathways. This results in potentiation of cell cycle arrest, growth inhibition in short term and long term assays and strong induction of apoptosis. Preliminary xenograft studies confirm that anti-ErbB3 mAbs in combination with BRAF and MEK inhibitors exert a more profound inhibition of tumor growth than individual treatments and this is paralleled by a potent downregulation of oncogenic signaling.

Conclusions: Feedback activation of ErbB3/AKT phosphorylation is a fast and common response of BRAF mutated melanoma cells to BRAF and/or MEK inhibitors. Our results suggest

that combinatorial treatment of BRAF/MEK inhibitors with anti-ErbB3 antibodies could represent a promising approach to reduce development of resistance in the clinic.

I 6
INVOLVEMENT OF ATP-BINDING CASSETTE TRANSPORTERS IN EARLY STAGES OF HEPATOCELLULAR CARCINOMA

Sandra Mattu [1] - Andrea Perra [1] - Marta Anna Kowalik [1] - Giovanna Maria Ledda-columbano [1] - Amedeo Columbano [1]

Department of Biomedical Sciences, University of Cagliari, Cagliari, Italy [1]

Introduction: The failure to chemotherapeutic approaches is one of the leading cause of mortality in hepatocellular carcinoma (HCC). This is presumably mainly due to a phenomenon known as "multiple drug resistance" (MDR), that involves several membrane transporters, the ATP-binding cassette (ABC) family, which catalyze the ATP-dependent efflux of cytotoxic drugs outside the cells. Several studies have shown up-regulation of ABC genes in experimental and human HCC; however, whether dysregulation of ABC genes occurs at early stages of the tumorigenic process remains elusive.

Materials and Methods: Since the study of early steps of HCC development in humans is hampered by the difficulty of diagnosing premalignant lesions, to circumvent this problem animal models are needed. The Resistant Hepatocyte (RH) rat model allows the identification of phenotypically distinct lesions along the various steps of hepatocarcinogenesis; moreover, it makes possible, on the basis of the positivity to the stem/progenitor cell marker KRT-19, to discriminate the lesions that will progress to HCC from those that will spontaneously regress. Therefore, this experimental protocol was adopted to investigate the role of *Abcb1*, a member of ABC family, in preneoplastic stages of the tumorigenic process.

Results: Our previous transcriptomic analysis has shown up-regulation of *Abcb1* expression all throughout the carcinogenic process. To assess the potential role of *Abcb1* in early stages, we analyzed its expression by qRT-PCR in preneoplastic rat lesions as early as 10 weeks after treatment with the carcinogen dietylnitrosamine (DENA). The results showed that Abcb1 was strongly up-regulated in KRT-19 positive lesions compared to KRT-19 negative lesions and to the liver from untreated rats. Since the regulation of ABC genes seems to involve microRNAs, we investigated whether *Abcb1* could be modified by miR-122. To this aim, the expression of miR-122 was determined by qRT-PCR in the same preneoplastic lesions analyzed also for Abcb1. The results showed a down-regulation of miR-122 expression in KRT-19 positive lesions vs. KRT-19 negative, highlighting an inverse correlation between *Abcb1* and miR-122 expression.

Conclusions: These results suggest that up-regulation of *Abcb1,* possibly as a consequence of down-regulation of miR-122, is a very early event and that it might be the mechanism contributing to the aggressiveness of KRT-19-positive lesions and their progression to HCC.

I 7
EPIGENETIC MECHANISMS OF DRUG RESISTANCE IN GLIOBLASTOMA

Massimo Romani [1] - Barbara Banelli [1] - Elisa Carra [2] - Federica Barbieri [2] - Roberto Wurth [2] - Federica Parodi [1] - Alessandra Forlsani [1] - Giorgio Allemanni [1] - Daniela Marubbi [2] - Tullio Florio [2] - Antonio Daga [1]

IRCCS AOU San Martino - IST, Hospital, Genova, Italy [1] - University of Genova, University, Genova, Italy [2]

Introduction: Glioblastoma (GBM) is constituted by cells with distinct phenotypic and molecular characteristics, that possess diverse differentiation potential and unique properties of invasiveness, proliferation, self-renewal and resistance to therapy.

The emergence of cell clones resistant to treatment is a key factor leading to tumor recurrence; therefore, understanding the mechanisms underlying chemoresistance in GBM has obvious clinical implications.

Materials and Methods: We generated primary GBM cells resistant to Temozolomide (TMZ), the drug of choice for GBM treatment, and we characterized these TMZ-R cells in term of growth and stemness characteristics, sensitivity to drugs, epigenetic asset, expression and functionality of genes involved in tumor progression and resistance to drugs.

Results: We demonstrated that TMZ resistance can be reverted by "drug wash out" suggesting the intervention of epigenetic mechanisms in drug resistance and supporting the idea of TMZ rechallenge in GBM patients after prior exposure to the drug. TMZ-R cells are slow-growing and refractory to apoptosis but exquisitely sensitive to HDAC inhibitors that synergize with TMZ and selectively kill TMZ-R cells. We did not detect alterations of the methylation and expression of the MGMT gene nor in the drug efflux mechanisms.

Many histone demethylase genes are altered in TMZ-R cells and we mimicked TMZ sensitivity and resistance by exogenously expressing or inactivating the KDM5A gene.

Epigenome-wide analysis showed that 1471 genes are differentially methylated in TMZ-R and sensitive GBM cells. Some of these genes are obvious candidates for further studies (i.e.: DNA repair genes).

Although stemness is generally associated with drug-resistance, we observed that TMZ-R cells transiently acquire morphologic and molecular characteristics of differentiation that are lost at drug wash out.

Conclusions: We demonstrated that TMZ resistance in GBM is driven largely by epigenetic mechanisms in a subset of slow-cycling and transiently partially differentiated cells that escape killing, overcome the G2 checkpoint and are capable of sustained clonal growth.

We showed also that, in vitro, drugs acting on the epigenome (SAHA or TSA) selectively kill the cells that are either intrinsically resistant or that rapidly acquire TMZ resistance. The strong synergism between TMZ and SAHA or TSA could be exploited to develop novel combined adjuvant therapies for this rapidly progressing and invariably lethal cancer.

I 8
GENE EXPRESSION PROFILING OF HUMAN MELANOMA CELL LINES AND CLINICAL SAMPLES DEFINES SUBSETS DIFFERING IN SUSCEPTIBILITY TO BRAFV600E AND PATHWAY-DIRECTED INHIBITORS

Matteo Dugo [1] - Gabriella Nicolini [1] - Gabrina Tragni [1] - Ilaria Bersani [1] - Antonella Tomassetti [1] - Silvana Canevari [1] - Andrea Anichini [1] - Marialuisa Sensi [1]

Fondazione IRCCS Istituto Nazionale dei Tumori, Hospital, Milano, Italy [1]

Introduction: Gene expression profiling can be used to classify tumors according to intrinsic features, often predictive of drug response. Constitutive activation of receptor tyrosine kinases (RTKs) plays important roles in melanoma (Me) biology as well as in drug resistance. We therefore investigated whether: a) novel subsets of tumors could be identified by integrating hierarchical clustering results of genes encoding RTKs with those of genes belonging to a previously defined Me-Phenotype-Specific-Expression (MPSE) signature; b) such identified subsets could possess clinical relevance by linking the refined phenotype to the sensitivity of several drugs directed against components of signaling pathways deranged in Me.

Materials and methods: We applied unsupervised hierarchical clustering using RTK and MPSE gene signatures to a discovery dataset of 187 Me cell lines collected from 5 independent studies. The identified subtypes were validated in independent datasets of 58 Me cell lines contained within the Cancer Cell line Encyclopedia database and of 361 Me clinical samples, retrieved from 8 publicly available datasets. ComBat was applied for batch effect removal. Prediction Analysis of Microarray algorithm with ten-fold cross-validation was used to derive the minimal subset of genes that characterize each subset. SubMap algorithm was used to compare subsets across the clinical and Me cell lines datasets. Validation of subset-specific expression of selected markers was performed by Western blot and immunohistochemistry on cultured Me lines and clinical samples, respectively. In vitro proliferation assays were used to test susceptibility to small molecule inhibitors.

Results: Transcriptional profiling of metastatic Me cell lines and clinical samples revealed the existence of distinct subsets of melanoma and a novel group was identified. Differential expression of relevant genes on in vitro cultured Me cell lines could recapitulate the different subsets. Me cell lines contained within the different subsets display different sensitivity to the BRAFV600E inhibitor PLX4720 and to other drugs targeting MAPK or PI3K/AKT pathways**.**

Conclusions: Successful development of targeted therapies will rely on defining patients that are most likely to benefit from drug candidates. The identification of distinct subsets of Me suggests that biological characteristics identified by gene expression profiling may aid in stratifying patients with respect to the choice of therapeutic agents**.**

Supported by AIRC

I 9
TARGETING ACQUIRED RESISTANCE TO TARGETED THERAPIES IN CUTANEOUS MELANOMA

Elisabetta Fratta [1] - Francesca Colizzi [1] - Maurizio Polano [2] - Aurora Rizzo [1] - Riccardo Dolcetti [1] - Luca Sigalotti [1]

Cancer Bioimmunotherapy Unit, Centro di Riferimento Oncologico I.R.C.C.S. National Cancer Institute, Aviano, Italy [1] - Experimental Oncology 1, Centro di Riferimento Oncologico I.R.C.C.S. National Cancer Institute, Aviano, Italy [2]

Introduction: Activating BRAF mutations, occurring in about half of cutaneous melanomas, are effectively targeted by specific inhibitors such as vemurafenib and dabrafenib, which have shown dramatic clinical responses in advanced patients. Despite their effectiveness, progression almost invariably occurs through an acquired resistance of neoplastic cells to these targeted agents. This behaviour prompts for an urgent identification of the resistance mechanisms, which could possibly allow defining new therapeutic options.

Materials and methods: sequential adaptation to increasing concentrations of vemurafenib was used to raise drug-resistant isogenic cell cultures from a panel of BRAF-typed, vemurafenib sensitive, cell lines generated from metastatic lesions surgically removed from melanoma patients at our Institute. Gene expression profiling, RNAsequencing, quantitative RT-PCR, receptor tyrosine kinase (RTK) arrays, western blotting and MTT vitality assays were used to compare resistant cells to their parental sensitive cells in the presence or absence of different small molecule inhibitors.

Results: vemurafenib resistant cultures were successfully generated from 8 metastatic melanoma cell lines. Gene expression profiling showed a significant upregulation of RTK mRNAs following acquisition of resistance. Quantitative RT-PCR confirmed upregulation of AXL and its ligand GAS6, EGFR and/or PDGFRβ in 6 out of 8 resistant cell cultures, with frequent co-expression of multiple RTK. Accordingly, increased phosphorylation of AXL, EGFR and PDGFRβ was observed in lysates from vemurafenib–resistant vs sensitive parental cells. Small-molecule-mediated inhibition of AXL and PDGFRβ, partially restored the sensitivity of resistant cells to vemurafenib, while no effect was observed upon inhibition of EGFR, despite significant reduction of its phosphorylation following treatment.

Conclusions: the data: i) further support the role of RTK activation in contributing to the acquired resistance to BRAF-targeted therapies; ii) identify for the first time AXL as an important player; iii) suggest for a common underlying mechanism leading to the concomitant upregulation of different RTKs; and iv) may suggest the use of combined targeted therapeutics to overcome the acquired resistance to BRAF inhibition.

I 10
TUMOR METABOLISM AND DOCETAXEL RESISTANCE IN PROSTATE CANCER

Maria Letizia Taddei [1] - Alberto Marini [1] - Luigi Ippolito [1] - Andrea Morandi [1] - Elisa Giannoni [1] - Paola Chiarugi [1]

Department of Experimental and Clinical Biomedical Sciences, University of Florence, Florence, Italy [1]

Introduction: Drug resistance is recognized as the primary cause of failure of chemotherapeutic treatment in most human cancers. Mounting evidences support the idea that deregulated cellular metabolism is linked to drug resistance in cancer therapy. Indeed, both components of the glycolytic and mitochondrial pathways are involved in altered metabolism linked to chemoresistance in several cancers. In this context, we characterized a PCa cell line resistant to docetaxel and we evaluated the phenotypic and metabolic behavior of this cell line, in order to identify the drug-induced metabolic adaptations conferring advantages in resistant cells.

Material and method: PC3 cell line resistant to docetaxel (Doce Res) was obtained by treating sensitive PC3 cells with increased doses of drug, until the final concentration of 10 nM. Western blot analysis was used to investigate the levels of EMT markers and key metabolic players in the PC3 and PC3 Doce Res cells. Reactive oxygen species were evaluated using the redox sensitive probe: 2',7'-dichlorofluorescein diacetate (DCF-DA), selectively responsive to hydrogen peroxide. Boyden assay was used to assess the invasive properties of PC3 and Doce Res cells. Radioactive assays were used to determine the levels of glucose, glutamine and lactate uptake and to monitor OXPHOS activity. Metformin, an inhibitor of mitochondrial respiratory chain complex I, was used to assess the dependence on mitochondrial respiration of the two different cell lines.

Results: Doce Res cells acquire a pro-invasive behavior and a down-regulation of E-cadherin, consistent with the acquisition of an EMT program and a pro-metastatic phenotype. Moreover, DoceRes cells show a decrease of intracellular ROS, NADPH level and proliferation compared to sensitive cells. These features are not linked to an induction of the pentose phosphate pathway, but are associated with an enhancement in the antioxidant response, mainly driven by increased expression of the transcription factor Nrf2 (Nuclear factor erythroid 2 related factor 2). Metabolic analysis in Doce Res cells reveals a shift toward OXPHOS, with a greater utilization of glucose, glutamine and lactate by mitochondrial respiration. In agreement, metformin, impairing mitochondrial complex I function, selectively decreases proliferation and invasiveness in resistant cells. Furthermore, stromal cancer associated fibroblasts, which cause a "reverse Warburg" phenotype in prostate cancer cells, are able to protect sensitive and resistant cell lines from docetaxel toxicity. In keeping, an approach based on re-expression of miR-205, able to shift metabolism from OXPHOS to a Warburg phenotype, induces an increase of docetaxel toxicity in prostate cancer cells.

Conclusion: Taken together, these findings suggest that chemoresistance to docetaxel induces an escape from Warburg metabolism with a potential involvement of OXPHOS to confer a metabolic advantage to these cells. We hypothesize that the impairment of mitochondrial function could be an attractive adjuvant therapy for several anticancer regimens.

I 11
COOPERATIVE INHIBITION OF CXCR1/2, TGF-ß, AND IL-1R SIGNALING PATHWAYS MODULATES IN VIVO ACQUIRED RESISTANCE TO ANTI-VEGF TREATMENT

Anna Tamburrino [1] - Carmine Carbine [1] - Geny Piro [2] - Federico Boschi [3] - Maria Mihaela Mina [2] - Marco Zanotto [1] - Giampaolo Tortora [2] - Davide Melisi [1]

Digestive Molecular Clinical Oncology Research Unit, University of Verona, Verona, Italy [1] - Laboratory of Oncology and Molecular Therapy, University of Verona, Verona, Italy [2] - Section of Anatomy and Histology, University of Verona, Verona, Italy [3]

Introduction: Resistance of tumors to antiangiogenic therapies is becoming increasingly relevant. We recently identified several proinflammatory factors, including interleukin (IL)-1, different CXC receptors (CXCR)1/2 ligands, and Tumor Growth Factor-beta (TGF-β), that were expressed at higher levels in murine models established in vivo to be resistant to the VEGF-specific antibody bevacizumab (Bev). These factors stimulated the recruitment of $CD11b^+$ proangiogenic myeloid cells, induced epithelial-to-mesenchymal transition (EMT) and, increased the aggressiveness of Bev-resistant tumors. Here, we hypothesized that the combined inhibition of these proinflammatory signaling pathways might reverse the resistance to anti-VEGF treatment.

Materials and methods: Bev-resistant COLO357FG (FGBR) orthotopic tumor bearing mice received Bev alone or in combination with the recombinant human IL-1 receptor antagonist anakinra, a monoclonal antibody against TGF-β Receptor type II (TGF-β RII) TR1, and the recombinant antibody binding CXCR1/2 ligands LSN3005541. Immunohistochemical analyses were carried out to determine the TGF-β– stimulated phosphorylation of Smad2, and the expression of the IL-1– induced protein IL-6, the proangiogenic myeloid cells marker CD11b, and the EMT markers E-cadherin and Vimentin.

Results: The effective inhibition of IL-1, TGF-b, and CXCR1/2 signaling pathways in FGBR cells was demonstrated in vitro by the reduction of the phosphorylation of Smad2 and p65.

In vivo, the combination of anakinra, TR1, and LSN3005541 with Bev reduced the tumor burden and significantly prolonged mice survival if compared with Bev alone.

Tumors from mice receiving the combination treatment demonstrated significantly lower expression of IL-6 and phosphorylation of p65 and Smad2 when compared with control.

FGBR cells treated in vitro with anakinra, TR1, and LSN3005541 plus Bev had significantly higher levels of E-cadherin and lower levels of vimentin, and exhibited significantly lower migration rates than did their Bev- treated controls.

Consistently, tumors from mice receiving the combination treatment demonstrated significantly higher expression of E-cadherin, lower levels of vimentin, and a significantly lower infiltration by CD11b+ cells when compared with Bev- treated controls.

Conclusions: This study suggests that inhibition of CXCR1/2, TGF-β, and IL-1R signaling pathways is a potential therapeutic approach to reversal of the acquired resistance to anti-VEGF treatment.

I 12
THE MULTIKINASE INHIBITOR SORAFENIB TARGETS MITOCHONDRIA AND SYNERGIZES WITH THE GLYCOLYSIS INHIBITOR 2-DEOXYGLUCOSE FOR CANCER CELL KILLING: A NEW HOPE FOR THE TREATMENT OF HEPATOCHOLANGIOCARCINOMA

Valentina Tesori [1] - Maria Ausiliatrice Puglisi [1] - Giovanni Battista Gasbarrini [2] - Antonio Gasbarrini [1]

UOC of Internal Medicine and Gastroenterology, Gemelli Hospital, Rome, Italy [1] - Research in Medicine Foundation, Onlus, Bologna, Italy [2]

Background: The multikinase inhibitor Sorafenib (SFB) is the gold-standard therapy for patients with advanced hepatocellular carcinoma (HCC). A recent study showed that SFB also exhibits a potent antitumor activity in a model of cholangiocarcinoma (CCC). However, no data are available regarding SFB effects against hepatocellular-cholangiocarcinoma (HCC-CC), a rare form of primary liver cancer with histopathologic features of both hepatocellular carcinoma and cholangiocarcinoma.

SFB inhibits tumor proliferation by targeting Raf kinase isoforms (V600E mutation), although it is believed that additional mechanisms account for its elevated anticancer activity. In particular, recent studies have pointed to mitochondrial damage as additional BRAF-independent mechanism underlying cell response to SFB.

AIMS: Aim of the present study was to investigate the effects of SFB in a rat model of HCC-CC, focusing on mitochondrial and metabolic targets.

Methods: Drug toxicity was evaluated through Propidium Iodide (PI) assay. Gene expression was investigated by microarray analysis. Protein expression was evaluated by western blotting. Intracellular energetic balance was assessed by ATP measurement. Mitochondrial activity was assessed by Oxygraph Respirometric Analysis. Mitochondrial potential was measured with the JC-1 probe.

Results: We observed that SFB elicited a raise of intracellular ROS, a severe decrease of oxygen consumption and intracellular ATP levels, consistent with mitochondrial damage. In order to assess the molecular mechanisms underlying the antitumor activity of the SFB in HCC-CC, we performed a microarray analysis of SFB treated HCC-CC cells. Gene expression profiling revealed a metabolic reprogramming toward aerobic glycolysis. Additionally, SFB cytotoxicity was strongly potentiated by the glycolytic inhibitor 2-Deoxyglucose and intracellular ROS could be pivotal in this process.

Conclusion: This study identifies novel and relevant aspects of SFB action on HCC-CC cells, including metabolic cell reprogramming towards a state of "glucose addiction" .These data could be exploitable in therapy and provide a rationale for treatment of HCC-CC with SFB.

In Vivo Models Of Cancer

L 1
DISSECTING THE ROLE OF CURCUMIN ON TUMOR GROWTH AND ANGIOGENESIS IN MOUSE MODEL OF HUMAN BREAST CANCER

Sabrina Bimonte [1] - Antonio Barbieri [2] - Giuseppe Palma [3] - Domenica Rea [2] - Antonio Luciano [4] - Claudio Arra [2] - Carlo Leonetti [5]

Istituto Nazionale Tumori Fondazione G. pascale, Istituto Nazionale Tumori Fondazione G. pascale, Naples, Italy [1] - Istituto Nazionale Tumori Fondazione G. pascale, Istituto Nazionale Tumori Fondazione G. pascale, naples, Italy [2] - Istituto di Endocrinologia e Oncologia Sperimentale del Consiglio Nazionale delle Ricerche L. Califano, Istituto di Endocrinologia e Oncologia Sperimentale del Consiglio Nazionale delle Ricerche L. Califano, naples, Italy [3] - Istituto Nazionale per lo studio e la cura dei Tumori "Fondazione G. Pascale, Istituto Nazionale per lo studio e la cura dei Tumori "Fondazione G. Pascale, naples, Italy [4] - Istituto Nazionale Tumori Regina Elena, Istituto Nazionale Tumori Regina Elena, rome, Italy [5]

Introduction: Breast cancer is considered the most common cancer for women worldwide and it is now the second leading cause of cancer-related deaths among females in the world. Since breast cancer is highly resistant to chemotherapy, novel strategies involving less toxic anticancer agents are necessary. Curcumin, an active component of turmeric (*Curcuma longa*), is one such agent which inhibits the angiogenesis and proliferation of a wide variety of tumor cells, through the modulation of various cell signalling pathways.

Material and Methods: Curcumin used for in *vitro* experiments was dissolved in dimethyl sulphoxyde (DMSO), diluted in DMEM and 10% FBS and added to MDA.MB231 cells in two different doses (5μg/ml and 7,5μg/ml). The proliferation and apoptosis of cells were determined by wound-healing and MTT assay. In *vivo* mouse model was generated by subcutaneous injection of MDA.MB231 in nude mice. We placed mice on diet containing curcumin at 0, 6% for 6 weeks. NF-κB (nuclear factor kappa B) activation in tumor samples was detected by electrophoresis mobility shift assays (EMSA). The expression pattern of cyclin D1, PECAM-1, MMP-9, p65 was determined by western blot analysis with specific antibodies.

Results: We demonstrated that curcumin in *vitro* inhibited the proliferation and enhanced apoptosis of MDA.MB231 cells. In *vivo* studies performed on xenograft mouse model of human breast cancer revealed that tumors of mice treated with curcumin were smaller than those observed in controls (P = 0, 004) and showed a down regulation of the transcriptor factor nuclear factor NF-κB and NF-κB- regulated gene products.

Conclusion: Our findings indicate that curcumin has an antitumor activity in human breast cancer, through the modulation of NF-κB pathway. Since curcumin is very well tolerated in human subjects and is assumed by food, our mouse model demonstrated that curcumin can be used as an adjuvant agent to chemotherapy in treatment of triple negative breast cancer.

L 2
ESTABLISHMENT OF A PLATFORM OF PATIENT-DERIVED TUMOR XENOGRAFTS (EOC-PDX) TO STUDY THE BIOLOGY AND THERAPY OF EPITHELIAL OVARIAN CANCER

Francesca Bizzaro [1] - Francesca Ricci [1] - Marta Cesca [1] - Federica Guffanti [1] - Alessandra Decio [1] - Carmen Ghilardi [1] - Patrizia Perego [2] - Robert Fruscio [3] - Paola Ostano [4] - Giovanna Chiorino [4] - Maria Rosa Bani [1] - Giovanna Damia [1] - Raffaella Giavazzi [1]

Department of Oncology, IRCCS-Istituto di Ricerche Farmacologiche Mario Negri, Milan, Italy [1] - Department of Pathology, San Gerardo Hospital, Monza, Italy [2] - Obstetrics and Gynecology Clinic, San Gerardo Hospital, Monza, Italy [3] - Cancer Genomincs Laboratory, Fondazione Edo ed Elvo Tempia Valenta, Biella, Italy [4]

Epithelial ovarian cancer (EOC) is the most lethal gynecological malignancy in western world. Current clinical guidelines recognize eight histological tumor subtypes, which are unique entities with distinct biological behavior and outcome. On the basis of genetic changes, a dualistic model of pathogenesis has been proposed: type I EOC includes genetically stable and relatively indolent, low grade tumors, type II are high-grade serous and endometrioid carcinomas, aggressive, genetically unstable and frequently mutated in TP53.

The aim of this study was to develop a panel of patient-derived EOC-xenografts (EOC-PDX) that preserve the characteristics of the original patient tumor and recapitulate the molecular and biological heterogeneity of the disease.

Thirty-four EOC-PDX were successfully established subcutaneously or intraperitoneally in nude mice. They comprised all the EOC subtypes and were histologically similar to the original patient tumor. After orthotopic transplantation in the bursa of mouse ovary, EOC-PDX disseminate into the organs of the peritoneal cavity and produce ascites. Genome-wide gene expression analysis and mutational status of EOC-PDX indicate a high degree of similarity with the original patient tumors and discriminate different subsets of xenografts. EOC-PDX are differently responsive to cisplatin and paclitaxel, resembling the clinical situation of ovarian cancer.

This panel of EOC-PDX that recapitulates the heterogeneity of ovarian cancer and retains the phenotypic and genomic characteristics of the original tumors is an ideal preclinical model to study the biology of ovarian cancer, identify tumor-specific molecular markers and develop novel treatment modalities.

L 3
A MOUSE MODEL THAT DEVELOPS LIVER CANCER IN A CIRRHOSIS BACKGROUND

Elisa Callegari [1] - Marco Domenicali [2] - Lucilla D'abundo [1] - Ferdinando Giannone [2] - Cristian Bassi [1] - Bahaeldin K. Elamin [3] - Barbara Zagatti [1] - Manuela Ferracin [1] - Francesca Fornari [4] - Laura Gramantieri [4] - Silvia Sabbioni [5] - Massimo Negrini [1]

Dipartimento di Morfologia, Chirurgia e Medicina Sperimentale, Università di Ferrara, Ferrara, Italy [1] - Unità di Semeiotica Medica, Policlinico S. Orsola-Malpighi, Dipartimento di Scienze Mediche e Chirurgiche, Universita di Bologna, Bologna, Italy [2] - Department of Microbiology, Faculty of Medical Laboratory Sciences, University of Khartoum, Khartoum, Sudan [3] - Centro di Ricerca Biomedica Applicata e Dipartimento di Medicina Interna, Policlinico S. Orsola-Malpighi e Università di Bologna, Bologna, Italy [4] - Dipartimento di Scienze della Vita e Biotecnologie, Università di Ferrara, Ferrara, Italy [5]

Introduction: Hepatocellular carcinoma (HCC) is one the most common cause of cancer-related mortality worldwide. In most of the cases, HCC occurs in a context of cirrhosis, which represents a pre-neoplastic condition. The development of animal models that could mimic this condition represented an important tool to test innovative approaches for preventing or curing liver malignancies. We recently generated a transgenic mouse model over-expressing an oncogenic microRNA, miR-221, in the liver: animals exhibited a strong predisposition to the development of HCC and a significant acceleration in developing liver neoplastic nodules after treatment with a carcinogen. In this study we exposed these mice to carbon tetrachloride (CCl4) administration, in order to establish liver cirrhosis in this animal model, and verify whether the animal develop cancer.

Materials and methods: Carbon tetrachloride (CCl4) was administrated by short inhalation cycles to *wild type* and transgenic TG221 animals, respecting the same conditions in both groups. During cirrhosis induction, all the animals were treated with phenobarbital to enhance CCl4 hepatotoxicity.

Results: Following CCl4 administration, all the animals developed a frank cirrhosis. However, only TG221 mice developed visible neoplastic lesions in 100% of the cases while wild type mice did not. The lesions appeared within 6 months of age and ranged from adenomas to dysplastic adenomas and early carcinomas.

Conclusions: The study established a reproducible mouse preclinical model of hepatocarcinogenesis in a background of cirrhosis, a condition that closer resembles the human condition. This model may be used for testing new approaches for preventing cancer development in a cirrhotic liver background

L 4
DEVELOPMENT OF LYMPHO/MYELOID FEATURES IN NOTCH3 DEPENDENT 'T CELL-ACUTE LYMPHOBLASTIC LEUKEMIA'

Paola Grazioli [1] - Claudia Noce [1] - Sofia Verkhovskaia [1] - Gaia Scafetta [2] - Alberto Gulino [1] - Isabella Screpanti [1] - Antonio Francesco Campese [1]

Dip.to Medicina Molecolare, Sapienza, Università di Roma, Roma, Italy [1] - Dip.to Scienze e Biotecnologie Medico-Chirurgiche, Sapienza, Università di Roma, Roma, Italy [2]

Introduction: Notch receptors deeply influence T cell differentiation and Notch3 constitutive activation inside T cell compartment of transgenic (*N3tg*) mice induces an acute form of T cell leukemia (T-ALL). Recently, a role of Notch signalling in some subset of myeloid tumors expressing T cell markers was suggested. Moreover, a cluster of T-ALL cases with overexpression of myeloid-related genes has been described. Finally, Notch signaling modulation has been related to alterations in differentiation/proliferation of myeloid cells. We aim to define a possible role of a T-cell specific deregulation of Notch signaling on inducing the acquisition of myeloid features both in cis, in-

side tumoral cells, and in trans by influencing the behaviour of myeloid cells.

Materials and methods: We used as in vivo model of T-ALL the *N3tg* transgenic mice, that overexpress the intracellular domain of Notch3 under the control of the *lck* proximal promoter, to drive the transgene expression inside immature tymocyte subset. $CD4^+CD8^+$ (DP) T cells, as well as $Mac1^+Gr1^+$ myeloid cells, were purified from spleen, bone-marrow and thymus of *N3tg* mice and *wt* controls, by 'FACS-assisted cell sorting', to obtain both RNA and total protein samples. Then RNA samples were processed for real-time RT-PCR assay and protein extracts were used in Western-blotting analysis.

Results: On one side, we revealed the overexpression of several transcripts involved in myeloid differentiation (e.g. CEBPalpha, Pu.1, Gata2, granulin, MPO) in peripheral $CD4^+CD8^+$ (DP) T tumoral cells from *N3tg* mice. We also identified a subset of DP *N3tg* T cells showing MPO and CD3 co expression, that specifically define T/myeloid blasts. On the other side, we observed in *N3-tg* mice a significant increase of $Mac1^+Gr1^+$ myeloid cells, with the appearence of Myeloid Derived Suppressor Cells. These cells do not express Notch3, suggesting an influence of this receptor on myeloid compartment in trans.

Conclusions: Altogether, our data reveal an unexpected myeloid trait of the Notch-dependent T-ALL, that may influence the disease outcomes and thus would have an important impact in the search of novel diagnostic tools and on the development of innovative multitargeted therapies.

L 5
ROLE OF P2X7 RECEPTOR IN TUMOR-HOST INTERACTION

Marina Capece [(1)] - Alessia Franceschini [(1)] - Alessandra Rotondo [(1)] - Domenica Corigliano [(2)] - Elena Adinolfi [(1)] - Francesco Di Virgilio [(1)]

Department of Morphology, Surgery and Experimental Medicine, UNIVERSITY OF FERRARA, FERRARA, Italy [(1)] - Department of Science of Health, UNIVERSITY OF MAGNA GRECIA, CATANZARO, Italy [(2)]

Introduction: The P2X7 receptor (P2X7R) is an ion channel belonging to the P2X purinergic family, activated by extracellular ATP. It is chiefly expressed by immune cells, such as macrophages and dendritic cells (DCs). It acts as pro-inflammatory receptor, mediating secretion of mature interleukin-1 beta (IL-1β) and as modulator of immune response.[1] Recently it has been characterized a growth-promoting activity of P2X7R especially in cancer cells.[2] P2X7R is overexpressed by many malignancies[2,3] and supports tumor growth[2] and metastatization *in vivo*[4].

As P2X7R is involved both in tumor growth and in host immune defence, it is challenging to predict which would be the final effect of targeting P2X7R in cancer therapy.

To unravel this question we induced experimental subcutaneous melanoma and lung metastatis formation in P2X7R-KO mice.

Materials and methods: B16 murine melanoma cells were subcutaneously or intravenously inoculated into wild type (wt) and P2X7R-KO syngeneic C57Bl/6. Tumor mass growth was monitored by in vivo caliper measurement, while metastatic spreading was followed by total body luminometer (IVIS Lumina) thanks to cell transfection with intracellular luciferase. Pharmacological treatments were administrated as intra peritoneal injection every two days and two different P2X7R antagonists were used.

Results: Tumor growth and metastatic diffusion were strongly accelerated in P2X7R-KO compared to wt mice. Immunohistochemical analysis of tumors revealed that lymphocytic and macrophagic infiltrate ($CD3^+$ or F4/80 positive cells, respectively) was almost abrogated in P2X7R-KO mice. This inability to recruit immune cells could be due to incapacity of P2X7R-less cells to respond to stimulation by cancer cells. In line with this prediction, IL-1β content of tumors from P2X7R-KO mice was drastically reduced compared to that of wt, IL-1β release from B16-stimulated DCs of P2X7R-KO mice was neglible and chemotaxis of P2X7R-null immune cells was nearly abrogated.

Conclusions: P2X7R is a non redundant factor in anti-cancer immunity.

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L 6
PRE-CLINICAL ASSESSMENT OF MIR-34A-BASED THERAPIES IN A CHRONIC LYMPHOCYTIC LEUKEMIA MOUSE MODEL

Lucilla D'abundo [(1)] - Elisa Callegari [(1)] - Antonella Bresin [(1)] - Cristian Bassi [(1)] - Roberta Roncarati [(1)] - Barbara Zagatti [(1)] - Elena Saccenti [(2)] - Manuela Ferracin [(1)] - Elena Miotto [(1)] - Giandomenico Russo [(3)] - Silvia Sabbioni [(4)] - Massimo Negrini [(1)]

Dipartimento di Morfologia, Chirurgia e Medicina Sperimentale, Università di Ferrara, Ferrara, Italy [(1)] - Sezione di Ematologia e Fisiopatologia dell'Emostasi, Azienda Università Ospedale di Ferrara, Ferrara, Italy [(2)] - Lab di Oncologia Molecolare, Instituto Dermopatico dell'Immacolata, Roma, Italy [(3)] - Dipartimento di Scienze della Vita e Biotecnologie, Università di Ferrara, Ferrara, Italy [(4)]

Introduction: Despite its indolent nature, chronic lymphocytic leukemia (CLL) is an incurable disease. The development of new drugs for CLL has been limited by the lack of stable CLL cell lines for in vitro testing. However, suitable animal models for CLL-like disease are available. A previously described TCL-1 transgenic mouse develops a chronic B-cell CD5+ leukemia similar to an aggressive form of human B-CLLs, which represents an useful in vivo tool to screen new drugs. Dysregulation of miRNAs plays an important role in the pathogenesis of CLL, thus they are attractive candidates as therapeutic targets for CLL. We investigated the use of miR-34a as a new therapeutic approach against CLL.

Materials and Methods: Mice were treated with 100 μg of specific single strand miR-34a mimic three times a week for 3 weeks. To analyze the progression of leukemia, the human TCL-1 gene was quantified from genomic DNA of murine blood cells by Real-Time PCR. In addition the apoptotic effects

of miR-34a on mice tumoral splenocytes were assessed by Muse™ Annexin V.

Results and Discussion: Mice developed an aggressive CD5+ B cell leukemia characterized by spleno- and hepatomegaly associated with high counts of white blood cells. In vivo treatments with miR-34a increase the apoptosis of leukemic spleen cells, which could be related to the modulation of multiple targets. In addition, a 3-weeks treatment with miR-34a mimics could elicit a measurable anti-leukemic effect and extend the overall survival.

Conclusion: Our pre-clinical assessment of miRNA-based therapies in a CLL mouse model proves an anti-leukemic activity of miRNA-34a as single agent, and suggests that the use of microRNAs could represent a novel potential therapeutic approach for the treatment of CLL.

L 7

C-KIT EXPRESSION, ANGIOGENESIS AND GRADING IN CANINE MAST CELL TUMOUR: A UNIQUE MODEL TO STUDY C-KIT DRIVEN HUMAN MALIGNANCIES

Rosa Patruno [1] - Rosa Angela Rubini [1] - Anna Grazia Fortunato [1] - Gennaro Capriuolo [1] - Claudia Gadaleta [2] - Marcello Introna [2] - Nicola Zizzo [2] - Michele Ammendola [3] - Domenico Ribatti [4] - Ilaria Marech [5] - Cosmo Damiano Gadaleta [5] - Girolamo Ranieri [5]

Department of Prevention, ASL BAT, Barletta, Italy [1] - Chair of Pathology, Veterinary Medical School, University of Bari, Valenzano, Italy [2] - Chair of Clinical Surgery, University of Catanzaro "Magna Graecia" Medical School, Catanzaro, Italy [3] - Department of Basic Medical Sciences, Neurosciences and Sensory Organs, Section of Human Anatomy and Histology, University of Bari Medical School, Bari, Italy [4] - Interventional Radiology Unit with Integrated Section of Translational Medical, Oncology National Cancer Research Centre "Giovanni Paolo II", Bari, Italy [5]

Introduction: Canine cutaneous mast cell tumour (CMCT) is a c-Kit driven tumour sharing similar c-Kit aberrations found in human gastrointestinal stromal tumour. CMCT is classified in three forms: well (G1), intermediately (G2) (a more benign diseases) and poorly(G3) differentiated (a malignant form). CMCT seems to be a useful spontaneous tumour model to study the role of mast cells in tumor angiogenesis and to evaluate *in vivo* model novel targeted therapies.

Materials and methods: Here we assess a correlation between c-Kit status, grading and angiogenesis in CMCTs to explore their potential significance in humans. C-Kit receptor (c-KitR) expression, microvascular density (MVD), mast cell granulated and degranulated status density (MCGD and MCDD respectively) were analyzed in 97 CMCTs, by means of histochemistry, immunohistochemistry double staining and image analysis system.

Results: Data showed that predominantly diffuse cytoplasmic and predominantly focal paranuclear (Golgi-like) c-Kit protein (PDC-cKit and PFP-c-Kit respectively) expression correlates with high MVD, G3 histopathological grade and MCDD. Moreover, predominantly cell membrane c-KitR

(PCM-c-KitR) expression status correlates with low MVD, G1-G2 histopathological grade and MCGD.

Conclusions: These findings underline the key role of c-Kit in the biopathology of canine MCTs, indicating a link between aberrant c-Kit expression, increased angiogenesis and higher histopathological grade. CMCT seems to be a model to study contributes of c-Kit activated MCs in tumour angiogenesis and to evaluate the inhibition of MCs activation by mean c-Kit tyrosine kinase inhibitors, currently translated in humans.

L 8

A SPECIFIC C-MET INHIBITOR (JNJ-38877605) REDUCES BONE METASTASES INDUCED BY KIDNEY CANCER STEM CELLS IN A HUMAN-IN-MICE MODEL

Ilaria Roato [1] - Livio Trusolino [2] - Lucia D'amico [1] - Giorgia Migliardi [2] - Roberta Pulito [1] - Timothy Perera [3] - Paolo M. Comoglio [2] - Riccardo Ferracini [4]

CeRMS, (Center for Research and Medical Studies), A.O. Città della Salute e della Scienza di Torino, Torino, Italy [1] - IRCC, University of Torino, Torino, Italy [2] - Janssen Research and Development, Janssen, Beerse, Belgium [3] - Department of Orthopedics, A.O. Città della Salute e della Scienza di Torino, Torino, Italy [4]

Introduction. Skeleton is a common site of metastasis for 20-35% of kidney cancer patients. Bone metastases from kidney cancer are particularly destructive, with a rate of skeletal complications higher than other tumors. The cross-talk between tumor cells and the bone microenvironment leads to bone metastasis. Recently a direct action of cancer stem cells (CSCs) in promoting bone metastasis formation has been demonstrated. Therapeutic strategies to block CSCs and their interaction with bone microenvironment are currently under investigation. One of the target candidate gene is c-MET, the hepatocyte growth factor receprtor (HGF), which has been demonstrated to be a mediator of the interaction between cancer cells and mesenchymal cells of the bone microenvironment, promoting bone metastasis. This work aims to the ability of a c-MET inhibitor, JNJ-38877605, to interfere with the bone metastatic process induced by kidney CSCs in a human-in-mice model of bone metastasis. Materials and Methods. NOD/SCID mice, previously implanted with a small fragment of human bone, were injected with kidney CSCs (CD105+), expressing luciferase, and their in vivo localization was monitored. Mice were daily treated or not with JNJ-38877605 for 90 days after their injection of the kidney CSCs. Implanted bone, lungs and blood were retrieved and analysed. In vitro cultures of human osteoclasts and osteoblasts, stimulated or not with JNJ-38877605, were performed. Results. Bone colonization by CSCs was detectable only in mice untreated with JNJ-38877605. Tumor cells were detectable in the bone microenvironment of untreated mice, whereas in treated mice CSCs did not metastasize the bone implant. Preliminary data derived from histomorphometry analysis on bone showed an increased bone turnover induced by the presence of CSCs and the treatment with JNJ-38877605. In particular, in the bone microenvironment of mice treated with JNJ-38877605 there was a reactive fibrous scarring, which is typically present in diseases characterised by an increased bone turnover. In vitro data reported an inhibition of osteoclastogenesis by JNJ-38877605. Since IL-11 and CCL20 might be important for the CSCs-induced bone metastasis, they were dosed in mice sera and their levels resulted reduced in mice treated with JNJ-38877605. Conclusions.

Our preliminary results highlighted the ability of this c-MET inhibitor to reduce bone metastasis formation and progression.

Infectious Agents And Cancer

M 1
CROSS TALK BETWEEN EBV AND TELOMERASE: THE ROLE OF TERT IN THE SWITCH OF LATENT/LYTIC CYCLE OF THE VIRUS

Silvia Giunco [1] - Andrea Celeghin [2] - Stefano Indraccolo [1] - Riccardo Dolcetti [3] - Anita De Rossi [4]

Istituto Oncologico Veneto-IRCCS, IOV-IRCCS, Padova, Italy [1] - Department of Surgery, Oncology and Gastroenterology-Section of Oncology and Immunology, University of Padova, Padova, Italy [2] - Cancer Bio-Immunotherapy Unit, CRO-IRCCS, National Cancer Institute, CRO-IRCCS, National Cancer Institute, Aviano (PN), Italy [3] - Department of Surgery, Oncology and Gastroenterology-Section of Oncology and Immunology / Istituto Oncologico Veneto-IRCCS, University of Padova / IOV-IRCCS, Padova, Italy [4]

Introduction: EBV-associated malignancies *in vivo*, as well as lymphoblastoid cell lines (LCLs), obtained *in vitro* by EBV infection of B cells, selectively express latent viral proteins and maintain the ability to grow indefinitely through inappropriate activation of TERT, the catalytic component of telomerase. Our studies demonstrated that a high level of TERT expression in LCLs prevents the EBV lytic cycle, which is instead triggered by TERT silencing (*Giunco et al, Clin Cancer Res 2013;19:2036*). As the lytic infection promotes the death of EBV-positive tumor cells, understanding the mechanism(s) by which TERT affects the latent/lytic status of EBV may be important for setting new therapeutic strategies. BATF, a transcription factor activated by NOTCH2, the major NOTCH family member in B cells, negatively affects the expression of BZLF1, the master protein of the viral lytic cycle; therefore, we analyzed the interplay between TERT, BATF, and NOTCH2 in LCLs.

Materials and methods: Cells expressing ectopic TERT were obtained by infecting TERT-negative LCL with a retroviral vector expressing TERT (pBABE-hTERT). Cells expressing ectopic NOTCH2 intracellular domain (NOTCH2-IC) were obtained by infecting TERT-negative LCL with retroviral vectors expressing NOTCH2-IC (pMigRI-ICN2 or pMSCVpuro-ICN2). TERT levels and activity were assayed by real-time PCR and TRAP assay respectively. NOTCH2 and BATF expression were analyzed by real-time PCR and western blot. Transcriptional activity of TERT on NOTCH2 promoter was assessed by luciferase assay on HC116 cells by cotrasfecting NOTCH2 promoter (pGL3-TATA-N2PR-2327/-99) and TERT (pEGFP-hTERT).

Results: LCLs with high levels of endogenous TERT showed higher NOTCH2 and BATF expression compared to LCLs with low level of TERT expression. Ectopic expression of TERT in LCLs with low/absent level of endogenous telomerase was accompanied by the increase of NOTCH2 and BATF at both mRNA and protein levels. By contrast, infection of LCLs with retroviral vectors expressing NOTCH2-IC did not induce changes in TERT transcription. Luciferase reporter assays demonstrated that ectopic expression of TERT activated NOTCH2 promoter in a dose-dependent manner, while NOTCH2-IC did not activate the TERT promoter.

Conclusions: TERT activates NOTCH2 at transcriptional level and modulates lytic/latent status of EBV through the NOTCH2/BAFT pathway.

M 2
SIGNIFICANT HIGH PREVALENCE OF ANTIBODIES REACTING WITH SIMIAN VIRUS 40 MIMOTOPES IN SERA FROM PATIENTS AFFECTED BY GLIOBLASTOMA MULTIFORME.

Mauro Tognon [1] - Fernanda Martini [1] - Elisa Mazzoni [1] - Massimo Gerosa [2] - John Charles Rotondo [1]

University, Ferrara, Ferrara, Italy [1] - University, Verona, Verona, Italy [2]

Introduction: Glioblastoma multiforme (GBM) is a rare tumor, which affects 1/100,000 individuals. GBM, although characterized by genotypic and phenotypic heterogeneities, invariably resists to conventional chemo- and radio-therapies. The extremely grim prognosis of GBM patients probably explains why such a rare tumor account for 2% of all cancer related deaths both in Europe and U.S.A.. GBM is considered, like other tumors, a genetic disease of the somatic cell due to an impressive series of alterations of the cellular genome, such as point mutations, gene amplifications, translocations, deletions, as well as epigenetic modifications, including methylation of specific genes. A limited percentage (5%) of GBM is linked to genetic predispositions, which are mainly associated with rare hereditary syndromes, such as Li-Fraumeni syndrome, Turcot syndrome, Peutz-Jegher syndrome, Neurofibromatosis 1 and 2. Simian Virus 40 (SV40), a small DNA tumor virus, has been found in GBM specimens by some studies, while other investigations did not confirm the association.

Materials and methods: An indirect enzyme-linked immunosorbent assay (ELISA) with two synthetic peptides mimicking SV40 antigens of viral capsid proteins 1-3 was employed to detect specific antibodies against SV40 in serum samples from GBM affected patients, together with controls represented by patients affected by breast cancer and normal subjects, with the same median age.

Results: Our data indicate that in serum samples from GBM affected patients (n = 44) the prevalence of antibodies against SV40 VPs antigens is statistically significant higher (34%, P=0.016 and P=0.03) than in the control groups (15%), represented by healthy subjects (n=101) and patients affected by breast cancers (n=78), respectively. Analyzed sera remain positive at a 1:160 or 1:80 dilution, indicating that the title of SV40 antibodies does not greatly differ in normal individuals and GMB patients. The SV40 cytopathic effect was abolished or hampered bu immnune sera suggesting that they were positive for neutralizing antibodies

Conclusion: Our data indicate that SV40, or a closely related yet undiscovered human polyomavirus, is associated with a subset of glioblastoma multiforme and circulates in humans. Our results can be transferred to the clinical oncology application to discriminate different types of the heterogeneous glioblastoma multiforme, which in turn may address an innovative therapeutic approach to this fatal cancer.

Innovative Therapeutic

N 1
ANTITUMOR EFFECTS OF HIV-PROTEASE INHIBITORS ON CERVICAL CANCER

Elisabetta Bandiera [1] - Chiara Romani [1] - Renata Tassi [1] - Paola Todeschini [1] - Laura Zanotti [1] - Laura Tassone [1] - Eliana Bignotti [1] - Germana Tognon [2] - Franco Odicino [2] - Enrico Sartori [2] - Antonella Ravaggi [1]

Laboratorio Nocivelli, Università di Brescia, Brescia, Italy [1] - Divisione di Ginecologia e Ostetricia, Università di Brescia, Brescia, Italy [2]

Introduction: Despite the use of screening programs and therapy, invasive cervical carcinoma (ICC) remains the fifth most deadly cancer among women worldwide. Recent data indicate that HIV-protease inhibitors (HIV-IPs), Indinavir, Ritonavir and Saquinavir affect tumor cell metabolism, such as proteasome activity. The aim of this study was to evaluate the antitumoral effects of these HIV-IPs in ICC-cell lines.

Material and Methods: For this study we used 6 ICC-cell lines: 2 obtained from patients and 4 commercially available (Caski, Hela, HT3 and C33A). The media containing drugs at different concentrations (5-80μM) or DMSO/H_20 (as negative control) were added to the cell culture every day for different times. Proliferation and clonogenicity were evaluated by crystal violet dye. Proteasomal activity was assessed using the Proteasome-Glo cell-based assay (Promega). Cell cycle was analyzed using propidium iodide DNA staining. BD System was used for invasion assays. For statistical analysis T-test was used.

Results: For all drugs and for all ICC-cell lines, the inhibition of cell growth was directly proportional to the drug concentration and to the exposure time. Saquinavir was more effective than Ritonavir and Indinavir in all CCC lines and at all times. The treatment whit Saquinavir 40μM for 96 hours inhibited cell proliferation by 90-100% ($p<0.05$).

Saquinavir is more effective than Ritonavir and Indinavir to modulate the proteasome activities in all ICC-cell lines. The treatment whit Saquinavir 80μM for 2 hours inhibited proteasome activities by 10-50% ($p<0.05$).

We selected Saquinavir, as the most effective drug, and Hela cells, as the more susceptible cell line, to proceed with cell invasion, cell cycle and clonogenicity analysis, using two drug concentrations corresponding to IC50 (calculated in proliferation assay) and to plasma peak level detectable in treated HIV patients (19 and 10μM respectively).

The treatment with Saquinavir for 96 hours inhibited cell invasion by 23% for Saq 10μM and 61% for Saq 19μM ($p<0.05$).

The treatment with Saquinavir for 24 hours determined a cell accumulation in G0-G1 phase of 5 and 10 for Saq 10 and 19μM respectively ($p<0.05$). The treatment with Saquinavir for 96 hours inhibited the clonogenicity by 39% for Saq 10μM and 90% for Saq 19μM ($p<0.05$).

Discussion: These results indicate that Saquinavir can be able to consistently reduce proliferation, proteasome activities, cell invasion, cell cycle and clonogenicity in ICC cell lines.

N 2
PRECLINICAL ACTIVITY OF THE LIPOSOMAL CISPLATIN LIPOPLATIN IN CERVICAL AND OVARIAN CANCER

Naike Casagrande [1] - Marta Celegato [1] - Cinzia Borghese [1] - Maurizio Mongiat [1] - Alfonso Colombatti [1] - Donatella Aldinucci [1]

Department of Experimental Oncology 2, CRO Aviano National Cancer Institute, Aviano PN, Italy [1]

Introduction: Cisplatin-based chemotherapy improves survival in cervical and ovarian cancer; however, treatment is associated with tumor resistance and significant toxicity.

Lipoplatin is a liposomal encapsulated form of cisplatin, able to evade immune surveillance and to concentrate passively in tumors after intravenous administration. Lipoplatin is one of the most promising liposomal platinum drug formulations under clinical investigation; it has been successfully administered in randomized phase II and III clinical trials of lung cancer showing high response rates and reduced toxicity compared to cisplatin.

Materials and methods: The activity and mechanism of action of lipoplatin were studied in HeLa, cisplatin-sensitive cervical cancer cells ME-180, its cisplatin-resistant clone R-ME-180, and in a panel of ovarian cancer cell lines with different histology origins and cisplatin-sensitivity (A2780, A2780cis, MDAH, OVCAR3, OVCAR5, SKOV3 and TOV21G). We used cell proliferation assay, flow cytometry, ELISA assay, cell migration, spheroids and tumor xenograft.

Results: Lipoplatin inhibited the proliferation of cisplatin sensitive and resistant cell lines. It exerted its cytotoxic effect inducing apoptosis and increasing reactive oxygen species (ROS). It inhibited the enzymatic activity of thioredoxin reductase, an enzyme involved in ROS detoxification and over-expressed in many tumor cells contributing to drug resistance. Lipoplatin reduced EGFR expression and inhibited both migration and invasion.

Multiple drug treatment is commonly used in chemotherapy. Lipoplatin demonstrated a synergistic effect when combined with doxorubicin, widely used in relapsed ovarian cancer treatment, and with the albumin-bound paclitaxel, abraxane.

Tumor therapy resistance has been attributed to cancer stem cells (CSC). Lipoplatin treatment reduced the expression of the CSC markers CD133 and ALDH in a dose-dependent manner

and inhibited spheroid formation. It decreased ovarian cancer formed spheroid growth, vitality and migration.

Finally, lipoplatin inhibited cisplatin-resistant R-ME-180 tumor xenografts growth of 70%. In OVCAR5 xenografts we observed a reduction of more than 90% of tumor volume, with minimal systemic toxicity, and without tumor progression after treatment suspension.

Conclusions: These promising preclinical data suggest lipoplatin as a novel chemotherapeutic agent for the treatment of cisplatin-resistant recurrent cervical and ovarian cancer.

N 3
THE REPURPOSED DRUG AURANOFIN INDUCES APOPTOSIS, INHIBITS NF-KB AND EXERTS A POTENT ACTIVITY AGAINST CLASSICAL HODGKIN LYMPHOMA TUMOR XENOGRAFTS

Marta Celegato [1] - Cinzia Borghese [1] - Naike Casagrande [1] - Maurizio Mongiat [1] - Alfonso Colombatti [1] - Donatella Aldinucci [1]

Department of Experimental Oncology 2, CRO Aviano National Cancer Institute, Aviano, PN, Italy [1]

Introduction: Classical Hodgkin lymphoma (cHL) is considered a highly curable disease; however, 20% of patients cannot be treated with standard first-line chemotherapy and have a bad outcome.

Therefore, new drugs or drug combinations are needed to reduce the toxicity of first-line treatments and to increase the efficacy for refractory/resistant patients.

Drug repurposing, the discovery of new activities for old clinically used drugs, has been proposed as an additional strategy for drug development.

Auranofin (AF), an oral gold-containing trietylphosphine, was previously approved for use in treating rheumatoid arthritis. Recently, AF was shown to inhibit thioredoxin reductase (TrxR), increase reactive oxygen species (ROS) and induce apoptosis in cancer cells. AF is now considered a repurposed drug for Cronic Lymphocytic Leukemia (CLL) and phase I and II clinical trials are ongoing at the University of Kansas.

Materials and methods: The activity and mechanism of action of AF was studied in a panel of cHL cell lines (L-1236, L-428, KM-H2, HDLM-2 and L-540) by cell proliferation assays, flow cytometry, ELISA assay, cell migration and tumor xenograft.

Results: AF exhibited a cytotoxic activity in all cHL cell lines, induced apoptosis, caspase 3 activation, both Bcl-2 and Bcl-xL down-regulation and Bax up-regulation and DNA fragmentation.

AF was able to stimulate intracellular ROS generation and to inhibit both selenoenzyme TrxR and proteasome activity.

Aberrant NF-kB activation is a common feature of cHL cells. AF treatment inhibited NF-kB activity and the expression of its target genes, the survival factors IRF4 and CD40.

AF reduced the expression of CD30 and the release of cytokines and chemokines involved in cross-talk and/or formation of the microenvironment: IL-6, IL-13, CCL5, CCL17, TGF-β, TNF-α and VEGF. Accordingly, AF treatment decreased the ability of cHL cell supernatants to recruit peripheral blood mononuclear cells.

Interestingly, AF demonstrated a synergistic effect when used in combination with doxorubicin, gemcitabine and cisplatin, three chemotherapeutic drugs widely used in cHL treatment and was able to overcome both stroma and soluble CD40 ligand mediated drug resistance.

Finally, AF treatment led to an almost complete reduction of L-540 tumor xenograft growth with a minimal systemic toxicity.

Conclusions: Our results indicate that the repurposing AF may represent a new low cost therapeutic strategy in refractory/relapsed cHL patients.

N 4
LENTI-TRAIL TRANSDUCED K562 CELLS PRODUCE HIGHLY PRO-APOPTOTIC, MEMBRANE TRAIL-ARMED EXOSOMES

Paola Squarcina [1] - Buerdek Maja [1] - Claudia Chiodoni [1] - Beatrice Bianchi [1] - Pamela Della Mina [2] - Laura Cantone [3] - Antonello Villa [2] - Agata Cova [1] - Mario Paolo Colombo [1] - Valentina Bollati [3] - Alessandro Massimo Gianni [1] - Licia Rivoltini [1] - Veronica Huber [1]

Fondazione IRCCS, Istituto Nazionale dei Tumori, Milan, Italy [1] - CONSORZIO M.I.A. Microscopy and Image Analysis, Università degli Studi di Milano Bicocca, Monza, Italy [2] - Department of Clinical Sciences and Community Health, Università degli Studi di Milano, Milan, Italy [3]

Introduction: Exosomes have been identified as key players in intercellular cross-talk. Since these vesicles, produced by almost all cell types, share the ability to convey signals and molecules to target cells, they might be exploited as therapeutic tool. In contrast to non-transformed cells, cancer cells might be sensitive to TRAIL-mediated cell death, particularly if the pro-apoptotic ligand is delivered as transmembrane molecule. Thus, we tested whether membrane TRAIL-armed exosomes could represent an efficient tool for delivering death signals to the tumor.

Material and Methods: The poorly immunogenic erythroblastoid cell line K562 was stably transduced with a lentiviral vector containing membrane-bound human TRAIL. After verifying stable surface expression of TRAIL on transduced cells during long-term culture and cell expansion, optimized culture conditions for standardized exosome production were set up. Exosomes were isolated by differential ultracentrifugations from conditioned media after 24h culture in serum-free conditions. Obtained exosome preparations were assessed for dimensions, quantity, exosomal nature and TRAIL expression by NTA, electronmicroscopy, flow cytometry, Western blot and ELISA. The functionality of membrane TRAIL expressed on the surface of exosomes was evaluated *in vitro* by Annexin/PI staining and Caspase 3 activation in flow cytometry of SUDHL4 B-cell lymphoma, KMS11 multiple myeloma and INT12 melanoma cells co-cultured with TRAIL exosomes.

Results: Expression of surface TRAIL on transduced K562 cells was stable during large scale expansion of cells for standardized exosome production. The absence of serum led to an increase of TRAIL mean fluorescence intensity (ΔMFI) from 50 to 300 on K562 cells. Isolated exosomes showed a mean dimen-

sion of 140nm, expressed CD63, Rab5B and Lamp-2 endosomal markers. Exosomal TRAIL was measured as 1ng/µg protein by ELISA and confirmed as 32kDa transmembrane TRAIL protein. *In vitro* experiments demostrated that exosomes induce TRAIL-mediated and dose-dependent apoptosis in SUDHL4 (90±10%, at 100µg/ml), KMS11 (50±14%) and INT12 melanoma (60±15%) cells. mTRAIL Pro-apoptotic activity is retained after freezing and thawing procedures.

Conclusions: Recipient cells stably transduced with membrane-bound human TRAIL by lentiviral vector release exosomes bearing high levels of functional TRAIL. mTRAIL exosomes can be produced and stored according to SOPs suitable for transfer into clinical setting, as a tool for delivering pro-apoptotic signals to tumor site.

N 5
MODULATING LIPID RAFTS TO SENSITIZE B LYMPHOMA CELLS TO TRAIL-INDUCED APOPTOSIS

Paola Matarrese [1] - Matteo Marconi [1] - Barbara Ascione [1] - Laura Ciarlo [1] - Rosa Vona [1] - Lucrezia Gambardella [1] - Tina Garofalo [2] - Valeria Manganelli [2] - Maurizio Sorice [2] - Silvia Laura Locatelli [3] - Carmelo Carlo-stella [3] - Walter Malorni [1]

Department of Theraputic Research and Medicine Evaluation, Istituto Superiore di Sanità, Rome, Italy [1] - Department of Experimental Medicine, Sapienza University, Rome, Italy [2] - Department of Oncology and Hematology, Humanitas Cancer Center, Rozzano (MI), Italy [3]

Introduction: Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) acts as an apoptosis inducer for cancer cells sparing non-tumor cell targets. However, several phase I/II clinical trials have shown limited benefits of this molecule. Here we investigated whether cell susceptibility to TRAIL ligation could be due to the presence of TRAIL death receptors (DRs) 4 and 5 in membrane microdomains called lipid rafts.

Materials and Methods: We performed a series of analyses, either by biochemical methods or fluorescence resonance energy transfer (FRET) technique, on normal cells (i.e. lymphocytes, fibroblasts, endothelial cells), on a panel of human cancer B cell lines as well as on $CD19^+$ lymphocytes from patients with B-chronic lymphocytic leukemia, treated with recombinant soluble TRAIL, specific agonistic antibodies to DR4 and DR5, or $CD34^+$ TRAIL-armed cells.

Results: We found that irrespective to the expression levels of DRs, a molecular interaction between ganglioside GM3, abundant in lymphoid cells, and DR4 was detected in lymphoid cells but was negligible in non-transformed cells and was strictly related to TRAIL susceptibility. Interestingly, lipid raft disruptor methyl-beta-cyclodextrin abrogated this susceptibility whereas the chemotherapic drug perifosine, which induced the recruitment of TRAIL into lipid microdomains, improved TRAIL-induced apoptosis. Interestingly, we also found that cell pretreatment with Minerval, a compound that induces an important increase in the levels of membrane sphingomyelin (SM) and diacylglycerol (DAG) altering lipid rafts composition, also sensitized B lymphoma cells to TRAIL. Accordingly, in *ex vivo* samples from patients with B-chronic lymphocytic leukemia, the constitutive embedding of DR4 in lipid microdomains was associated *per se* with cell death susceptibility, whereas its exclusion was associated with TRAIL resistance.

Conclusions: These results provide a key mechanism for TRAIL-sensitivity in B cell malignances: the association, within lipid microdomains, of death receptor DR4, but not DR5, with a specific ganglioside, i.e. the monosialoganglioside GM3.

These data strongly suggest that lipid microdomains could exert a catalytic role for DR4-mediated cell death and that pharmacological modulation of lipid rafts could represent a useful strategy to sensitize lymphoma cells to the cytokine TRAIL.

N 6
PEPTIDE-NUCLEIC ACIDS TARGETING MIR-221 IN HUMAN GLIOBLASTOMA CELLS

Eleonora Brognara [1] - Enrica Fabbri [1] - Elena Bazzoli [2] - Giulia Montagner [1] - Claudio Ghimenton [3] - Albino Eccher [3] - Cinzia Cantù [4] - Alex Manicardi [5] - Nicoletta Bianchi [1] - Alessia Finotti [1] - Giulia Breveglieri [1] - Monica Borgatti [1] - Roberto Corradini [5] - Valentino Bezzerri [6] - Giulio Cabrini [6] - Roberto Gambari [1]

Department of Life Sciences and Biotechnology, University of Ferrara, Ferrara, Italy [1] - Department of Neuroscience/Division of Neurology/Neurooncology, University Hospital of Verona, Verona, Italy [2] - Department of Pathology and Diagnostics, University Hospital of Verona, Verona, Italy [3] - Laboratory of Molecular Pathology, University Hospital of Verona, Verona, Italy [4] - Department of Chemistry, University of Parma, Parma, Italy [5] - Department of Pathology and Diagnostics, Laboratory of Molecular Pathology, University Hospital of Verona, Verona, Italy [6]

Introduction: Peptide nucleic acids (PNAs) have been largely used as very efficient tools for alteration of gene expression. PNA and PNA-based analogues were proposed as antisense molecules targeting mRNAs, triple-helix forming molecules targeting eukaryotic gene promoters, artificial promoters, decoy molecules targeting transcription factors. Recently, PNAs were demonstrated to be able of altering biological functions of microRNAs, both *in vitro* and *in vivo*. The oncomiR miR-221 is highly expressed in human gliomas, as confirmed in samples of low and high grade gliomas, as well in the cell lines U251, U373 and T98G. Among miR-221 targets, $p27^{Kip1}$ mRNA appears to be one of the most interesting, together with other miR-221 targets, such as PUMA, ICAM-1, TIMP-3 and PTEN.

Materials and methods: In order to alter the biological functions of miR-221, a peptide nucleic acid targeting miR-221 (R8-PNA-a221) was produced and linked to an arginine-rich peptide (R8) to facilitate uptake by glioma cells. The effects of R8-PNA-a221 were analyzed in U251, U373 and T98G glioblastoma cell lines.

Results: R8-PNA-a221 was found to strongly inhibit miR-221 expression. In addition, the effects of R8-PNA-a221 on $p27^{Kip1}$ (a target of miR-221) were analyzed in U251 and T98G cells by RT-qPCR and by Western blotting. No change of $p27^{Kip1}$ mRNA content occurs in U251cells in the presence of PNA-a221 (lacking the R8 peptide), whereas significant increase of $p27^{Kip1}$ mRNA was observed with the R8-PNA-a221. A clear increment of $p27^{kip1}$ protein expression in the sample treated with R8-PNA-a221 was detected. In addition, R8-PNA-a221

was found to increase TIMP-3 expression (another target of miR-221) in T98G cells

Conclusions: Our results suggest that PNAs against oncomiRNA miR-221 might be proposed as useful tools for the development of experimental treatment of human gliomas (Brognara et al. *J Neuro-oncol* 2014).

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N 7
MEMBRANE TRAIL-ARMED EXOSOMES AS A NOVEL AND EFFECTIVE ANTI-TUMOR THERAPY

Veronica Huber [1] - Claudia Chiodoni [1] - Paola Squarcina [1] - Giorgio Mauri [1] - Laura Botti [1] - Ivano Arioli [1] - Barbara Vergani [2] - Antonello Villa [2] - Mario Paolo Colombo [1] - Alessandro Massimo Gianni [1] - Licia Rivoltini [1]

Fondazione IRCCS, Istituto Nazionale dei Tumori, Milan, Italy [1] - CONSORZIO M.I.A. - Microscopy and Image Analysis, Università degli Studi di Milano - Bicocca, Monza, Italy [2]

Introduction: Exosomes are nanovesicular structures produced by virtually all cell types, which share the ability to deliver signals and molecules to target cells. Exosomes might be exploited as therapeutic tool in cancer, to convey defined signals to tumor site. In the present study we tested whether membrane TRAIL-armed exosomes could transfer death signals to tumor cells in murine xenograft models.

Material & Methods: Membrane TRAIL-armed exosomes were fractioned in large scale from conditioned media of K562 cells, stably transduced with human TRAIL. Exosomes produced from K562 cells transduced with mutNGFR were used for comparison. After ascertaining their pro-apoptotic activity *in vitro*, TRAIL exosome preparations were tested *in vivo*, in SCID mice subcutaneously injected with SUDHL4 B cell lymphoma, KMS11 multiple myeloma and INT12 melanoma cells. Tumor growth was monitored by caliper measurement and VEVO ultrasound. In some cases animals were sacrificed 24h after the last treatment for *ex vivo* flow cytometry analyses of tumor apoptosis and immunohistochemistry (HE and Tunel staining). To evaluate the homing of TRAIL exosomes to tumor site *in vivo*, exosomes were labeled with the fluorescent dyes PKH26 or SP-DiOC$_{18}$(3) prior to injection. Mice were treated intra-tumorally and i.v. and sacrificed 24h later. Tumor lesions were extracted for confocal microscopy, *ex vivo* apoptosis and immunohistochemistry analysis.

Results: The treatment of tumor bearing mice with TRAIL exosomes reflected the sensitivity of the tumor cells to TRAIL exosomes measured *in vitro*. Intra-tumor injection of TRAIL exosomes, at the dose of 200μg/mouse, for 4 times every 48h, led to a significant reduction of SUDHL4 growth, with 70±10% inhibition with respect to mice injected with control exosomes or PBS. KMS11 and INT12 melanoma tumor growth was also reduced by 40±8% and 40±10%, respectively. Systemic administration of mTRAIL exosomes at the same dose and schedule resulted in an early growth arrest of SUDHL4 tumors, detectable after the second injection and retained for a week upon treatment suspension. Growth inhibition or perivascular necrosis were detected in INT12 and KMS11 lesions, respectively, although to a less extent than in SUDHL4 model. No toxicity on vital organs, including liver, was instead measurable. Homing of TRAIL exosomes to tumor site was demonstrated by the detection of fluorescent exosomes in extracted SUDHL4 tumor lesions 24h after i.v. injection.

Conclusions: TRAIL exosomes appear as an effective tool for the induction of apoptosis of cancer *in vivo*. They might thus become a feasible tool for delivering pro-apoptotic signals to tumor site and contribute to disease control.

Molecular Epidemiology And Chemoprevention

O 1
FENRETINIDE (4HPR) IS ABLE TO TARGET PARENTAL AND CANCER-INITIATING MEDULLOBLASTOMA CELLS

Barbara Bassani [1] - Antonino Bruno [1] - Katiuscia Dallaglio [2] - Arianna Pagani [3] - Elisa Principi [1] - Douglas Noonan [3] - Adriana Albini [2]

Fondazione MultiMedica Onlus, Fondazione MultiMedica Onlus, Milan, Italy [1] - IRCCS "Tecnologie Avanzate e Modelli Assistenziali in Oncologia", Arcispedale S. Maria Nuova, Reggio Emilia, Italy [2] - Department of Biotechnology and Life Sciences, University of Insubria, Varese, Italy [3]

Introduction: Medulloblastoma (MB) is a neuroectodermal tumor arising in the cerebellum that represents the most frequent malignant childhood brain tumor. Current treatments for MB include surgery and combination radio- and chemo-therapy, leading to severe side effects, thus novel therapeutic strategies are urgently needed. N-(4-Hydroxyphenyl)retinamide (4HPR, fenretinide), a synthetic analog of all-trans retinoic acid, has emerged as a promising and well-tolerated cancer chemoprevention and chemotherapy agent.

Materials and methods: Primary and metastatic medulloblastoma cell lines (MB-DAOY and ONS-76, respectively) were used to assess the ability of 4-HPR to affect cell proliferation and survival. Flow cytometry analysis was performed to assess 4-HPR induction of apoptosis and oxygen reactive species (ROS) production, as well as cell cycle effects, on MB cell lines. Moreover, functional analysis to determine whether 4-HPR is able to decrease MB chemotaxis and invasion were performed. The main pathways affected by 4-HPR treatment, including b-catenin/wint3a, were determined by western blot analysis. The ability of 4-HPR to target MB- cancer-stem-like cells was evaluated by flow cytometry and quantitative real-time PCR and to inhibit MB tumor growth in vivo in nude mice.

Results: We show that 4HPR induces caspase-dependent cell death in (DAOY and ONS-76) medulloblastoma cell lines associated with increased ROS generation, suggesting that free radical intermediates might be directly involved. 4HPR induces cell cycle arrest in G1/S phase, inactivated β-catenin and inhibited migration and invasion of MB cells. We observed inhibition of

transcription factor activation in both cell lines, affecting pathways frequently over-expressed in MB. 4HPR inhibited spheroid formation by both DAOY and ONS-76, known to be enriched in the tumor stem/initiating cell component, in terms of size, invasive/migratory properties as well as Oct-4, Sox-2 and Nanog expression levels. This inhibition was associated with decreased levels of $CD133^+$ and $ABCG2^+$cells, markers of cancer initiating cells. *In vivo* tumor growth of MB cell lines was inhibited by 4HPR.

Conclusions: Our data suggest that 4HPR also targets the tumor initiating cell population. Since 4HPR exerts low toxicity, it could represent a valid molecule in the management of human MB.

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O 2
ANTI ANGIOGENIC AND ANTI TUMOUR ACTIVITIES OF OLIVE MILL WASTEWATERS

Teresa Rossi [1] - Antonino Bruno [2] - Annarita Cantelmo [2] - Barbara Bassani [2] - Sara Canali [2] - Adriana Albini [1]

IRCCS "Tecnologie Avanzate e Modelli Assistenziali in Oncologia", Arcispedale S. Maria Nuova, Reggio Emilia, Italy [1] - Fondazione MultiMedica Onlus, Fondazione MultiMedica Onlus, Milan, Italy [2]

Introduction: Angiogenesis is a crucial event for cancer progression, since a cancer-associated bloodstream provides nutrients and oxygen to cancer cells and represent the "roadways" through transformed cells can invade distant organs and tissues. Several diet derived compounds have been reported to exert anti-oxidant, anti-proliferative, anti-angiogenic and pro-apoptotic effects in a variety of cancers, including leukemia, prostate, breast, colon, brain, melanoma, and pancreatic tumors. Further, they also affect cellular metabolism, targeting cancer cells though their metabolic derangements as well.

The consumption of extra virgin olive oil represents an important constituent of the Mediterranean diet. Compared to other vegetable oils, the presence of several phenolic antioxidants, including hydroxytyrosol, in olive oil is believed to prevent the occurrence of a variety of pathological processes, including cancer. While the strong antioxidant potential of these molecules is well characterized, their anti-angiogenic activities remain unknown. Here we assessed the anti-angiogenic and anti-tumor properties exerted by extracts from olive mill wastewaters (OMWs) *in vitro* and *in vivo*.

Material and methods: OMWs ability to affect cell proliferation and survival were evaluated on human umbilical vein endothelial cells (HUVECs) and murine C26 colorectal carcinoma cells with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, while the induction of apoptosis and reactive oxygen species were assessed by flow cytometry. Further, functional studies evaluated the capacity of OMWs to interfere with endothelial cell tube formation, migration and invasive activities were performed by morphogenesis and Boyden chamber assays. Finally, the inhibition of angiogenesis and tumor cell growth was evaluated *in vivo*, by the matrigel sponge assay and on tumor xenograft growth.

Results: OMWs were able to inhibit both HUVEC and C26 cell growth in a dose dependent manner, exerting a stronger inhibitory effect as compared with purified hydroxytyrosol alone. This effect was directly associated with the induction of apoptosis and oxygen reactive species (ROS) on HUVECs. Moreover, OMWs were able to inhibit HUVEC migration and invasive abilities in a dose dependent manner. Finally, OMWs inhibited tumor angiogenesis and C26 tumor cell growth *in vivo*.

Conclusions: Taken together, OMWs, which represent a waste product from olive oil industry, could represent a valid source of polyphenols to be used for and angiopreventive approaches with natural compounds.

O 3
DIET DERIVED FLAVONOIDS: ANALYSIS OF ANTI-ANGIOGENIC ACTIVITY AND POTENTIAL ROLE IN TUMOR PREVENTION OF A BEER HOP DERIVATIVE

Cristina Gallo [1] - Teresa Rossi [1] - Sally Maramotti [1] - Candida Bonelli [1] - Adriana Albini [2]

Laboratory of Translational Research, IRCCS-Arcispedale Santa Maria Nuova, Reggio Emilia, Italy [1] - Department Of Research And Statistics Infrastructure And Laboratory Of Translational Research, Irccs-Arcispedale Santa Maria Nuova, Reggio Emilia, Italy [2]

Introduction: The process of carcinogenesis is sustained by new vessel formation and chemoprevention of angiogenesis (angioprevention) represents a possible strategy to block/reverse the progression of tumors. Chemopreventive drugs are molecules preventing or retarding cancer progression and development. Recently, diet flavonoids have been recognized as chemo- and angiopreventive agents. Among these, Xanthohumol (XN), is the principal prenylated chalcone of the female inflorescence of the hop plant (*Humulus lupulus* L.) with chemo/angiopreventive properties. The biological effects of the chalcones mostly depend on their chemical structure, whose variations influence their anti-tumor effects. In order to identify novel potential chemo/angio-preventive agents, we analysed the effects of eight different synthetic derivatives of XN on tumor cells and normal primary endothelial cells (ECs) proliferation. AMP-activated protein kinase (AMPK), an energy and metabolic sensor, is a key target in chemoprevention, also involved in the regulation of angiogenesis. We therefore investigated the involvement of AMPK in XN effects on ECs.

Materials and methods: Tumor cell lines (A375 (Malignant Melanoma), MCF7 (Breast Carcinoma), PC3-DU145 (Prostate cancer), H29 (Colorectal cancer)), were used as *in vitro* models of highly vascularized and hormone-responsive tumors. Human Umbilical Vein Endothelial Cells (HUVEC), were used as the gold standard for ECs analysis.

We tested the anti-proliferative properties of XN synthetic derivatives by MTT assay. The effects of XN derivatives were compared to those of XN "master molecule", by calculating IC50 values (half maximal inhibitory concentration) at 96h. To assess the molecular mechanism underlying XN effects on ECs proliferation, HUVEC were stimulated with 10uM XN and

lysed after 5-10-30 min and 1 h. AMPK phosphorylation at Thr172 was evaluated by Western blotting.

Results: Overall all synthetic derivatives of XN display a lower IC50 value at 96h as compared to XN master molecule.

Further, XN activated AMPK in a time-dependent manner with a peak of activation after 5 minutes of exposure, up to 1h. Activation of AMPK signalling pathway by XN was confirmed ACC phosphorylation at Ser-79 at the same timepoints.

Conclusions: Since XN and some derivatives are able to inhibit tumor cell proliferation, without compromising ECs functions, they might be good candidates for both tumor chemoprevention and angio-protection. Moreover, preliminary results shown that AMPK seems to be a new target of XN in angioprevention.

O 4
EFFECTS OF METFORMIN ON ENDOTHELIAL AND CANCER CELLS

Tiziana Petrachi [1] - Katiuscia Dallaglio [1] - Antonino Bruno [2] - Cristina Masini [1] - Ulrich Pfeffer [3] - Francesco Bertolini [4] - Douglas M. Noonan [5] - Adriana Albini [6]

Translational Research Laboratory, IRCCS "Tecnologie Avanzate e Modelli Assistenziali in Oncologia", Arcispedale S. Maria Nuova, Reggio Emilia, Italy [1] - Scientific and Technologic Park, IRCCS MultiMedica, MILANO, Italy [2] - Integrated Molecular Pathology, IRCCS AOU San Martino, IST Istituto Nazionale per la Ricerca sul Cancro, GENOVA, Italy [3] - Laboratory of Hematology-Oncology, European Institute of Oncology (IEO), Milano, Italy [4] - Department of Biotechnology and Life Sciences, University of Insubria, Varese, Italy [5] - Department of Research and Statistics Infrastructure, IRCCS "Tecnologie Avanzate e Modelli Assistenziali in Oncologia", Arcispedale S. Maria Nuova, Reggio Emilia, Italy [6]

Background: Epidemiological evidence has suggested that metformin, an anti-hyperglycemic agent commonly used in the treatment of type 2 diabetes, is a potential cancer preventive agent. Anti-angiogenesis represents a key mechanism in cancer prevention, a concept termed angioprevention. Since conflicting data concerning the anti-angiogenic action of metformin are emerging, we elucidate the effects of metformin, on endothelial and tumor cells as well as on angiogenesis. Further, since metformin activates the energy and stress sensor AMP activated kinase (AMPK), we evaluated the involvement of AMPK in these mechanisms.

Materials and methods: We used Human Umbelical Vein Endothelial cells (HUVEC) to test the effects of metformin *in vitro*. We used *in vitro* assays to analyze HUVEC viability, proliferation, expression and functional analysis as well as *in vivo* assays and transcriptomic approaches. In order to test AMPK involvement in this mechanism, we transfected HUVE cells with an AMPK specific siRNA.

Results: We show that metformin inhibits endothelial cell ability to organize into capillary-like networks; this effect is partially dependent on the energy sensor AMPK. Gene expression and proteins profiling revealed paradoxic effects on several angiogenesis associated factors. We found induction of VEGF, COX2 and CXCR4 at the mRNA level and down-regulation of ADAMTS1. Interestingly, antibody array analysis showed essentially opposite regulation of numerous angiogenesis-associated proteins in endothelial and breast cancer cells. We also show that endothelial production of cytochrome p450 family member CYP1B1 was up-regulated by tumor cell supernatants (breast and prostate cancer), while metformin blocked this effect by acting on AMPK. Metformin anti-angiogenic activity was exerted through inhibition of ERK1/2 activation, even in the presence of VEGF, while blocking AMPK activity abrogated this effect. Metformin inhibited angiogenesis induced by VEGF in matrigel pellets *in vivo* and contrasted the increase in microvessel density in obese mice on a high fat diet. Further, it down-regulated the number of endothelial precursor cells from white adipose tissue in obese mice.

Conclusions: Our data show that metformin has an anti-angiogenic activity *in vitro* and *in vivo*, which is associated with a contradictory enhancement of chemokines and other inflammatory pro-angiogenic mediators, as well as a different regulation in endothelial and tumor cells.

Molecular Markers Of Diagnosis, Prognosis And Response To Therapy

P 1
ACTIVITY OF A NEW RUTHENIUM COMPLEX ON A MODEL OF BREAST CANCER

Gretta Veronica Badillo Pazmay [1] - Maura Montani [1] - Fabio Marchetti [2] - Corrado Di Nicola [2] - Riccardo Pettinari [3] - Claudio Pettinari [3] - Alba Hysi [4] - Stefano Ferraro [5] - Cristina Marchini [6] - Manuela Iezzi [4] - Giulio Lupidi [7] - Augusto Amici [6]

School of Bioscience and Veterinary Medicine, University of Camerino, Camerino (MC), Italy (1) - School of Science and Technology, University of Camerino, Camerino, Italy (2) - School of Pharmacy, University of Camerino, Camerino, Italy [3] - Department of Medicine and Aging Sciences. Aging Research Center (CeSI)Università di Chieti. Via dei Vestini,31 –66100 Chieti [4] - Scuola di Scienze Ambientali- Università di Camerino. via S.Agostino 1, 62032 Camerino (MC) [5] - School of Bioscience and Veterinary Medicine, Via Gentile III da Varano 62032 Camerino (MC) [6] - School of Pharmacy, Via S. Agostino 1, 62032 Camerino (MC) [7]

Introduction: Ruthenium shows patterns of antitumor activity and clinical toxicity that are different from those of platinum. In addition, some ruthenium complexes although displaying low cytotoxicity against primary tumors have greater antimetastatic effects mediating the inhibition of metastatic progression[1]. Our efforts are aimed at testing this agent and its derivatives, in a rich group of cell lines representing the complexity of cancer disease and detecting possible mechanism of action [2-4].

Methods: NAMI-A was prepared according to a patented procedure (Mestroni et al., 1998); Cisplatin was obtained by Sigma Chemical Co. (St. Louis, MO); UNICAM1 complex was prepared into our Inorganic Chemistry section of the University of Camerino. We evaluated the effects UNICAM1, NAMI-A and

Cisplatin on cell viability and motility through MTT and Wound-healing assays, respectively. We also investigated the effects on Tumor Growth; by i.p. treatment, on the body weight, retention of Ru and Pt in tissues of interest (histological analysis) and in serum.

Results: UNICAM1, IC50 value, is about 100 times higher than Cisplatin one, demonstrating the absence of toxicity. UNICAM1 proved to inhibit cell motility, with an inhibition of 60% and 75% after 24 and 48h, respectively. In vivo experiments, showed an inhibitory effect of UNICAM1 on tumor growth (q1x6 and q3X4 treatments); particularly with the q3x4 treatment, that looks like Cisplatin effect. In addition, we recovered the presence of Ru (UNICAM 1 and NAMI-A) and Pt (Cisplatin) traces in Serum vs time, and in the kidneys and liver after ~1 month of final dose; demonstrating a high clearance ability for UNICAM1. The body weight decrease was monitored obtaining a physiological course of growth with UNICAM 1 which looks like the group of animals without chemotherapeutic treatment.

Conclusion: The success of Pt-drugs has prompted research on other transition metals, which has led to the identification of gold and ruthenium compounds with high antitumor and antimetastatic activity. Consequently, metallodrugs appear to have the potential to achieve powerful new therapeutic effects, which cannot be induced by organic compounds. It is likely that this field will retain a high level of research interest. New synthesis and studies of metallodrugs are projected to improve the quality of life of patients and play a key role in chemotherapy and radiotherapy within this century.

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P 2

SLAMF1/CD150 IS A SIGNALING RECEPTOR EXPRESSED BY A SUBSET OF CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS CHARACTERIZED BY A FAVORABLE PROGNOSIS.

Cinzia Bologna (1) - Roberta Buonincontri (1) - Sara Serra (1) - Tiziana Vaisitti (1) - Valentina Audrito (1) - Davide Brusa (1) - Davide Rossi (2) - Marta Coscia (3) - Gianluca Gaidano (2) - Silvia Deaglio (1)

Department of Medical Sciences, University of Turin and HuGeF, Turin, Italy (1) - Division of Hematology, Department of Translational Medicine, "Amedeo Avogadro" University of Eastern Piedmont, Novara, Italy (2) - Division of Hematology, AO Città della Salute e della Scienza, Turin, Italy (3)

Human SLAMF-1 (signaling lymphocytic activation molecule) is expressed on hematopoietic cells where it acts as a co-stimulatory molecule through self-interactions. Moreover, it performs as a microbial sensor, regulating bacterial phagosome functions through an ubiquitous cellular autophagic machinery. We investigated the role of SLAMF-1 in human B cells, exploiting chronic lymphocytic leukemia (CLL) as a model. CLL is characterized by the expansion of a monoclonal population of mature B lymphocytes, with a highly variable clinical course.

We demonstrated that SLAMF-1 is expressed at variable levels by CLL cells and marks the subset characterized by a good prognosis. Silencing of SLAMF1 expression in Mec-1 cell line led to a down-modulation of pathways connected to cell death, intracellular vescicle formation and recirculation, as determined by global gene expression analysis. Moreover, the apoptotic response to fludarabine treatment observed in control Mec-1 cells (constitutively SLAMF-1^{+}) was completely lost in silenced cells. Consistent with previous findings, fludarabine activated autophagy in the Mec-1 cells. However, in SLAMF1^{-} cells no modulation of the autophagic flux was highlighted, suggesting that deletion of the molecule is responsible for this phenomenon. In line with this finding, fludarabine responses were different in CLL patients divided according to SLAMF-1 expression, as well as in SLAMF-1^{high} vs ^{low} cells separated through cell sorting from the same patient. Functional experiments confirmed that the engagement of the receptor initiates a signaling cascade that involves the direct interaction with the adaptor molecule Eat-2 and that converges on the activation of the MAP kinases. Prolonged engagement of SLAMF-1 led to the appearance of autophagic vesicles and to the increase of LC3B formation. The modulation of autophagy was mediated by ROS and by the sequential phosphorylation of Jnk1/2 and Bcl-2: the final result was the release of Beclin-1 from Bcl-2 and the consequent assembly of the autophagic complex, including Vps34.

Taken together, these results suggest that SLAMF-1 could represent a novel marker for the subset of CLL patients with an indolent clinical course. These results also suggest a link between the activation of the autophagic process and a milder form of the diseae, with a better response to fludarabine treatment.

P 3
ACTIVATED D16HER2 HOMODIMERS AND SRC KINASE SIGNALING AXIS IS A PREDICTOR OF TRASTUZUMAB BENEFIT.

Lorenzo Castagnoli [1] - Manuela Iezzi [2] - Valentina Ciravolo [1] - Gaia Cristina Ghedini [1] - Tiziana Triulzi [1] - Patrizia Gasparini [1] - Roberta Zappasodi [3] - Patrizia Nanni [4] - Elda Tagliabue [1] - Serenella Pupa [1]

Fondazione IRCCS - Istituto Nazionale dei Tumori, Dept. Experimental Oncology and Molecular Medicine, Milan, Italy [1] - Aging Research Centre, G. d'Annunzio University, Chieti, Italy [2] - Fondazione IRCCS - Istituto Nazionale dei Tumori, "C. Gandini" Medical Oncology, Bone Marrow Transplantation Unit, Milan, Italy [3] - Department of Experimental Pathology, University of Bologna, Bologna, Italy [4]

Introduction: Almost 90% of HER2 positive breast cancers patients (BCs) express the splice variant of HER2 lacking of exon 16 (d16HER2). This deletion promotes the formation of stable activated d16HER2 homodimers. The oncogenic activity and Trastuzumab susceptibility of d16HER2-positive mammary tumors, as well as the relationship of d16HER2 with wild type (WT) HER2-driven pathological and clinical features in human HER2-overexpressing BCs, await clarification. Toward this aim, we compared survival and tumor multiplicity of transgenic (tg) mouse models for the human d16 and WT HER2 isoforms and Trastuzumab activity in mice orthotopically transplanted with derived mammary tumor cell lines transgenically expressing d16 and WT HER2. We also analyzed functional relationship between activated d16HER2 and Src kinase (pSrc) and evaluated in HER2-positive BCs a potential association between the expression of d16HER2 and pSrc and their impact on prognosis.

Materials and methods: We evaluated: d16HER2- and WTHER2-driven tumorigenesis and Trastuzumab activity in tg and parental FVB mice; downstream signaling axes by western blot of spontaneous d16 and WT HER2 tumor lysates; expression of both HER2 isoforms and pSrc in murine and human specimens by immunofluorescence using confocal microscopy. We performed a gene expression analysis of human HER2-positive BCs treated adjuvantly with Trastuzumab on the Illumina Whole-Genome DASL® platform and data were analyzed according to d16HER2 and pSrc expression levels using GSEA bioinformatic tool.

Results: We revealed that d16HER2 significantly accelerates mammary tumorigenesis ($p<0.001$) and responds more efficiently to Trastuzumab ($p<0.001$) compared to WTHER2. Analysis of signaling downstream of d16HER2 and WTHER2 revealed that only activated d16HER2 is significantly functional and directly linked to expression of pSrc. In HER2-positive BCs we found a significant correlation between high levels of d16HER2 and pSrc ($p=0.0016$), suggesting that expression of activated d16HER2 is mirrored in high Src activity, consistent with results in tg mice. HER2-positive BCs who expressed high pSrc-d16HER2 levels exhibited the greatest benefits of Trastuzumab treatment ($p=0.022$).

Conclusions: Our data provide evidence that d16HER2-positive tumors are significantly responsive to Trastuzumab and that high expression of activated d16HER2 and Src represents a signaling axis marking Trastuzumab susceptibility.

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P 4
ROLE OF ZNF224 IN CHRONIC LYMPHOCYTIC LEUKEMIA

Elena Cesaro [1] - Giorgia Montano [1] - Michela Ciano [1] - Dario Bruzzese [2] - Paola Costanzo [1]

Department of Molecular Medicine and Medical Biotechnologies, University of Naples Federico II, Naples, Italy [1] - Department of Medical Statistics, University of Naples Federico II, Naples, Italy [2]

Introduction: Chronic Lymphocytic Leukemia (CLL) is an indolent non-Hodgkin's lymphoma and the most common type of leukemias in adult. It is characterized by the accumulation of lymphocytes with impaired apoptosis.

ZNF224 is a member of the KRAB-zinc finger family of transcription factors, that inhibits the transcription of its target gene through the interaction with KAP1, the universal co-repressor of KRAB-ZFPs. KAP1 in turn recruits activities modifying the chromatin structure to silence gene expression. Also, the transcriptional repression activity of ZNF224 required the methylation of H4R3 by PRMT5, a type II protein arginine methyltransferase, implicated in signaling and transcription regulation. Morever, ZNF224 acts as a transcriptional co-regulator of of the zinc finger protein Wilms'tumor 1 (WT1), thus playing a critical role in the regulation of apoptotic events in chronic myelogenous leukemia. In this study we examine the function of ZNF224 in B cell chronic lymphocytic leukemia. We analyzed the expression levels of ZNF224 and Cyclin D3, a protein with oncogenic potential and a key cell cycle regulatory component, in CLL patients.

Materials and methods: The study was conduced on peripheral blood sample obtained from 60 CLL patients. Patients either were untreated or had received no treatment for leatest one years before the study. A ZNF224 and Cyclin D3 mRNA levels were estimated by Real-Time PCR using SYBR Green

Results: We observed that CLL cells obtained from patient samples exhibited higher level of ZNF224 and Cyclin D3 than lymphocyte from healthy donors ($p<0,001$). Morever, we observed a positive correlation between ZNF224 and Cyclin D3 expression and between ZNF224 and lymphocyte number (ALC) in CLL patients, while, there was no association between ZNF224 and the lymphocyte doubling time (LTD), thus leading us to speculate a survival role for ZNF224 in malignant B-cells. Furthermore, we observed a decrease of ZNF224 by fludarabine treatment, a drug widely used in the treatment of CLL, both in leukemia cells than in CLL patients. This reduction is accompanied by a significant increase of apoptosis in leukemia cells.

Conclusions: Our data suggest that high levels of ZNF224 expression are probably related to B cell malignancies, through the transcriptional regulation of specific genes involved in cell cycle control, as CyclinD3, and/or survival. Further studied are needed to elucidate the role of ZNF224 the pathogenesis of chronic lymphocytic leukaemia, thus paving the way to the development of new therapeutic approach in leukemia.

P 5

EXPRESSION AND PROGNOSTIC ROLE OF CXCR4/CXCL12/CXCR7 AND MTOR PATHWAYS IN NEUROENDOCRINE TUMORS (NETS)

Luisa Circelli [1] - Concetta Sciammarella [2] - Antongiulio Faggiano [2] - Salvatore Tafuto [3] - Luciano Pezzullo [4] - Elsa Guadagno [5] - Maria Luisa Del Basso De Caro [5] - Fabiana Tatangelo [6] - Nunzia Simona Losito [6] - Giovanni Botti [1] - Francesco Izzo [3] - Stefania Scala [1] - Annamaria Colao [2]

Oncological Immunology, National Cancer Institute "Fondazione G.Pascale", Naples, Italy [1] - Department of Clinical Medicine and Surgery, University of Naples,"Federico II", Naples, Italy [2] - Department of Abdominal Oncology, National Cancer Institute "Fondazione G.Pascale", Naples, Italy [3] - Thyroid and Parathyroid Surgery Unit, National Cancer Institute "Fondazione G.Pascale", Naples, Italy [4] - Department of Functional and Biomorfological Science, University of Naples,"Federico II", Naples, Italy [5] - Department of Pathology, National Cancer Institute "Fondazione G.Pascale", Naples, Italy [6]

Background: Neuroendocrine tumors (NETs) are rare and heterogeneous neoplasms with variable biological behavior. The incidence of NETs is about 1-5 cases/100,000/year with a progressive increase of the incidence and prevalence. NETs are optimally treated with surgery and somatosatin analogs (SSA's) to control symptoms but are relatively insensitive to systemic chemotherapy. As a result,patients with advanced unresectable NETs have a poor prognosis. In 2011, two targeted therapies, sunitinib and everolimus were approved in the subset of progressive pancreatic NETs (pNETs). The chemokine receptor CXCR4 has been shown to signal on mTOR pathway in gastric and renal cancer. CXCR4 interacts with the chemokine CXCL12 to exert proliferative and chemotactic effects. CXCL12 was shown to recognise with high affinity the orphan receptor CXCR7. To identify possible prognostic factors and new therapeutic targets in NET patients, the role of the axis CXCR4-CXCL12-CXCR7 and mTor pathway was evaluated.

Methods: 61 human NET were included into the study: 40 gastro-entero-pancreatic (GEP),21 medullary thyroid cancer (MTC). The mRNA was extracted from fresh /paraffin-embedded tissue and CXCR4, CXCL12 and CXCR7 was determined by qRT-PCR.CXCR4,CXCL12 and CXCR7 and mTOR pathway (mTOR,p-mTOR,p-p70S6K,p-4eBP1) was evaluated by immunohistochemistry (IHC) in our cohort. Expression of CXCR4/CXCR7 was also evaluated on NCIH727 (Bronchial-NET), BON (P-NET) and TT (Medullary-Thyroid) cell lines by qRT-PCR and confirmed by Western-Blotting.

Results: CXCR4, CXCR7 and CXCL12 mRNA was significantly overexpressed in tumour as compared to normal tissue, $p<0.001$, $p<0.009$ and $p<0.0013$, respectively. The IHC score of CXCR4($p<0.001$), p-mTOR($p<0.05$), p-4EBP1($p<0.01$), p-S6K1 ($p<0.05$) was significantly higher in G1/G2 tumours, while CXCR7 and CXCL12 score was higher but not significantly in G3 tumour. Preliminary prognostic evaluation suggest that CXCR4 ($p<0.001$), CXCR7 ($p<0.01$), CXCL12 ($p<0.01$), mTOR ($p<0.01$), p-mTOR ($p<0.05$) and p-S6K1 ($p<0.01$) correlate with unfavorable prognosis. CXCR4/CXCR7 expression levels on NET cells are comparable to positive controls.

Conclusions: CXCR4/CXCL12/CXCR7 and mTOR pathways are expressed in NETs and might represent a prognostic factor in these tumors. Concomitant inhibition of CXCR4 and mTOR pathways may improve effectiveness and overcome resistance

P 6

SERUM BIOMARKERS IDENTIFICATION BY NANOPARTICLE TECHNOLOGY IN METASTATIC SOFT TISSUE SARCOMA

Amalia Conti [1] - Claudia Fredolini [2] - Maria Serena Benassi [1] - Davide Tamburro [2] - Giovanna Magagnoli [1] - Weidong Zhou [2] - Lance Liotta [2] - Piero Picci [1] - Alessandra Luchini [2]

Laboratory of Experimental Oncology, Istituto Ortopedico Rizzoli, Bologna, Italy [1] - Center for Applied Proteomics and Molecular Medicine, George Mason University, Manassas, Virginia, United States [2]

Introduction: Soft Tissue Sarcoma (STS) are extremely rare tumors. They are less than 1% of all adult cancers, and 7-10% of pediatric ones. They are also very complex and varied as there are approximately 50 different benign and 50 malignant histotypes.

The identification of circulating cancer biomarkers advances the possibility for an early detection, it is important in the patients' stratification into distinct risk subgroups, to better monitor tumor progression or response to therapy, guiding physicians in choosing the best treatment. However, at present there are no laboratory tests that permit a reliable early detection of STS or to indicate their dissemination. Discovery of new serum protein biomarkers is especially needed for those Soft Tissue Sarcomas which are associated with advanced stage at presentation and poor survival.

Materials and methods: Proteomic technologies are used for global profiling and identification of disease-associated markers in biological fluids, such as serum. The low-molecular-weight proteome (<30 kDa) is considered as a rich source of new potential biomarkers, that often can escape the detection because of the presence of thousands of very abundant proteins in serum. In order to determine whether low-abundant serum proteins (<30 kDa) can be measured and useful for prognostic stratification of STS patients, we analyzed a discovery set of sera from non-metastatic and metastatic STS patients using poly(N-isopropylacrylamide-co-vinylsulfonic acid) hydrogel core-shell nanoparticles with incorporated Cibacron Blue F3G-A. These nanoparticles selectively entrap low molecular weight proteins on the bases of size exclusion and affinity chromatography, protecting them from enzymatic degradation and amplifying the analyte concentration for mass spectrometry (MS) detection.

Results: Differentially abundant candidate peptidome biomarkers that appear to be specific for detection of metastatic compared to non-metastatic STS have been identified revealing the potential utility for this new methodology. Subsequently, the biomarkers identified by MS have been validated on a second set of sera of metastatic and non-metastatic STS patients.

Conclusions: Our data suggest that new proteomic technology in combination with careful statistical analysis appears to be useful to identify novel biomarkers for Soft Tissue Sarcoma. However, subsequent experiments will be crucial for their clinical utility and functional studies.

P 7

SERUM MASS-SPECTROMETRY TEST IN FIRST LINE ADVANCED NSCLC PATIENTS TREATED WITH STANDARD CHEMOTHERAPY REGIMES.

Maria Giovanna Dal Belllo [1] - Carlo Genova [1] - Erika Rijavec [1] - Anna Truini [1] - Angela Alama [1] - Simona Coco [1] - Irene Vanni [1] - Giulia Barletta [1] - Federica Biello [1] - Julia Grigorieva [2] - Krista Meyer [2] - Heinrich Roder [2] - Francesco Grossi [1]

U.O.S. Tumori Polmonari, IRCCS A.O.U. San Martino-IST-Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy [1] - Biodesix, Biodesix, Boulder, United States [2]

Introduction: The mass-spectrometry based serum test VeriStrat® (VS) was shown to be prognostic in various therapies and tumor types and predictive of differential overall survival (OS) benefit for erlotinib vs. chemotherapy (CT) in second line NSCLC setting. The mechanism of action is yet unknown, but thought to be related to the host response of an organism to a tumor. Performance of the test in CT is of clinical interest. We investigated the role of VS in 1st line advanced NSCLC patients (pts) treated with Cisplatin (Cis) or Carboplatin (Carbo) plus Pemetrexed (P).

Materials and methods: VS classification was available for 55 eligible stage IV, pts with non-squamous histology; pts were classified as VS Good (VSG) or VS Poor (VSP), VS testing of pretreatment serum samples was done blinded to clinical data. OS and progression-free survival (PFS) were analyzed by Kaplan-Meier method and compared using log-rank p-values; Cox models were used in multivariate analysis. Association with categorical variables was analyzed by Fisher's exact test.

Results: 36 (65%) pts were classified as VSG and 19 (35%) as VSP. In the overall population, median PFS was 5.7 months (mo) for VSG vs.1.4 mo for VSP (hazard ratio (HR) 0.37 [0.19-0.72], p=0.002); adjusted HR (AHR) 0.38 [0.17-0.86], p=0.021). Median OS was 10.8 mo for VSG vs. 3.4 mo for VSP (HR 0.23 [0.11-0.51], p < 0.001; AHR 0.12 [0.04-0.37], p<0.001). A similar relationship was found in both treatments: In CarboP median PFS in VSG and VSP was 3.8 mo and 2.0 mo respectively (HR 0.38 [0.15-0.94], p=0.030); median OS was 10.8 mo in VSG and 3.4 mo in VSP (HR 0.26 [0.09-0.72], p=0.006). In CisP median PFS was 6.1 mo in VSG and 1.2 mo in VSP (HR 0.40 [0.14-1.12], p=0.070), median OS was 12.3 mo in VSG, 3.8 mo in VSP (HR 0.17 [0.04-0.69], p=0.005).When compared within VS groups, no statistically significant differences between CarboP and CisP was found either for PFS (VSG: p=0.508, VSP: p=0.718) or OS (VSG: p=0.466 , VSP: p=0.522). VS was significantly associated with disease control rate (p=0.003) and trended towards significance for objective response (p=0.085).

Conclusions: In Platinum-based doublet CT, VSP pts had much shorter PFS and OS than VSG . The behavior was similar in CisP and CarboP arms. Further research is needed to find alternative treatments to improve outcomes for VSP pts.

ClinicalTrials.gov Identifier NCT02055144.

P 8

CXCR4/CXCL12/CXCR7 AND TLR2-4 MEDIATED INFLAMMATION IN COLORECTAL LIVER METASTASES

Crescenzo D'alterio [1] - Guglielmo Nasti [2] - Marianeve Polimeno [1] - Alessandro Ottaiano [2] - Luisa Circelli [1] - Fabiana Tatangelo [3] - Francesco Izzo [4] - Rosario Vincenzo Iaffaioli [2] - Stefania Scala [5]

Department of Oncological Immunology, National Cancer Institute, "G. Pascale", Naples, Italy, Napoli, - [1] - Department of Gastrointestinal Medical Oncology, National Cancer Institute, "G. Pascale", Naples, Italy, Napoli, Italy [2] - Department of Pathology, National Cancer Institute, "G. Pascale", Naples, Italy, Napoli, Italy [3] - Hepatobiliary Surgery Unit, National Cancer Institute, "G. Pascale", Naples, Italy, Napoli, Italy [4] - Department of Oncological Immunology, National Cancer Institute, "G. Pascale", Naples, Italy, Napoli, Italy [5]

Introduction: About 65% of all patients with colorectal cancer (CRC) develop distant metastasis, the liver being the most common site (40%) and their presence accounts for at least two thirds of all CRC deaths. It is estimated that approximately 25% of patients with liver-limited disease are resectable with curative intent at the time of detection. Use of first-line appropriate conversion therapy for patients with initially unresectable liver-limited disease may allow subsequent resection in a further 24–54% of patients. Inflammation has emerged as a non-mutational driver of tumor development and progression. Emerging data suggest the central role of chemokine CXCL12 and its cognate receptors CXCR4 and CXCR7 in CRC. influencing aspects such as proliferation, migration and immunoescape. A key signaling mechanism of inflammation utilizes the Toll-like receptor (TLR) family, that recognize a range of microbial agents and endogenous macromolecules released by injured tissue. In the inflammation that occurs during tumorigenesis, the TLR-driven tissue response may promote neoangiogenesis and tumor growth by poorly defined mechanisms. Emerging data suggest that the Toll-like receptor 2 and 4 in the intestinal epithelium of patients with CRC are up-regulated.

Materials and methods: CXCR4, CXCR7, CXCR12, TLR2 and TLR4 expression was determined in 27 biopsies from patients with CRC derived liver metastases (CRLM) uniformly subjected to neoadjuvant treatment according to the regimen Folfiri + Avastin (Nasti, BJC).

Results: Interestingly the follow up discriminated patients in three different risk categories. The CXCR4-CXCL12-CXCR7 and TLR2 and 4 expression was evaluated through real-time quantitative (qRT-PCR), Immunohistochemistry and Western-blot. CXCR4, CXCR7 and CXCL12 and TLR4 differentially expressed in the three prognostic categories being decreased in the tumor tissue patients displaying poor prognosis.

Conclusions: Ongoing studies are characterizing the interactions with the primary tumors and evaluating the expression of other markers modulating the immune response such as the presence of PD-1 expressing lymphocytes.

P 9
CLINICAL RELEVANCE OF CIRCULATING ENDOTHELIAL CELLS AS PROGNOSTIC AND EARLY PREDICTIVE BIOMARKER OF RESPONSE TO ANTI-ANGIOGENIC THERAPY IN RECTAL CANCER PATIENTS

Elena Di Gennaro [1] - Paolo Delrio [1] - Alessandra Leone [1] - Tania Moccia [1] - Eleonora Cardone [1] - Carmen Romano [1] - Antonio Avallon [1] - Alfredo Budillon [1]

Istituto Nazionale per lo Studio e la Cura dei Tumori, "Fondazione Giovanni Pascale"- IRCCS – Italia, Naples, Italy [1]

Background: The benefits of combining bevacizumab (BEV) plus chemotherapy have thus far been rather modest, stimulating interest in developing novel effective combination schedule as well as valid predictive biomarkers.

Recent clinical trials suggested the circulating endothelial cells (CECs) as promising surrogate biomarkers of therapeutic activity of regimens including anti-angiogenic drugs. In this regard, we assessed, by 4-colors flow cytometry, the basal level and the kinetic of CECs count in patients enrolled in a phase II study (BRANCH) evaluating an experimental administration of the anti-VEGF antibody BEV, with preoperative chemo-radiotherapy (BEV 4 days before chemo-radiotherapy), in poor-prognosis locally advanced rectal cancer patients (LARC).

Results: We observed a significant increase of CECs level, defined as CD45-/ CD31+/CD34+, at baseline in cancer patients compared to healty donors, indipendently of pathological response defined as tumor regression grade (TRG). In details, median CECs at baseline in 41 cancer patients was 1.24/µl, range 0-6.01, while in 37 healty donors median was 0/µl, range 0-3.97 ($p < 0.001$).

Interestingly, basal CECs count did not correlate with prognostic markers such as CEA, circumferential resection margin and class of risk.

Moreover, we demonstrated a correlation between CECs level during treatment and pathological response. Specifically, in TRG1-2 patients CECs were significantly reduced on day 10 *vs* baseline (median 0.42/µl, range 0-1.74; $p < 0.001$). This pattern was not statistically significant in TRG3-4 patients ($p = 0.073$). On the contrary, preoperative CECs levels were not predictive of pathological response, in both TRG1-2 and TRG3-4.

To assess the role of CECs as predictive marker of pathological response to anti-angiogenic therapy, we analyzed also CECs count in LARC patients treated only with short course radiotherapy or with chemotherapy alone. Statistical analyses are ongoing.

Conclusions: Overall, our data revealed a potential clinical relevance of CECs count as a surrogate biomarker for the early (day 10 of treatment) selection of responding rectal cancer patients to anti-angiogenic therapy.

P 10
ROLE OF TGF BETA-1 IN REGULATING PANCREATIC NEUROENDOCRINE TUMORS CELL VIABILITY

Simona Falletta (1) - Teresa Gagliano (1) - Erica Gentilin (1) - Carmelina Di Pasquale (1) - Katiuscia Benfini (1) - Mariaenrica Bellio (1) - Ettore Degli Uberti (1) - Maria Chiara Zatelli (1)

Dipartimento di Scienze Mediche- Sezione di Endocrinologia, Università di Ferrara, Ferrara, Italy (1)

Introduction: Neuroendocrine tumors (NETs) are relatively rare neoplasms arising from neuroendocrine cells spread in the respiratory and gastro-entero-pancreatic epithelium. NETs are very heterogeneous and differ largely based on organ origin, biological pathway, grade of differentiation and proliferation rate. The role of transforming growth factor beta-1 (TGF beta-1) in neuroendocrine tumours biology is currently largely unknown. Previous studies indicate that the TGF beta-1 signaling pathway is tumor suppressive in most non-transformed epithelial cell lines. In contrast, many human carcinomas are refractory to the growth–inhibitory effects of TGF beta-1.

Aim: To investigate whether TGF beta-1 may modulate cell viability and apoptosis activation in neuroendocrine tumor of the pancreas and to understand whether TGF beta-1 may influence the effects of therapeutic molecules currently used in the management of NETs.

Methods: 10 primary cultures obtained from surgical samples of pancreatic NETs (PPC) were treated with TGF beta-1 and/or Everolimus, a mTOR inhibitor. Cell viability and caspase activity were evaluated.

Results: We found that in 2 PPC TGF beta-1 reduced cell viability by 50% with a concomitant increase in apoptotic rate. In 3 PPC TGF beta-1 induced a slight increase in cell viability by 30% with a concomitant decrease in apoptotic rate. TGF beta-1 did not modify cell viability in 5 PPC, but reduced apoptotic rate by 20-60%. However, in the latter group, the combination of TGF beta-1 and Everolimus induced a significant decrease in cell viability, which was not observed under treatment with Everolimus alone.

Conclusions: In conclusion, TGFbeta-1 was found to reduce cell viability of a sub-group of PPC. In addition, our data suggest a possible cooperation between TGF beta-1 and Everolimus in inducing growth arrest. Further studies are necessary to understand TGF beta-1 related functional context in PCC.

P 11
SEARCH OF NEW POTENTIAL BIOMARKERS FOR PROSTATE CANCER BY METABOLOMIC ANALYSIS

Daniela Farioli [1] - Katiuscia Dallaglio [1] - Martina Chiappelli [1] - Cristina Masini [1] - Matteo Conti [2] - Antonino Bruno [3] - Douglas M. Noonan [4] - Adriana Albini [5]

Translational Research Laboratory, IRCCS Arcispedale S. Maria Nuova, Reggio Emilia, Italy [1] - Central Laboratory, Department of Haematology, Oncology, and Laboratory Medicine, Sant'Orsola-Malpighi Hospital, Bologna, Italy [2] - Scientific and technological Pole, IRCCS MultiMedica, Milano, Italy [3] - Department of Biotechnology and Life Sciences, Univer-

sity of Insubria, Varese, Italy [4] - Department of Research and Statistics Infrastructure, IRCCS Arcispedale S. Maria Nuova, Reggio Emilia, Italy [5]

Introduction: Prostate cancer (PCa) is one of the most frequently diagnosed cancers in men and is a main cause of morbidity and mortality. A number of algorithms or nomograms primarily based on tissue pathology and PSA (Prostate-specific antigen) levels are available to diagnose prostate cancer and predict clinical outcome, which can range from relatively indolent to highly aggressive. Although these prediction tools are generally suitable, the optimal value of PSA for screening and detection of PCa is controversial. New molecular biomarkers are needed to improve PCa detection and classification of individual prognosis or risk of progression. Among markers for cancer diagnosis, prognosis and therapy management, molecules linked to metabolic alterations appear particularly promising. Surface Activated Chemical Ionization-Electrospray-mass spectrometry (SACI-ESI) is a novel technique in chromatography-mass spectrometry, and allows a reduction in chemical noise and an increase in ionization efficiency in LC/MS analysis of metabolites in human serum samples.

Materials and methods : Patients were male undergoing a prostate biopsy for PCa diagnosis at the Urology Unit of the MultiMedica Castellanza. Serum samples from 28 patients with prostate cancer and 30 age matched controls (with negative biopsy) were analyzed with SACI-ESI technique, coupled with the NIST (National Institution of Standards and Technology) database technology.

Results: The SACI-NIST approach identified a group of molecules related to fatty acids metabolism (decanoyl carnitine, octanoyl carnitine, cis5-tetradecanoyl carnitine), which have shown statistically significantly differential expression between controls and prostate cancer patients.

Conclusions: The SACI-NIST approach identified molecules characteristic of pathological samples thus demonstrating its potential use in the clinical setting of prostate cancer biomarkers. These data are preliminary and future studies will be performed on a larger cohort of patient samples in order to validate the diagnostic potential of the biomarkers panel identified. Samples, obtained from a serum Biobank that will be created at IRCCS-ASMN Hospital in Reggio Emilia, will be analized by two systems, SACI-NIST at MultiMedica Hospital and GC-MS (Gas chromatography-Mass spectometry) at IRCCS-ASMN Hospital, in order to assess the consistency and robustness of results.

P 12
QUANTIFICATION OF CIRCULATING MICRORNAS BY DROPLET DIGITAL PCR: COMPARISON OF EVAGREEN AND TAQMAN BASED CHEMISTRIES

Manuela Ferracin [1] - Elena Miotto [2] - Elena Saccenti [3] - Laura Lupini [2] - Irene Salamon [2] - Elisa Callegari [2] - Maria Vittoria Zanzi [2] - Paolo Carcoforo [2] - Giorgio Cavallesco [2] - Massimo Negrini [1]

Dept. Morphology, Surgery and Experimental Medicine and Laboratory for the Technologies of Advanced Therapies (LTTA), University of Ferrara, Ferrara, Italy [1] - Dept. Morphology, Surgery and Experimental Medicine, University of Ferrara, Ferrara, Italy [2] - Dept. Medical Sciences, University of Ferrara, Ferrara, Italy [3]

Introduction: Digital PCR shows great promises for the quantification of diagnostic biomarkers. To this end, Taqman droplet digital PCR (ddPCR) system has been successfully used for DNA mutations and copy number or gene expression assessment, including microRNAs (miRNAs) absolute quantification. Recently, a new ddPCR system able to detect DNA-binding dye chemistry was developed but it has never been tested for miRNA quantification.

Materials and methods : Taqman miRNA assays (Applied Biosystems) and miRCURY LNA miRNA assays (Exiqon) were performed on QX200 ddPCR system (BioRad) using Taqman and EvaGreen detection chemistries respectively. Two different miRNA assays were performed in ten plasma RNAs to detect the absolute number of copies of circulating miRNA.

Results: DdPCR conditions were optimized to run Exiqon miRNAs assays on EvaGreen ddPCR system. Reproducibility across PCR and reverse transcription replicates were evaluated, as well as precision and dynamic range of EvaGreen assay. Quantification of two different targets (one endogenous miRNA and one synthetic miRNA) in cancer samples plasma was performed using Taqman and EvaGreen ddPCR with comparable results.

Conclusions: LNA miRNA assays were optimized for ddPCR instrument and provided reliable results with EvaGreen ddPCR over four orders of target cDNA amount. Circulating miRNA quantification obtained with Taqman- or EvaGreen-based ddPCR provided comparable results. We demonstrated the feasibility and ability of both approaches to provide an absolute miRNA quantification in human plasma.

P 13
CLU TRANSCRIPT VARIANTS AS POTENTIAL BIOMARKERS FOR THE THYROID CANCER

Paolo Fuzio [1] - Nicoletta Nuzziello [2] - Anna Napoli [3] - Anna Ciampolillo [4] - Serafina Lattarulo [4] - Angela Pezzolla [4] - Sabino Liuni [2] - Francesco Giorgino [4] - Eugenio Maiorano [3] - Elda Perlino [2]

Institute of Biomedical Technologies, National Research Council (CNR), Bari, Italy [1] - Institute of Biomedical Technologies, National Research Council (CNR), 70126 Bari, Italy [2] - Department of Emergency and Organ Transplantation, Section of Pathological Anatomy, University of Bari Aldo Moro, 70124 Bari, Italy [3] - Department of Emergency and Organ Transplantation – Section of Endocrinology, University of Bari Aldo Moro, 70124 Bari, Italy [4]

Introduction. Clusterin (CLU) is a glycoprotein with a nearly ubiquitous tissue distribution and an apparent involvement in biological processes ranging from neurodegeneration in Alzheimer's disease to cancer initiation and progression. The functions of CLU have been an elusive goal and it has been ascribed to multiple and sometimes contradictory processes. Part of this ambiguity probably results from existence of 2 functionally divergent isoforms, sCLU and nCLU, that play a crucial role in the balance between cell proliferation and death. In the light of recent findings, it is clear that the characterization of CLU and its functional role have not been well established yet.

Materials and methods/Results. To identify the possible CLU variants, we first inspected the CLU entries in the Alternative Splicing Prediction Data Base (http://t.caspur.it/ASPicDB/index.php). These inspections revealed multiple possible CLU mRNA variants, two of which, identified by the Signature ID c7175b345e:9, 1057fea355:9, overlap with sequences of the NCBI database with accession number NM_001831, CLU1 and NR_038335 CLU2 and stood out by having substantially more sequence support than the others in ASPicDB in silico analysis. In this light we decided to investigate *in vivo* these CLU transcript variants expression by using the neoplastic thyroid tissues obtained form patients affected by thyroid papillary carcinoma.

Conclusions. The analysis of the ratio of the two CLU variants measured by qPCR showed a specific increase of the CLU2 transcript variant in the thyroid cancer, confirming the crucial role of CLU in the balance between cell proliferation and death and suggesting a possible use as biomarker also in the thyroid cancer.

P 14
THE GRAB ALL- ASSAY: A MULTI-PARAMETRIC APPROACH TO INDENTIFY HETEROGENIC CIRCULATING TUMOR CELLS

Giulia Gallerani [1] - Pietro Fici [2] - Dino Amadori [2] - Wainer Zoli [2] - Francesco Fabbri [2]

Department of Medical and Surgical Sciences (DIMEC), and Pathology Unit, Felice Addari Institute, University of Bologna, Bologna, Italy [1] - Biosciences Laboratory, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (I.R.S.T.), Meldola (FC), Italy [2]

Introduction: An increasing number of studies ascertains that circulating tumour cells are a heterogeneous population in which cells have different degrees of metastatic potential, thanks to the cell plasticity phenomenon known as epithelial-mesenchymal transition (EMT) also. CTC heterogeneity has not been fully investigated yet due to incomplete multi-parametric studies and limited experimental models.

This study aimed to set up a multi-parametric characterization assay to identify and phenotypically select rare and heterogeneous cell populations simultaneously.

Materials and methods: We performed different experiments using cancer (MCF7 and MDA-MB231) and normal (MCF10A and hTERT) cell lines to closely mime CTC heterogeneity. We evaluated the basal cell phenotype and after EMT induction. In order to study the ability of the assay to evaluate the whole process of transition EMT was induced in MCF10A cells by TGF-beta treatment and in hTERT cells by forced expression of EMT inducers (TWIST1, ZEB1, ZEB2). Single cell analysis was conducted by DEPArray. We assigned a specific phenotypic tag to four different fluorescence channels. The epithelial tag consisted of anti- EpCAM, panCKs, E-cadherin antibodies, while the stem/mesenchymal tag of anti- N-cadherin, CD44v6, ABCG2 antibodies. Nuclear and CD45 staining were evaluated by the remaining two channels. CD44/CD24 and E-cadherin/Vimentin determination by flow cytometry and IF microscopy were performed to confirm the EMT status of the processed cells.

Results/Conclusions: Data showed that the assay is feasible, capable to analyze the phenotypic tags by DEPArray using a multiple staining without aspecific signals and to pinpoint subpopulations of cells during EMT.

Preliminary experiments carried out on whole blood from metastatic cancer patients showed the ability of the Grab all-assay to identify subpopulations of CTC with different epithelial/stem/mesenchymal phenotypes.

P 15
SELENOPROTEINS INVOLVEMENT EVALUATION IN HEPATOCELLULAR CARCINOMA BY TRANSCRIPTOMICS AND IMMUNOHISTOCHEMISTRY STUDIES

Eliana Guerriero [1] - Francesca Capone [1] - Marina Accardo [2] - Giovanni Di Bernardo [3] - Stefano Guariniello [4] - Giovanni Colonna [4] - Giuseppe Castello [1] - Susan Costantini [1]

Istituto Nazionale Per Lo Studio E La Cura Dei Tumori "Fondazione Giovanni Pascale", IRCCS, Napoli, Italy [1] - Dipartimento di Salute Mentale e Fisica- sez. Anatomia Patologica, Seconda Università degli Studi Di Napoli, Napoli, Italy [2] - Dipartimento di Medicina Sperimentale, Seconda Università degli Studi Di Napoli, Napoli, Italy [3] - Dipartimento di Biochimica, Biofisica e Patologia Generale, Seconda Università degli Studi Di Napoli, Napoli, Italy [4]

Introduction: Hepatocellular carcinoma (HCC) is a leading cause of cancer-related death worldwide and its incidence is expected to raise further in coming years due to the increased exposure to different risk factors such as genetic mutations and viral infections. Recently we compared gene expression profiles related to HepG2 cell line (as HCC model) and normal hepato-

cytes cells by microarray technology providing a human hepatoma cells gene signature showing gene expression changes as a consequence of liver cancer in the absence of genetic mutations or complications by viral infection. Today considerable evidence has linked cancer and oxidative damage. Since the selenium (an essential trace element for humans and animals) is able to fight the oxidative damage and it exerts its biochemical and metabolic effects through selenoproteins, we have focused our attention on them and their involvement in HCC.

Materials and methods: In this study we performed a global analysis of the selenotranscriptome expression in HepG2 cell line compared to the normal human hepatocytes by quantitative Real Time RT-PCR. Therefore, functional analysis and interactomics studies were carried out by IPA tool to evaluate the ability of these genes to correlate as well as to evidence the metabolic pathways in which they are involved. Moreover, to verify the results obtained by transcriptomics studies on HCC cells, we investigated the expression of some selenoproteins by immunohistochemistry in liver tissues of HCC and hepatitis C virus-related cirrhosis patients and evaluated their expression scoring.

Results: Our data showed that in HepG2 cell line there are five up-regulated genes (GPX4, GPX7, SELK, SELM, SEP15) and that are connected in the same network named "Amino acid metabolism, protein synthesis and small molecule biochemistry" that presents some nodes that bind to selenoprotein mRNAs. Moreover, immunohistochemistry studies evidenced for the first time an increase of SELM, GPX4 and GPX7 expression in HCC liver tissues.

Conclusions: In conclusion, in this work we propose i) a signature of selenoprotein mRNAs specific for human hepatoma cells, and ii) the evaluation of the selenoproteins expression as putative markers for HCC on which to focus future studies to understand if they can be used to improve the HCC prognosis/diagnosis.

P 16
EVALUATION BY MULTICOLOR FLOW CYTOMETRY OF CIRCULATING ENDOTHELIAL CELLS AS PROGNOSTIC AND PREDICTIVE BIOMARKER IN HEALTHY DONORS AND COLORECTAL CANCER PATIENTS

Alessandra Leone [(1)] - Maria Serena Roca [(1)] - Carlo Vitagliano [(1)] - Francesco Bianco [(1)] - Silvia De Franciscis [(1)] - Gianluca Rotta [(2)] - Ernesta Cavalcanti [(1)] - Rosa Azzaro [(1)] - Annamaria Diodato [(1)] - Carmela Cacciapuoti [(1)] - Guglielmo Nasti [(1)] - Antonio Avallone [(1)] - Alfredo Budillon [(1)] - Elena Di Gennaro [(1)]

Istituto Nazionale per lo Studio e la Cura dei Tumori, 'Fondazione Giovanni Pascale'- IRCCS – Italia, Naples, Italy [(1)] - BD, Biosciences, Buccinasco (MI), Italy [(2)]

Several reports demonstrated that circulating endothelial cells (CECs) count in the peripheral blood of cancer patients correlates with prognosis and represents a promising tool for selecting patients who might benefit from anti-angiogenic therapies. Recent clinical trials suggested that the anti-VEGF antibody, bevacizumab (BEV), is able to decrease CECs in patient's peripheral blood. However, there is no standardized methodology of CECs analysis or consensus on their phenotype. Moreover, published data on this subject are often conflicting and obtained from heterogeneous studies that evaluate patients with different tumors and/or stages.

In collaboration with other Italian research groups, we have counted CECs, through a standardized methodology, in the peripheral blood of healthy donors defining a reference range in these subjects (range 0-30 cells/ml). In details, blood sample collection and CECs analysis have been performed, by 7-color flowcytometry, twice for each donor with an interval of at least 90 days in 42 healthy donors enrolled by our group. Notably, in order to reach a congruous number of samples, the other centers involved in the project enrolled the same numbers of healthy individuals, following the same protocol and by using same reagents and instruments.

Based on our previous experience and our preliminary data on the evaluation of CECs in cancer patients we hypothesize that CECs count could represent an important prognostic and predictive biomarker in colorectal cancer (CRC). Moreover, we have compared CECs count in healthy individuals with the basal levels measured in naïve (untreated) CRC patients. Actually, we have enrolled 80 metastatic stage IV and 20 stage I, II and III CRC patients and analyses are ongoing. Furthermore, in CRC metastatic patients we are analyzing also CECs level at different time points after BEV-based chemotherapy in order to define if CECs could represents a prognostic and predictive biomarker of clinical efficacy of anticancer treatment including anti-angiogenic agents.

Overall, our study could represent the basis for the validation of CECs count as new potential diagnostic, prognostic and/or predictive cancer biomarkers and thus could help to select the patients most likely to benefit from anti-angiogenic therapies, and/or to identify possible mechanisms of resistance.

P 17
ISOLATION AND CHARACTERIZATION OF URINARY EXOSOMES FROM PROSTATE CANCER PATIENTS

Donatella Lucchetti [(1)] - Valentina Palmieri [(2)] - Francesco Pinto [(3)] - Alessandro Calarco [(3)] - Marisa Farina [(1)] - Caterina Fanali [(1)] - Maria Svelto [(1)] - Federica Calapà [(1)] - Alma Boninsegna [(1)] - Alessandro Sgambato [(1)]

Pathology Institute, Università Cattolica Del Sacro Cuore, Roma, Italy [(1)] - Physics Institute, Università Cattolica Del Sacro Cuore, Roma, Italy [(2)] - Urology Institute, Università Cattolica Del Sacro Cuore, Roma, - [(3)]

Introduction: microparticles are heterogeneous membrane-bound sacs that are shed from the surfaces of cells into the extracellular environment and are involved in extracellular communication. Current research interest focuses primarily on the study of exosomes, nanovesicles that originate from the endosomal compartment, with a diameter size of about 30-100 nm. The molecular profile of exosomes largely mirrors that of the cells from which they originated, offering a rich source for the discovery of new biomarkers. Presently, the PSA is the most widely used biomarker for early diagnosis and monitoring of progression and recurrence in prostate cancer. PSA is not able to accurately predict the disease evolution and to identify patients that surely benefit from surgery. Therefore new biomarkers are needed for the diagnosis and prognosis of prostate cancer. Several studies suggest that high concentrations of exoso-

mes can be detected in biological fluids of cancer patients. Given the non-invasive nature of the collection of biological fluids, the analysis of exosomes contents may be useful in the identification of new biomarkers in prostate cancer.

Material And Methods: in this study we recruited 10 patients affected by prostate cancer and collected urine samples before and after surgery. The correct isolation of exosomes from urine samples was confirmed by Dynamic Light-Scattering, Transmission Electron Microscopy, and Western-Blot (WB) analysis through the expression of the exosomal marker CD63. For the quantitative evaluation of exosomes the Bradford Assay was adopted. The analysis of their content in terms of protein and RNA was carried out respectively by WB and nested RT-PCR.

Results: protocols for isolating urinary exosomes by differential centrifugation and to extract proteins and RNA contents have been developed. Urinary exosomes isolated have significant inter-individual variations in patients affected by prostate cancer, in terms of exosome quantity and expression of the stem cell marker CD133. Using a nested RT-PCR based approach, we have shown that urinary tumour exosomes carry genetic information specific for Prostate Cancer Antigen (PCA3).

CONCLUSIONS: we suggest that the quantitative and molecular analysis of circulating exosomes may represent a useful strategy for the identification of novel diagnostic and prognostic biomarkers in prostate cancer. Further study are needed to confirm their possible use as biomarkers.

P 18
PROTEOMIC ANALYSIS OF LIPID RAFTS ISOLATED FROM PRIMARY AND METASTATIC MELANOMA CELLS OF THE SAME PATIENT

Matteo Marconi [1] - Serena Camerini [2] - Marialuisa Casella [2] - Chiara Tommasino [1] - Tina Garofalo [3] - Maurizio Sorice [3] - Barbara Bellei [4] - Paola Matarrese [1] - Marco Crescenzi [2] - Walter Malorni [1]

Department of Theraputic Research and Medicine Evaluation, Istituto Superiore di Sanità, Rome, Italy [1] - Department of Cell Biology and Neurosciences, Istituto Superiore di Sanità, Rome, Italy [2] - Department of Experimental Medicine, Sapienza University, Rome, Italy [3] - Laboratory of Cutaneous Physiopathology,, San Gallicano Dermatologic Institute IRCCS, Rome, Italy [4]

Introduction: Metastatic spread of malignant melanoma is often associated with cancer progression with poor prognosis and survival. To move to a new era of therapeutic decisions based on highly specific tumor profiling, the discovery and validation of new prognostic and predictive biomarkers in melanoma is critical. It is generally acknowledged that changes in the plasma membrane might account for the biological behavior of tumors. The dynamic nature of membrane along with an uneven distribution of lipids leads to the formation of specialized membrane domains where proteins can selectively be included or excluded. In this regard, the dynamic and preferential clustering and packing of sphingolipids and cholesterol into moving platforms, named as lipid rafts, form membrane domains that act as scaffolds for the attachment of specific proteins and for the proper functioning of a number of signaling cascades. Lipid rafts harbor several signaling routes that promote cell survival and proliferation, and thereby they could play a role in the development of cancer. For these reasons we decided to analyze the protein content of lipid rafts in primary and metastatic melanoma cells by proteomic analysis. In fact, proteomic studies have generated numerous datasets of potential diagnostic, prognostic, and therapeutic significance in human cancer.

Materials and methods: In order to identify possible mechanisms necessary for tumor progression in melanoma by excluding the genetic variability we analyzed cells freshly isolated from a primary and a metastatic melanoma lesion of the same patient. Lipid raft-containing sucrose-gradient fractions from primary and metastatic melanoma cells were gel fractionated and analyzed by liquid chromatography-tandem mass spectrometry.

Results: The protein contents of the analyzed fractions from the two cell lines were compared. A significantly higher number of mitochondrial proteins localized into the lipid rafts of metastatic, compared to primary melanoma cells. In particular, metastatic cell rafts were found enriched in proteins of the complexes I and IV of the respiratory chain.

Conclusions: The functional significance of this specific accumulation is not yet clear. However, our results point at mitochondrial membranes as one focus for changes occurring during melanoma progression to metastasis.

P 19
CIRCULATING TUMOR CELLS (CTCS) AS PREDICTIVE OF CLINICAL OUTCOME IN PATIENTS WITH ADVANCED CHEMOREFRACTORY, RAS WILD-TYPE COLORECTAL CANCER (CRC) TREATED WITH CETUXIMAB OR PANITUMUMAB

Valeria Musella [1] - Eleonora Di Buduo [1] - Filippo Pietrantonio [1] - Patrizia Miodini [1] - Ilaria Bossi [1] - Roberto Iacovelli [1] - Elisa Sottotetti [1] - Antonia Martinetti [1] - Maria Grazia Daidone [1] - Vera Cappelletti [1]

Fondazione IRCCS Istituto Nazionale dei Tumori, Istituto Nazionale dei Tumori, Milan, Italy [1]

Cetuximab and panitumumab, plus chemotherapy or in chemorefractory disease improve outcome of patients with *RAS* wt advanced CRC. Further biomarkers to drive "negative" selection of patients are needed to identify the subset of non responders. CTCs might fulfill such a need. We investigated the prognostic role of baseline-and during anti-EGFR treatment CTCs status in 38 patients with advanced, chemorefractory, *RAS* wt CRC.

The study approved by the IRB, included: consented patients with advanced *RAS* wt CRC, with measurable disease and treated with cetuximab-irinotecan or panitumumab after failure of standard chemotherapy. Blood was collected at baseline, early (at 2-4 weeks) and every 8-10 weeks. CTCs were isolated with the AdnaGEN Colon Select kit and detected with the AdnaGEN colon Detect kit. Samples were CTC+ if at least one of genes (*EGFR*, *EPCAM*, *CEA*) was above defined thresholds. To investigate predictive value, besides single time-point CTC status, CTC changes profile were classified as unfavourable if patients were CTCs+/- at baseline, but consistently CTCs+ at early

and/or first reassessment time-points, or as favourable if patients were defined as CTCs+/- at baseline, but CTCs- at early assessment and confirmed in the later re-assessment.

Response rate and disease control rate were 26% and 40%, while median PFS (mPFS) and OS (mOS) were 2.7 (95% CI, 2.3-3.2) and 7.7(95% CI, 3.2-12.2) months (mos), respectively. At baseline, early and late assessment times 50%, 32% and 46% of patients respectively were defined as CTC-positive. A CTC unfavourable profile was observed in 54% of patients.

Outcome was not significantly related to baseline CTC status (CTC+ vs. CTC-: mPFS, 3.2 vs 2.7 mos, HR=0.96; 95% CI, 0.48-1.93; p=0.938; mOS, 6.1 vs 10.1 mos, HR=1.75; 95% CI, 0.73-4.23; p=0.21).

In patients with CTC+ early status mPFS was 2.0 vs 4.0 mos for CTC- patients (HR=3.3; 95% CI, 1.48-7.36; p=0.004). Similarly mOS was 4.7 for CTC+ patients compared to 11.7 mos in CTC- patients (HR=2.86; 95% CI, 1.05-7.73; p=0.039).

Kaplan–Meier curves showed shorter PFS and OS when CTC changes were unfavourable compared to favourable (mPFS, 2.1 vs 7.2 mos; HR 6.10; 95% CI, 2.49-14.96; p<0.001; mOS, 4.7 mos vs >18; HR 8.06; 95% CI, 2.54-25.59; p<0.001).

Baseline CTC status in patients receiving anti-EGFR MoAb as ≥ 3rd line treatment line for *RAS* wt CRC is not prognostic in chemorefractory CRC, whereas early CTCs status and modulation predict outcomes anticipating imaging response.

P 20
A SPECIFIC AND SENSITIVE METHOD FOR THE INDIVIDUATION OF EGFR MUTATIONS IN CIRCULATING FREE TUMOR DNA FROM NSCLC PATIENTS

Raffaella Pasquale [1] - Anna Maria Rachiglio [1] - Claudia Esposito [1] - Alessandra Sacco [1] - Riziero Esposito Abate [1] - Simona Bevilacqua [2] - Cristin Roma [1] - Antonella De Luca [2] - Nicola Normanno [2]

INT Fondazione Pascale–CROM, Pharmacogenomic Laboratory, Mercogliano (AV), Italy [1] - Istituto Nazionale Tumori "Fondazione G. Pascale"- IRCCS, Cell Biology and Biotherapy Unit, Naples, Italy [2]

Introduction: Non-small cell lung cancer (NSCLC) patients with activating mutations of the epidermal growth factor receptor (EGFR) gene benefit of treatment with EGFR-tyrosine kinase inhibitors (EGFR TKIs). EGFR testing is usually performed on tissue, but a significant fraction of patients do not have available sufficient amount of material for mutational analysis. Liquid biopsy, i.e. circulating free tumor DNA (cftDNA), is emerging as an alternative source for the identification of predictive biomarkers. However, previous studies have suggested an approximately 40% sensitivity of the Therascreen EGFR kit to detect somatic mutations in liquid biopsies.

Methods: The Therascreen EGFR RGQ PCR kit, based on Amplification Refractory Mutation System (ARMS) allele-specific PCR and Scorpions technologies, was used to detect somatic mutations in the EGFR gene. Specific DNA extraction protocols; a dedicated workflow of analysis; and appropriate deltaCt thresholds for each specific EGFR mutation were identified for analysis of plasma samples (plasma-Therascreen). Serial dilutions of mutant DNA in wild-type DNA obtained from the NSCLC cell lines H1975, that carries the p.L858R mutation, and H1650, that has the exon 19 deletion p.E746_A750, were used to optimize the analytical protocol.

Results: Blood samples were obtained from 87 NSCLC patients with known EGFR mutational status based on analysis performed with routine diagnostic techniques on tissue samples. Plasma was isolated immediately after blood drawing and cftDNA was extracted. Sixty-one patients were wild-type and 26 EGFR-mutant (18 with exon 19 deletions; 6 with exon 21 p.L858R; 2 with exon 20 insertions) according to tissue analysis. None of the 61 EGFR wild-type patients resulted to have EGFR mutations when ctfDNA was analyzed with the plasma-Therascreen workflow, thus indicating a 100% specificity of the test that did not show any false positive result. In contrast, analysis of liquid biopsies from EGFR mutant patients identified the same EGFR mutation reported in the corresponding primary tumor in 18/26 patients, proving an analytical sensitivity of 69.23%.

Conclusions: We developed a specific and sensitive method for detection of EGFR mutations in plasma-derived cftDNA of NSCLC patients. This method might allow identification of EGFR variants in patients that have no tissue available for mutational analysis.

P 21
DUAL TUMOR SUPPRESSING AND PROMOTING FUNCTION OF NOTCH1 SIGNALING IN HUMAN PROSTATE CANCER

Karine Lefort [1] - Paola Ostano [2] - Maurizia Mello Grand [2] - Maria Scatolini [3] - Valerie Calpini [1] - Giovanna Chiorino [2] - Gian Paolo Dotto [1]

Department of Biochemistry, University of Lausanne, Epalinges, Switzerland [1] - Laboratory of Cancer Genomics, Fondazione "Edo ed Elvo Tempia Valenta", Biella, Italy [2] - Laboratory of Molecular Oncology, Fondazione "Edo ed Elvo Tempia Valenta", Biella, Italy [3]

Introduction: Adenocarcinomas of the prostate arise as multifocal heterogeneous lesions as the likely result of genetic and epigenetic alterations and deranged cell-cell communication. Notch signaling is an important form of intercellular communication with a role in growth/differentiation control and tumorigenesis that is highly cell-type and context dependent. Contrasting reports exist in the literature on the role of this pathway in prostate cancer development.

Materials and methods: Global gene and miRNA expression analysis was performed on prostate cancer tissue samples obtained from 67 patients and on biopsies from 10 healthy patients. Immortalized normal prostate epithelial cells and prostate cancer cells were used for functional evaluation of Notch1 activity.

Results: We report here that Notch1 gene expression is significantly reduced in a substantial fraction of human prostate cancers, while it is unaffected or even increased in others. Down-modulation of Notch1 expression and activity in immortalized normal prostate epithelial cells increases their proliferation potential, while increased Notch1 activity in prostate cancer cells, where endogenous Notch1 expression is decreased, suppresses growth and tumorigenicity. However, global analysis of gene

expression shows that increased Notch1 expression in these cells, while counteracting to a significant extent the abnormal program of gene expression that is characteristically altered in clinically occurring tumors, is enhancing some other aberrant aspects of this program.

Conclusions: Our present findings indicate that Notch signaling is likely to exert an important growth and tumor suppressing function in the prostate. In fact, while control of p21Cip1/WAF1 expression by increased Notch1 activity through a Smad3-dependent mechanism and control of miR-205 can explain the growth inhibitory effects, other target genes, like UHRF1, are concomitantly up-regulated by Notch, and this can lead to enhanced tumorigenic behavior, once cells have escaped from Notch growth inhibition.

P 22
PROGNOSTIC VALUE OF ABC TRANSPORTERS IN EWING SARCOMA

Michela Pasello [1] - Marilù Fanelli [2] - Valentina Mularoni [2] - Massimo Serra [2] - Katia Scotlandi [1]

CRS Development of Biomolecular Therapies, Experimental Oncology Laboratory; PROMETEO Laboratory, STB, RIT Department, Istituto Ortopedico Rizzoli, Bologna, Italy [1] - Pharmacogenomics and Pharmacogenetics Research Unit - Experimental Oncology Laboratory, Istituto Ortopedico Rizzoli, Bologna, Italy [2]

Introduction: Ewing Sarcoma (ES) is the second most common bone tumor occurring in children and young adults. Mediators of chemoresistance are still poorly defined in ES. Conventional ABC transporters, such as ABCB1 (MDR1) have been only sporadically described in ES biopsies and do not appear as crucial mediators of prognosis. In this study we analyzed the prognostic value of other 20 ABC transporters, with additional roles with respect to lipid metabolism and/or neural differentiation.

Materials and methods: Gene expression of 20 ABC transporters was determined by RT-PCR in two different series of primary ES samples (training set: 30 pts; validation set: 68 pts) and in human ES cell lines. Association with patient outcome was determined by log-rank test and Kaplan-Meier survival curves. In vitro analysis of migration, growth in anchorage independent conditions, proliferation and sensitivity to chemotherapeutic agents were done in human ES cell lines. Sensitivity to chemotherapeutic agents was expressed as IC50.

Results: Among the 20 ABC transporters that were analyzed in the training set, only the gene expression of ABCA2, ABCA6, ABCA7, ABCB9 and ABCC4 was associated with differential prognosis. Of these, the expression of ABCA6 and ABCA7 was confirmed to be a significant indicator of tumor progression when the analysis was extended to a new series of 68 ES patients with primary localized tumor at diagnosis (validation set): high expression of ABCA6 or ABCA7 was associated with a better prognosis. In vitro, correlative studies on cell lines showed that the high expression of ABCA7 was associated with better response to Doxorubicin, but not with parameters of cell malignancy.

Conclusions: The study showed that ABCA6 and ABCA7 have impact on ES progression. In vitro studies indicated that ABCA7 expression affects the drug response rather than cell malignancy. However, since high expression of ABCA7 is related to better prognosis, a role different from the canonical function of drug extruders should be supposed for these ABC transporters. Further investigation is needed to understand their biological role in ES.

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P 23
PANCREATIC CANCER: IDENTIFICATION OF NEW PUTATIVE BIOMARKERS FOR EARLY DIAGNOSIS IN A SPONTANEOUS TRANSGENIC MOUSE MODEL

Valentina Pasquale [1] - Erica Dugnani [1] - Daniela Liberati [1] - Paolo Marra [2] - Tamara Canu [2] - Antonio Esposito [2] - Lorenzo Piemonti [1]

Division of Immunology, Transplantation, and Infectious Diseases - DRI - Diabetes Research Institute, beta-cell biology, San Raffaele Scientific Institute, Milano, Italy [1] - Experimental Imaging Center, San Raffaele Scientific Institute, Milano, Italy [2]

Introduction: Pancreatic ductal adenocarcinoma (PDC) is a deadly disease, indeed, even surgery contributes little to survival if PDC is not diagnosed at an initial stage. Diabetes associated to PDC was described and it often manifests several months before diagnosis. Our aim is to find endocrine/metabolic biomarkers of early PDC development in a state-of-art mouse model.

Materials and methods: Candidate markers were investigated using LSL-KrasG12D/+;LSL-Trp53R172H/+;Pdx-1-Cre (KPC) mouse, a genetically-engineered model that develops PDC with 100% penetrance. To overcome the problem of asynchronous development of PDC in KPC, 7T-MRI was used to classify mice in actual pathological stage: stage 1, normal (n=20); stage 2, cystic degeneration(n=16); stage 3, small tumor (n=8) and stage 4, advanced pancreatic cancer (n=7). On KPC mice and age-matched Pdx-1-Cre controls (Cre), we performed metabolic test (OGTT), in vivo phenotyping of adipose tissue deposits (7T-MRI) and serum endocrine/metabolic profile (xMAP technology and clinical biochemistry).

Results: i) Glucose metabolism: no significant difference was observed in not-fasting and 4 hours fasting glycaemia between KPC and Cre; indeed the AUC in OGTT decreased in KPC mice ($p<0,02$). ii) Despite similarity in the weight, MRI studies revealed in KPC with pancreatic cystic degeneration (stage 2) a significant decrease of abdominal fat volume (AFV) in compared to control group ($p=0.001$). iii) Serum levels of 29 endocrine/metabolic biomarkers were assessed during disease progression. Fourteen of these analytes had a statistically significant variations associated with PDC risk (Cre vs stage 2,3,4): albumin, direct bilirubin, leptin, LDL, uric acid, ghrelin, IL6, peptide YY (PYY), TNF-α, gastric inhibitor polypeptide, glucagon, MCP1 (p value <0,01) and iron and glucagon (p value <0,05). To evaluate the added predictive ability, we ranked these 14 factors on the base of the area under ROC curve (AUC): leptin, albumin and PYY showed AUC>0,85 with $p<0,001$.

Conclusions: Unlike human, the development of PDC is not associated with an hyperglycemic status, while an increased in glucose consumption was observed. Our results highlight a crosstalk between early PDC and fat compartments, metabolism and endocrine system, and supply new biomarkers to be validated in high risk PDC human population.

P 24
QUANTIFICATION OF MICRORNAS PLASMA LEVELS FOR COLORECTAL ADENOCARCINOMA DETECTION AND MONITORING

Lisa Perilli [1] - Caterina Vicentini [2] - Marco Agostini [1] - Silvia Pizzini [3] - Marco Pizzi [4] - Edoardo D'angelo [1] - Stefania Bortoluzzi [3] - Susanna Mandruzzato [1] - Enzo Mammano [1] - Massimo Rugge [4] - Donato Nitti [1] - Aldo Scarpa [2] - Matteo Fassan [2] - Paola Zanovello [1]

University of Padova, University of Padova, Department of Surgery, Oncology and Gastroenterology, Padova, Italy [1] - ARC-NET Research Centre, University of Verona, Verona, Italy [2] - University of Padova, University of Padova, Department of Biology, Padova, Italy [3] - University of Padova, Department of Medicine, Surgical Pathology and Cytopathology Unit, Padova, Italy [4]

Introduction: Numerous studies have demonstrated that aberrant expressions of specific microRNAs (miRNAs) are involved in many cancer types including colorectal cancer (CRC). Moreover, several reports demonstrated miRNAs as stably detectable in plasma, thus indicating their quantification as a new tool for cancer detection and monitoring. We recently demonstrated that miR-182 was one of the most up-regulated DEMs (Differentially Expressed miRNAs) in primary CRC compared to normal colon mucosa. We also indicated that this miRNA is a negative regulator of ENTPD5 gene expression in tumor cell lines.

Materials and methods : To further confirm its involvement in CRC development and progression, miR-182 expression was evaluated by qRT-PCR and *in situ* hybridization in 20 tubular adenomas, 50 CRC, and 40 CRC liver metastases. As normal controls, we considered 50 samples obtained from patients with irritable bowel syndrome, or tumor-matched normal colon mucosa. To evaluate miR-182 as a novel biomarker, plasma samples from 51 CRC patients and controls were tested by qRT-PCR. Moreover, the down-regulation of ENTPD5 protein levels was investigated by immunohistochemical analysis.

Results: We observed a significant overexpression of miR-182 in CRC primary tumor compared to normal colon mucosa, which is also maintained in CRC liver metastases. Plasma miR-182 concentrations were significantly higher in CRC patients than in healthy controls or patients with colon polyps at endoscopy. Moreover, miR-182 plasma levels were significantly reduced in post-operative samples after radical hepatic metastasectomy, compared to pre-operative samples. In ENTPD5 immunohistochemical analysis we observed that normal colocytes featured strong cytoplasmic staining whereas a significantly and progressively lower expression was detectable along with dedifferentiation of the histologic phenotype, and in CRC liver metastases.

Conclusions: Our results strengthen the involvement of miR-182 dysregulation in colon mucosa transformation, confirm the negative regulation of ENTPD5 gene and demonstrate that circulating miR-182 could be used as a biomarker for screening CRC and monitoring tumor relapse during the follow-up.

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P 25
PREDICTIVE BIOMARKERS OF TRABECTEDIN (TR) AND PARP-1 INHIBITOR SYNERGISM IN PRECLINICAL MODELS OF MESENCHYMAL TUMORS (MTS).

Ymera Pignochino [1] - Federica Capozzi [1] - Carmine Dell'aglio [1] - Marta Canta [1] - Marco Basiricò [1] - Annalisa Lorenzato [1] - Lorenzo D'ambrosio [1] - Danilo Galizia [1] - Maria Serena Benassi [2] - Massimo Aglietta [1] - Giovanni Grignani [1]

Candiolo Cancer Institute, IRCCS, University of Torino Medical School, Candiolo, TO, Italy [1] - Laboratory of experiemntal Oncology, Istituti Ortopedici Rizzoli, Bologna, Italy [2]

Introduction. Metastatic or relapsing MTs are an unmet medical need. We developed a preclinical platform for exploring activity and predictive biomarkers of synergism between TR, a specific DNA-damaging agent, and olaparib (OL), inhibitor of PARP-1 a key-factor of DNA-repair machinery. We speculated that TR-induced DNA damage could be perpetuated by PARP-1 inhibition and this effect could be more potent in tumor with peculiar contest of DNA- repair-gene status and expression.

Material and Methods. We tested cell viability after 72 h treatment with scalar doses of TR and OL against 20 different MT cell lines. We studied cell cycle and apoptosis by flow cytometry. We checked DNA damage by pH2AX expression and by comet assay. RT-PCR, western blot, immunocytochemistry were used to evaluate mRNA and protein expression of DNA repair system components. Statistical analysis included Pearson's correlation (r), t distribution and p-value. Stable silencing and overexpression of PARP-1 and BRCA-1 were done with lentiviral vectors. Subcutaneous and orthotopic MT models were obtained in NOD/SCID mice and tumor growth was monitored by caliper and by in vivo imaging.

Results. TR and OL blocked cell cycle at G2/M checkpoint, induced apoptosis and DNA damage. Cell viability tests revealed a strong synergism of TR-OL ($0.16 \leq$combination index≤ 0.84 average $=0.58$) in 18/20 cell lines. Nonetheless, a 15-fold range of sensitivities among MT histotypes occurred. Synergism significantly correlates with the expression of PARP-1 ($r=0.56$, $p=0.012$), RAD51 ($r=0.71$, $p= 0.00067$) and BRCA1 ($r=0.62$, $p= 0.004$). Down-modulation of PARP-1 by silencing significantly reduced TR-OL synergism in sensitive cells (33±3%), while stable overexpression of PARP-1 and BRCA-1 in less sensitive cells increase synergistic effect (30±3%, and 23,8 ±2%, respectively $p<0.05$), confirming their functional role. In TR-OL- treated xenografts reduced tumor volume, metastases spreading and cell proliferation, increased apoptosis and DNA damage, were observed if compared to controls and single agents.

Conclusions. We demonstrated that the TR-OL displayed effective antitumor and antimetastatic activity in preclinical models

of MTs and are more synergic in cells with high PARP-1, BRCA-1 and RAD51 expression. These findings pave the way to the clinical evaluation of this novel therapy and its predictive biomarkers in MTs. Next, these positive results could be exploited in more common cancers (e.g ovary and breast).

P 26
TAK1-REGULATED EXPRESSION OF BIRC3 IS RESPONSIBLE FOR CHEMORADIOTHERAPY (CRT) RESISTANCE IN ESOPHAGOGASTRIC JUNCTION (EGJ) ADENOCARCINOMA

Geny Piro [1] - Simone Giacopuzzi [2] - Maria Bencivenga [2] - Giuseppe Verlato [3] - Carmine Carbone [4] - Valeria Merz [4] - Anna Tamburrino [4] - Giovanni De Manzoni [2] - Giampaolo Tortora [1] - Davide Melisi [4]

Laboratory of Oncology and Molecular Therapy, University of Verona, Verona, Italy [1] - Department of Surgery, University of Verona, Verona, Italy [2] - Department Public Health and Community Medicine, University of Verona, Verona, Italy [3] - Digestive Molecular Clinical Oncology Research Unit, University of Verona, Verona, Italy [4]

Introduction: Preoperative CRT followed by surgery is the most common approach for patients with resectable esophageal and EGJ cancers. Based on the histology, patients with adenocarcinoma consistently demonstrated significantly lower rate of pathological complete response if compared with patients with squamous cell carcinoma, thus the need for accurate biomarkers to select the patients with esophageal and EGJ adenocarcinoma most likely to benefit from preoperative CRT has become even more critical. We recently demonstrated that the MAP3K TGF-b-activated kinase-1 (TAK1) is responsible for the resistance to the proapoptotic effect of chemotherapeutic agents by increasing the transcription of the member of the inhibitor of apoptosis proteins family BIRC3 in preclinical models of pancreatic cancer. Here, we hypothesized that the TAK1-regulated expression of BIRC3 might be responsible for the resistance to CRT in EGJ adenocarcinoma.

Materials and methods: TAK1 kinase activity was targeted in Flo-1 and Kyae-1 esophageal cells by using (5Z)-7oxozeaenol. To test the effect of reducing BIRC3 expression on the resistance to CRT, Flo-1 and Kyae-1 cells were treated with increasing doses of cisplatin, 5-fluorouracil, paclitaxel, or radiotherapy in combination with (5Z)-7oxozeaenol. Drug interactions were studied for synergism according to Chou and Talalay method. BIRC3 expression was measured in 32 pretreatment biopsies from patients with EGJ adenocarcinoma receiving neoadjuvant CRT by Real-Time PCR. Tumor response was evaluated by Tumour regression grade (TRG) and by Size-based Pathological Response (SPR) scores. Correlation between BIRC3 expression and treatment response was analyzed by ROC curve analysis.

Results: In vitro, (5Z)-7oxozeaenol significantly reduced Birc3 expression in Flo-1 and Kyae-1 esophageal cells. Simultaneous exposure to sublethal equitoxic doses of chemotherapeutic agents or radiotherapy, plus (5Z)-7oxozeaenol resulted in a strong synergistic anti-proliferative effect. Patients with EGJ adenocarcinoma expressing higher levels of BIRC3 at baseline had a significantly poorer treatment response than did those with lower expression, indicating that BIRC3 expression significantly correlates with response to preoperative CRT (AUC-ROC = 0.777 and 0.807 for SPR and TRG, respectively).

Conclusions: TAK1-regulated expression of BIRC3 may be a valid biomarker to predict resistance to CRT in EGJ adenocarcinoma patients.

P 27
DEREGULATION OF NLRP3 INFLAMMASOME IN CHRONICH LYMPHOCYTIC LEUKEMIA: A NEW POSSIBLE TARGET?

Erica Salaro [1] - Alessia Rambaldi [1] - Alessia Franceschini [1] - Francesca Amoroso [1] - Simonetta Falzoni [1] - Francesco Cavazzini [2] - Antonio Cuneo [2] - Francesco Di Virgilio [1]

Department of Morphology, Surgery and Experimental Medicine Section of Experimental pathology, Oncology and Biology, University of Ferrara, Ferrara, Italy [1] - Department of Medical Sciences, Section of Hematology, Azienda Ospedaliero-Universitaria, Arcispedale Sant'Anna, Ferrara, Italy [2]

Introduction: B-cell chronic lymphocytic leukemia (B-CLL) is one of the most common leukemia in adults, resulting in the accumulation of $CD5^+$ B lymphocytes in the blood and the bone marrow.[1] Recent evidence have shown that different types of tumors overexpress the P2X7 receptor, an ATP-gated ion-channel thought to be involved in tumor growth.[2] It is known that P2X7 receptors is one of the most potent activator of NLRP-3 Inflammasome, a multiprotein complex that operate as a platform for the maturation and secretion of proinflammatory cytokines such as IL-1 b and IL-18.[3] Recently, experimental evidence showed that NLRP3 Inflammasome is deregulated in hepatic parenchymal cells during liver cancer progression.[4] Moreover patients affected by CLL have lower plasma levels of IL-1 b than healthy subjects.[5] The aim of this study is to explore the role of NLRP3 Inflammasome in CLL.

Materials and methods : We have analysed PBMC from 20 CLL patients, determined NLRP3 and ASC mRNA and protein levels. We have used the THP-1 silenced for NLRP3, and HEK293-NLRP3 for study cell proliferation *in vitro*. Finally, RAMOS and THP-1 cell line model, transiently transfected for NLRP3 were used to investigated the possible cytostatic effect of NLRP3.

Results: Lower levels of NLRP3 were expressed in CLL patients compare to healthy subjects. Reduction of NLRP3 levels by shRNA transfection increased proliferation of THP-1 cells. Transfection of NLRP3 into HEK293 reduced cell proliferation. Finally, preliminary data suggest that the overexpression of NLRP3 in THP-1 and RAMOS cell lines, may induce death by apoptosis.

Conclusions: This study shown that NLRP3 expression negatively affect cell proliferation, and that NLRP3 is down modulated in CLL patients. Thus, NLRP3 might be a novel prognostic marker in CLL.

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P 28
THE PROGNOSTIC ROLE OF MIR-34A IN OSTEOSARCOMA

Marika Sciandra [(1)] - Cristina Baricordi [(1)] - Valentina Chiadini [(1)] - Andrea Grilli [(1)] - Piero Picci [(1)] - Davide Donati [(2)] - Stefano Ferrari [(3)] - Katia Scotlandi [(1)]

CRS Development of Biomolecular Therapies, Laboratory of Experimental Oncology, Rizzoli Orthopaedic Institute, Bologna, Italy [(1)] - III Orthopaedic Clinic, Bologna University, Rizzoli Orthopaedic Institute, Bologna, Italy [(2)] - Chemotherapy Section, Rizzoli Orthopaedic Institute, Bologna, Italy [(3)]

Introduction: Osteosarcoma (OS) is the most common primary bone malignancy arising in children and young adults. The broad range of molecular alterations as well as the redundancy of autocrine loops and kinase activations have hampered any effective application of targeted therapies and therapy is still entrapped to conventional treatments. In this context, the identification of new biomarkers to predict the response to chemotherapy emerges as a compelling need. This study explores the prognostic role of miR-34a in OS.

Materials and methods: a cohort of 135 high grade (grade IV) OS patients homogenously treated were evaluated for miR-34a expression by qPCR. Kaplan-Meier survival curves and log rak test were used to verify associations with prognosis. For *in vitro* studies, OS cell lines were exposed to miR-34a mimics followed by treatments with conventional drugs (doxorubicin, cisplatin, and methotrexate) to evaluate effects on cell growth (MTT assay or vital count with Tripan blue). Expression of miR34a was analyzed also in resistant variants of U-2 OS or Saos-2 cell lines.

Results: miR-34a was less expressed in OS clinical samples compared to normal controls. High expression of miR-34a was found to be associated to better event-free survival (EFS) but not to overall survival (OVS). *In vitro*, when miR-34a expression was enforced cells were less proliferative and were sensitized to chemioterapeutic drugs. Consistently, cells resistant tochemotherapeutics displayed a lower expression of miR-34a with respect to the respective parental cell lines.

Conclusions: miR-34a expression is a promising and validated biomarker that may define the sensitivity of OS cells to conventional chemotherapeutic agents. Consistently miR-34a expression predicts the EFS, but not OVS, in clinical samples and its detection may help to stratify patients and refer them to more appropriate treatments. Granted by: AIRC to KS (IG14049) and EU Provabes.

P 29
EARLY RELAPSE UPON TRASTUZUMAB TREATMENT IS PREDICTABLE BY TRANSCRIPTOME ANALYSIS OF PRIMARY HER2-POSITIVE BREAST CANCER

Tiziana Triulzi [(1)] - Loris De Cecco [(1)] - Aleix Prat [(2)] - Marco Sandri [(1)] - Marta Giussani [(1)] - Maria Luisa Carcangiu [(3)] - Silvana Canevari [(1)] - Serena Di Cosimo [(4)] - Sylvie Menard [(1)] - Manuela Campiglio [(1)] - Elda Tagliabue [(1)]

Fondazione IRCCS Istituto Nazionale dei Tumori, Department of Experimental Oncology and Molecular Medicine, Milan, Italy [(1)] - Vall d'Hebron Institute of Oncology, Translational Genomics Group, Barcelona, Spain [(2)] - Fondazione IRCCS Istituto Nazionale dei Tumori, Department of Pathology, Milan, Italy [(3)] - Fondazione IRCCS Istituto Nazionale dei Tumori, Department of Oncology, Milan, Italy [(4)]

Background. Trastuzumab, a recombinant humanized monoclonal antibody directed to the HER2 protein, has shown survival benefits in women with HER2+ breast cancer, but some patients relapse after treatment underscoring the need to identify patients for whom chemotherapy + trastuzumab is adequate versus those requiring additional drugs. To search factors predictive of relapse of HER2+ breast cancer patients treated with adjuvant trastuzumab we performed whole gene expression profile of a series of HER2+ breast tumors.

Methods. RNA from formalin-fixed paraffin embedded HER2+ tumors, including 23 relapsed and 30 non-relapsed patients, with similar clinic-pathological characteristics and a median follow-up of 4 years from adjuvant trastuzumab, were profiled on the Illumina platform. Consensus clustering and a Cox's proportional hazard model were used to identify samples partition and to construct a model for relapse risk, respectively. Public available datasets were used to validate subtypes and risk-model.

Results. Unsupervised analysis identified 3 stable tumor subtypes and the one (HER2-III) characterized by high ERBB2 and low ESR1 expression and high immune-response genes expression showed the best clinical outcome (p=0.0001). Estimate of the association between gene expression and relapse free survival (RFS) enabled to develop a 41-gene model (TRAstuzumab Risk model: TRAR), that stratified patients into two groups (TRAR high and low), with a 4-year RFS probability of 14.5% in TRAR high versus 87% in TRAR low tumors (HR= 8.33, 95% CI=3.53-18.18, p<0.0001). TRAR low and HER2-III tumors resulted to belong mainly to the HER2 enriched subtype, as evaluated by PAM50 classification. *In silico* analysis on a dataset containing 132 HER2+ breast cancers treated with chemotherapy alone revealed an inverse association between groups and prognosis, with an overall survival probability of 81% in TRAR high and 56% in TRAR low group (p=0.0249). Moreover, a lower TRAR score was found in tumors that respond to trastuzumab-based neoadjuvant treatment in two public datasets, suggesting that our model is specific for trastuzumab benefit.

Conclusion. Results provide compelling evidence that the identification of the tumor molecular portrait in HER2+ disease predicts early relapse in patients treated with adjuvant trastuzumab, and that tumors with the highest activation of HER2 signals are those enriched in immune pathways and most sensitive to trastuzumab.

Supported by AIRC

P 30
FC GAMMA RECEPTOR IIIA POLYMORPHISMS CORRELATED WITH ANTIBODY DEPENDENT CELL MEDIATED CYTOTOXICITY (ADCC) -ANTI EGFR ANTIBODIES INDUCED AND CLINICAL OUTCOME IN METASTATIC COLORECTAL CANCER PATIENTS

Anna Maria Trotta [1] - Alessandro Ottaiano [2] - Carmen Romano [2] - Serena Zanotta [1] - Guglielmo Nasti [2] - Chiara De Divitiis [2] - Anna Nappi [2] - Rosario Vincenzo Iaffaioli [2] - Stefania Scala [1]

Oncological Immunology, National Cancer Institute "Fondazione G.Pascale", Naples, Italy [1] - Department of Abdominal Oncology, National Cancer Institute "Fondazione G.Pascale", Naples, Italy [2]

Introduction: One of the mechanism of the anti-EGFR monoclonal antibodies in the treatment of metastatic colorectal cancer (mCRC) is the antibody-dependent cell-mediated cytotoxicity (ADCC) in which the Fc region of the antibody binds to the Fc gamma receptors (FcγR) expressed by immune cells. Single nucleotide polymorphisms was reported for FcγRIIa and FcγRIIIa and correlated to the patients outcome. The present study investigated the association between single nucleotide polymorphisms of FcγRIIa and FcγRIIIa, in vitro ADCC and clinical outcome in mCRC patients treated with anti-EGFR antibodies.

Materials and **methods**: Interleukin-2 (IL-2) activated PBMCs (LAK) cells from 70 patients with mCRC enrolled in the CALLAS study (observational study on the treatment of mCRC designed to examine the role of baseline of K-ras) were evaluated for in vitro ADCC cetuximab-mediated (SRB assay and CytoTox96) against HT-29 (human colon cancer cell line EGFR+). Genotyping of FcγRIIa- H131R and the FcγRIIIa-V158F was performed automatic sequencing. FcγRIIIa gene expression was determined by quantitative real-time PCR.

Results: The genotypes frequencies were distributed as follow: 41%HH, 43%HR and 16%RR for FcγRIIa ; 26%VV, 43%VF and 31%FF for FcγRIIIa being the genotype frequencies in Hardy-Weinberg Equilibrium. These frequencies did not significantly differ between the mCRC and healthy subjects (p= 0.626 , p=0.613 respectively). Patients carrying the FcγRIIIA genotypes V/V or V/F and FcγRIIa H/H or H/R induced a higher ADCC- cetuximab mediated compared to F/F and R/R carrying patients. The presence of a V and a H allele correlated with enhanced ADCC ($p<0.001$ and p=0.011 respectively). FcγRIIIa mRNA expression was determined in 34 mCRC patients. Although the FcγRIIIa transcript level was higher in patients with FcRIIIa-158 V/V or V/F compared to F/F, analysis of variance showed no significant differences (p=0.240). Based on these results, we correlated enhanced ADCC-cetuximab mediated and outcomes of cetuximab-based therapy in patients with mCRC. Actually only 22 patients (32%) received anti-EGFR antibody based regimen. Preliminary data showed a significant association between clinical outcome and enhanced ADCC cetuximab-mediated (p=0.017).

Conclusions: The study is currently recruiting consecutive mCRC patients evaluating FcγR polymorphisms, the effective in vitro cetuximab-ADCC activity and biological tumor features could have a prognostic/ predictive power?

P 31
CDCP1 AS A MARKER OF AGGRESSIVENESS IN TRIPLE-NEGATIVE BREAST CANCER

Federica Turdo [1] - Marianna Sasso [1] - Patrizia Casalini [1] - Patrizia Gasparini [1] - Francesca Bianchi [1] - Luca Forte [1] - Roberto Agresti [2] - Ilaria Maugeri [2] - Gabriella Sozzi [1] - Elda Tagliabue [1] - Manuela Campiglio [1]

Fondazione IRCCS - Istituto Nazionale dei Tumori, Dept . Experimental Oncology and Molecular Medicine, Milan, Italy [1] - Fondazione IRCCS - Istituto Nazionale dei Tumori, Division of Surgical Oncology-Breast Unit, Milan, Italy [2]

Triple-negative breast cancer (TNBC) is an aggressive breast cancer subtype defined by the lack of known markers and of ER, PgR, and HER2 receptor expression. To date, no therapies other than chemotherapy are available. In an effort to identify specific markers responsible for aggressiveness of TNBCs, we used post-surgery wound-healing fluids (WHFs) from breast cancer patients, known to be extremely enriched in growth factors and cytokines as a tool mimicking the post-surgery host microenvironment reported to favor early relapse of aggressive breast tumors. Gene expression profiling (GEP) on the Illumina platform using TNBC cell lines derived after stimulation with WHFs identified the transmembrane non-catalytic receptor CDCP1 (CUB domain-containing protein 1) as the most significantly up-modulated gene. CDCP1 was found basally overexpressed in 7 of 8 TNBC cell lines. Its silencing in the most highly expressing TNBC lines (MDA-MB-231 and BT-549) strongly impaired both their migration (~70%) and invasion (~50%) ability but did not affect their *in vitro* proliferation, suggesting a role for CDCP1 in TNBC dissemination. IHC analysis of CDCP1 in 126 human primary TNBC FFPE specimens revealed intense membrane staining in 60% of cases. CDCP1 expression was found to be a risk factor that significantly reduces both DFS (log- rank p=0.0115) and DDFS (log-rank p=0.0063) of TNBC patients. FISH analysis of FFPE sections from 63 of the 126 TNBC cases to test for possible genetic alterations underlying CDCP1 overexpression using a pool of 3 BAC (bacterial artificial chromosome) clones that cover the CDCP1 gene at chromosome 3p21.31 and a commercial chromosome 3 enumeration probe, showed that 26 (41%) cases presented 3 to 20 signals for CDCP1 accompanied by gains of chromosome 3; polysomy strongly correlated with CDCP1 protein expression levels.

Our data identify CDCP1, overexpressed due to genetic alteration, as a marker of extremely aggressive TNBCs, suggesting its candidacy as a target of novel therapeutic strategies against this disease.

Supported by AIRC

P 32
VON HIPPEL-LINDAU GENE MUTATIONS PROMOTE NATURAL KILLER CELLS ACTIVITY IN CLEAR-CELL RENAL CELL CARCINOMA (RCC) PATIENTS .

Serena Zanotta [1] - Sabrina Cecere [2] - Sara Santagata [1] - Crescenzo D'alterio [1] - Maria Napolitano [1] - Renato De Domenico [2] - Renato Franco [3] - Gaetano Facchini [2] - Nicola Longo [4] - Sisto Perdonà [2] - Sandro Pignata [2] - Stefania Scala [1]

Oncological Immunology, National Cancer Institute "Fondazione G.Pascale", Naples, Italy [1] - Department of Urology, National Cancer Institute "Fondazione G.Pascale", Naples, Italy [2] - Department of Pathology, National Cancer Institute "Fondazione G.Pascale", Naples, Italy [3] - Genitourinary Oncology and Rare Cancer Center, University of Naples "Federico II", Naples, Italy [4]

Introduction: Approximately 75% of clear cell RCCs manifest biallelic von Hippel-Lindau gene (VHL) inactivation. The biological and prognostic effect of the mutation is still undefined. It was previously demonstrated that VHL gene status regulates NK activity in vitro (Perier et al. Oncogene 2011). To investigate on the effect of VHL gene status on NK activity in RCC patients, the VHL mutational status of primary tumor was correlated to the lytic potential of patients peripheral NK cells.

Material and Methods: 33 patients with primary RCC were enrolled. VHL mutational status was determined by PCR amplification and direct sequencing on DNA extracted from tissue. Patients whole blood was tested in vitro against VHL-wild type (SN12C and CAKI) and VHL- mutated (A498 and O786 cells) human RCC cells. NK mediated cytotoxicity was evaluated through cell-surface mobilization of CD107a. NK cells actively degranulating were identified as CD3- CD56+ CD107a+ by flow cytometry. CD56 and CD16, the inhibitory receptor KLRB1 and of the activating receptors DNAM1, NKp30, NKp40, NKp44 and NKG2D expression were evaluated through quantitative RT-PCR.

Results: Out of 33 primary RCC patients analyzed for VHL mutational status 19 (57%) resulted mutated. When NK cells from RCC patients carrying VHL mutations were challenged in vitro toward VHL- mutated human RCC cells the number NK-CD107a+ cells was significantly higher ($p<0.05$), conversely when NK cells from RCC patients carrying VHL-wild type were challenged in vitro toward VHL- mutated or VHL-wild type human RCC cells the percentage of NK-CD107a+ was comparable ($p= 0.7$).

To investigate on NK function the expression of NK specific receptors was evaluated in 33 RCC patient samples (NK identification markers (CD56, CD16) versus NK functional status (DNAM1, NKG2D, NKp30, NKp44). Preliminary data suggested the NK CD56 and CD16 gene expression was unchange between VHL-WT versus VHL-mutated , the NK markers of activation, DNAM1 and NKG2D were overexpressed in VHL mutated tumor tissues ($p<0.05$).

Conclusions: NK cells isolated from RCC-VHL mutated cancer patients showed higher cytotoxic activity in vitro suggesting that VHL genetic status may regulate the recognition and lysis of RCC cells by NK cells. This is the first time that a differential NK activity is shown toward RCC patients and identify in NK cells another target to improve the therapy with antiangiogenic drugs in RCC patients.

Nanotechnology

Q 1
MULTICOMPONENT NANOVECTORS FOR DELIVERY OF THERAPEUTIC MOLECULES IN CANCER

Armando Cevenini [1] - Alessandro Parodi [2] - Sm Khaled [3] - Iman K Yazdi [3] - Matteo Ciancaioni [4] - Xueavu Liu [3] - Christian Celia [4] - Nicoletta Peluso [1] - Rosa Peltrini [1] - Stefania Orrù [5] - Luigi Del Vecchio [6] - Ennio Tasciotti [3] - Mauro Ferrari [3] - Francesco Salvatore [1]

CEINGE-Biotecnologie Avanzate, s.c.a r.l., Naples, Italy [1] - Fondazione SDN, IRCCS, Naples, Italy [2] - Department of Nanomedicine, Houston Methodist Research Institute, Houston, TX, United States [3] - Department of Pharmacy, University of Chieti-Pescara "G. d'Annunzio", Chieti, Italy [4] - Department of Movement Sciences and Wellness, University of Naples "Parthenope", Naples, Italy [5] - Department of Molecular Medicine and Medical Biotechnology, University of Naples "Federico II", Naples, Italy [6]

Introduction: Nanotechnology is an emerging approach to ameliorate biodistribution and side effects of anti-cancer therapeutics. Nanoparticles (NP) can i) protect therapeutic molecules from reticuloendothelial clearance, ii) transport them to the site of action minimizing their influence on normal tissues and iii) enhance drug concentration and effects in target tissues and cells, allowing the use of low doses. However a single component nanovector cannot accomplish all those duties; therefore, our efforts are focused on the production and optimization of multi-component drug-delivering nanosystems (made of silica, silicon, polymers, or lipids) and on the evaluation of their efficacy. Here we present: 1) an hybrid NP (HNP) composed by a core of silica and by a cationic poly(2-diethylaminoethyl methacrylate) hydrogel shell; and 2) a multistage nanovector (MSV), constituted by two stages: a stage 1 mesoporous silicon particle (S1MP) that accommodates, in his porous structure, stage 2 NPs (S2NP) consisting of oxaliplatin-loaded liposomes.

Materials and methods: NP hydrodynamic diameter and z-potential were measured by dynamic and electrophoretic light scattering. Loading and release of siRNA and liposomes were measured by spectrofluorimetry. Cytotoxicity of NPs was measured by MTT assay. Lysosomal activity was analyzed by Neutral Red assay. Intracellular delivery of siRNA was measured by fluorescence microscopy. Gene silencing efficacy of siRNA-loaded HNP was assessed by Real-time PCR and Western blot. Cell lines: MDA-MB-231 (breast cancer(BC)); HUVEC (primary endothelial cells); CACO-2 and HCT-116 (colorectal cancer (CRC)).

Results and Conclusions: We demostrated that HNPs are pH-responsive, they swell at acidic pH and have a buffering activity due to a proton sponge effect which allow their lisosomal escape. Moreover they can be loaded with a large amount of siR-

NA and show efficient siRNA cytoplasmic delivery and high gene silencing efficacy in a BC cell line.

In order to assemble a MSV for CRC therapy, we loaded into S1MP two selected formulations of S2NPs which showed fast biphasic release kinetics. Moreover, these liposomes showed a moderate oxaliplatin-indepent cytotoxicity in HUVECs and an high oxaliplatin-dependent reduction of cell growth in two CRC cell lines.

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Q 2
ANTI-TUMOR ACTIVITY OF A NEUROBLASTOMA-SPECIFIC PEPTIDE CONJUGATED TO NANOCARRIERS

Loi Monica [1] - Gianluca Bottoni [2] - Alice Bartolini [3] - Daniela Di Paolo [1] - Laura Emionite [4] - Angelina Sacchi [5] - Flavio Curnis [6] - Michela Massollo [2] - Cristina Gagliani [7] - Silvia Bruno [7] - Alessandro Gori [8] - Renato Longhi [8] - Michele Cilli [4] - Carlo Tacchetti [9] - Angelo Corti [6] - Gianmario Sambuceti [2] - Mirco Ponzoni [1] - Serena Marchiò [3] - Fabio Pastorino [1]

Experimental Therapy Unit, Laboratory of Oncology, Istituto Giannina Gaslini, Genoa, Italy [1] - Nuclear Medicine Unit, IRCCS Azienda Ospedaliera Universitaria San Martino–IST Istituto Nazionale per la Ricerca sul Cancro, Genoa, Italy [2] - Laboratory of Tumor Microenvironment, Institute for Cancer Research at Candiolo (IRCCS), Candiolo (TO), Italy [3] - Animal Facility, IRCCS Azienda Ospedaliera Universitaria San Martino–IST Istituto Nazionale per la Ricerca sul Cancro, Genoa, Italy [4] - Division of Molecular Oncology, San Raffaele Scientific Institute, Mlan, Italy [5] - Division of Molecular Oncology, San Raffaele Scientific Institute, Milan, Italy [6] - Department of Experimental Medicine, University of Genoa, Genoa, Italy [7] - Istituto di Chimica del Riconoscimento Molecolare, CNR, Milan, Italy [8] - Department of Experimental Medicine

Introduction. The identification of peptide ligands specific for solid tumors is expected to provide targeting moieties to improve delivery and to decrease toxicity of chemotherapy. We have recently identified the peptide HSYWLRS as a specific ligand for neuroblastoma (NB), a childhood tumor mostly refractory to current therapies.

Experimental procedures. The capability of peptide HSYWLRS to recognize NB cells was evaluated by coupling Qdot fluorescent nanoparticles with HSYWLRS or its scrambled version (SCR). NB cell association and internalization of HSYWLRS-targeted liposomes were tested by FACS and confocal microscopy studies. Therapeutic experiments in mice orthotopically injected with luc-trasfected NB cells and treated with HSYWLRS-targeted, doxorubicin-loaded liposomes (HSYWLRS-SL[DXR]) were performed. Anti-tumor efficacy was evaluated by BLI imaging. *In vivo* imaging was also performed by injecting mice with a bolus of fluorodeoxyglucose during a list mode acquisition lasting one hour using a dedicated micro-PET system. After framing rate optimization, tumor glucose consumption was measured using Patlak graphical approach and normalizing the slope of regression line for serum glucose level.

Results. FACS analysis showed that HSYWLRS-Qdot and SCR-Qdot bound NB cells in a dose-dependent manner, however with different intensity, being HSYWLRS-Qdot the more potent. The binding of HSYWLRS-Qdot was efficiently inhibited by an excess of HSYWLRS, but not by control SCR peptide. In contrast, the binding of SCR-Qdot was not inhibited neither by an excess of SCR nor by HSYWLRS peptide, suggesting that the binding of SCR-Qdot is not specific. Again, the specific peptide-driven binding of HSYWLRS-targeted liposomes (HSYWLRS-SL) to NB cells was inhibited by an excess of HSYWLRS peptide. Preliminary *in vivo* results obtained by BLI and micro-PET devises indicated that HSYWLRS-SL[DXR] decrease tumor growth through a reduction of tumor glucose consumption, leading to an enhanced life span in treated mice.

Conclusion. Our findings demonstrate that HSYWLRS peptide recognizes NB cells and is functional in the design of nanocarriers with therapeutic efficacy paving the way to its clinical development.

Non-coding Rnas

R 1
RECONSTRUCTION OF MICRORNA/GENES TRANSCRIPTIONAL REGULATORY NETWORKS OF MULTIPLE MYELOMA THROUGH IN SILICO INTEGRATIVE GENOMICS ANALYSIS

Luca Agnelli [1] - Andrea Bisognin [2] - Enrica Calura [2] - Katia Todoerti [3] - Martina Manzoni [4] - Paolo Martini [2] - Gabriele Sales [2] - Chiara Romualdi [2] - Stefania Bortoluzzi [2] - Antonino Neri [4]

Department of Clinical Sciences and Community Health, University of Milan, Milan, Italy [1] - Department of Biology, University of Padova, Padova, Italy [2] - Laboratory of Pre-Clinical and Translational Research, IRCCS-CROB, Referral Cancer Center of Basilicata, Rionero in Vulture (PZ), Italy [3] - UOC Hematology, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy [4]

Introduction. The identification of deregulated miRNA in multiple myeloma (MM) has progressively added a further level of complexity to MM biology. Herein, we take virtue of *in silico* integrative genomics analysis to generate an unprecedented global view of the transcriptional regulatory networks modulated in MM and to define microRNAs impacting in regulatory circuits with potential functional and clinical relevance.

Materials ands Methods. miRNA and gene expression profiles in four large proprietary (Lionetti M, 2009; Wu P, 2013) and public (Gutierrez N, 2010; Zhou Y, 2010) representative MM datasets, available from retrospective and prospective clinical trials and encompassing a total of 305 patients at diagnosis, were analyzed by means of *MAGIA2* method (Bisognin A, 2012), to identify mixed circuits (triplets) involving miRNA/transcript/transcription factor (TF). *Micrographite* computational procedure (Calura E, 2014) was then applied to identify

highly modulated miRNA-gene networks in the disease, in terms of differential strength of inferred interactions, and highlight those pathways enriched with miRNA–target gene interactions stemming from expression data analysis and from predicted/validated interactions from public databases (TargetScan, TarBase and miRecords).

Results. Matched miRNA and genes/transcripts expression profiles of 4 MM dataset (including 40, 52, 60 and 153 cases) were combined with a curated set of miRNA-target predictions, thus allowing to identify critical circuits involved in MM, and the results obtained were overlapped to find common triplets. Two circuits emerged as common to at least two datasets, including hsa-mir-200c-3p, the TF *ATF2* and the autophagy receptor *NBR1*, and the other hsa-mir-497-5p (that we have previously found associated with treatment response in plasma cell leukemia – Lionetti et al, 2013), the Rho-like GTPase *ARHGEF9* and the TF *NFE2L1*. Integrated pathway analyses performed on miRNA and gene expression data of the 153 MM patients included in MRC Myeloma IX trial, stratified according to their hyperdiploid (HD) status, allowed to generate a meta-pathway composed by the miRNA/genes and the interactions that mainly characterize HD in MM.

Conclusions. Our analysis on 4 independent MM datasets allowed to define a preliminary but comprehensive picture of regulatory networks involving genes and miRNAs with putative roles in MM tumor biology, and identify specific elements and interactions thereof that are worth further investigation.

R 2
TARGETING ABERRANT HDAC4 ACTIVITY IN MULTIPLE MYELOMA (MM) THROUGH A MIR-29B-BASED THERAPEUTIC STRATEGY

Nicola Amodio [1] - Marzia Leotta [1] - Eugenio Morelli [1] - Maria Angelica Stamato [1] - Lavinia Raimondi [1] - Anna Maria Gullà [1] - Maria Teresa Di Martino [1] - Teresa Calimeri [1] - Marco Rossi [1] - Antonino Neri [2] - Pierosandro Tagliaferri [1] - Pierfrancesco Tassone [1]

Department of Experimental and Clinical Medicine, University Magna Graecia of Catanzaro, Catanzaro, Italy [1] - Department of Clinical Sciences and Community Health, University of Milan, Milan, Italy [2]

Introduction: Gene transcription within the tumor cell and its microenvironment can be affected by epigenetic modulation in histones, and inhibition of histone deacetylases represents a promising therapeutic option in MM. MicroRNAs are non-coding RNAs that control gene expression; a subclass of miRNAs, named epi-miRNAs, exerts anti-tumor activity by targeting effectors of the epigenetic machinery. We recently demonstrated a key role of miR-29b as epi-miRNA through the targeting of DNA-methyltransferases in MM. *In silico* search of miR-29b targets further clarifying its epi-miRNA function, unveiled the histone deacetylase HDAC4. Here, we aimed at characterizing HDAC4 expression, function and its regulation by miR-29b in MM.

Materials and methods: HDAC4 mRNA and protein levels were assessed in 11 MM cell lines by qRT-PCR and western blotting. miR-29b and HDAC4 levels in MM patient cells were obtained from our microarray datasets (GSE17498, GSE13591). Silencing of HDAC4 was obtained by lentiviral shRNAs; overexpression of miR-29b was achieved by synthetic mimics. Apoptosis and autophagy were analyzed by Annexin V and MDC staining respectively, and by assessing levels of active caspases or LC3A/B.

Results: HDAC4 mRNA, protein levels and activity were found upregulated in MM cells as compared to normal controls. The analysis of our microarray dataset indicated HDAC4 overexpression in cancer samples and its inverse correlation with miR-29b. Transfected miR-29b mimics down-regulated HDAC4 mRNA and protein in MM cells, and inhibited the 3'UTR of HDAC4 cloned in a luciferase vector. Stable silencing of HDAC4 induced growth inhibition, caspase 3/7-dependent apoptosis and autophagy in U266 and KMS11 cells, along with miR-29b up-regulation; interestingly, HDAC4 depletion potentiated dexamethasone, bortezomib and vorinostat anti-myeloma activity. Of note, the pan-HDAC inhibitor vorinostat also triggered apoptosis and autophagy in MM cells, along with the induction of miR-29b and the down-regulation of HDAC4 and of other miR-29b targets. miR-29b itself promoted beclin-1 induction and LC3A/B cleavage, which were abrogated by ectopic HDAC4. Finally, miR-29b overexpression potentiated, whereas its inhibition dampened, apoptosis and autophagy elicited by vorinostat.

Conclusions: High HDAC4 activity is a feature of MM cells and can be efficiently targeted through a miR-29b-based therapeutic strategy; moreover, miR-29b is a novel effector of vorinostat activity in MM cells.

R 3
REPLACEMENT OF TUMOR-SUPPRESSOR MIR-486-5P INHIBITS LUNG TUMORIGENESIS BY TARGETING CANCER STEM CELLS

Mattia Boeri [1] - Orazio Fortunato [1] - Massimo Moro [1] - Carla Verri [1] - Mavis Mensah [1] - Davide Conte [1] - Luca Roz [1] - Gabriella Sozzi [1]

Fondazione IRCCS Istituto Nazionale dei Tumori, Fondazione IRCCS Istituto Nazionale dei Tumori, Milano, Italy [1]

The development of novel therapies directed to biological targets has modified the therapeutic approach for lung cancer, but the frequent insurgence of resistance mechanisms due to cancer heterogeneity limits their capacity to reduce lung cancer mortality. Cancer stem cells (CSCs) are responsible for tumor formation and its heterogeneity, and they are also inherently resistant to the cytotoxic effect of chemotherapy because of their low proliferation rate and resistance mechanisms. Therapies aimed to target CSCs could represent a useful strategy to radically cure cancer.

MicroRNAs (miRNAs) are small non-coding RNAs that modulate gene activity and are aberrantly expressed in most types of cancer. Many papers have recently reported about the therapeutic use of miRNA mimics and inhibitors in order to restore the physiological miRNA levels in tumor cells and to treat lung cancer. In previous studies we reported that mir-486-5p behaves like a tumor-suppressor miRNA, being down-regulated in tumors compared to normal lung tissue and in plasma samples of lung cancer patients with poor prognosis.

In this study we first confirmed that the transient overexpression of mir-486-5p using miRNA mimics reduced migration, invasion, proliferation properties and increased apoptosis

in NCI-H460, LT73, A549 p53 wild-type lung cancer cells. Moreover, *in vivo* experiments using xenograft models showed that the transient transfection of the miRNA mimic inhibits completely tumor growth in all cell lines. Conversely, in the H1299 p53-null lung cancer cell line we observed a reduction of tumor growth *in vivo*, but not *in vitro*.

Interestingly, in all *in vivo* models tumor growth was inhibited up to 60 days, also when the transfected miRNA was not present anymore, suggesting an activity of mir-486-5p on CSCs. Accordingly, i*n vitro* experiments revealed that the mir-486-5p over-expression resulted in decreased levels of the CSCs' markers in all cancer cell lines. We also demonstrated that mir-486-5p activity on CSCs was achieved through the regulation of specific targets in the PI3K-AKT. Further experiments using patient derived xenografts (PDXs) are still ongoing in order to confirm the effect of mir-486-5p in models that closely represent the original human tumor.

Our finding supports that mir-486-5p may act as a tumor suppressor miRNA regulating crucial pathways, setting the bases for a therapeutic use of this miRNA for lung cancer treatment.

R 4
A PRECLINICAL STUDY ON MIR-181B AS THERAPEUTIC AGENT FOR CLL IN EU-TCL1FL-TG MOUSE MODEL

Antonella Bresin [1] - Elisa Callegari [1] - Lucilla D'abundo [1] - Caterina Cattani [2] - Cristian Bassi [1] - Maria Grazia Narducci [2] - Elisabetta Caprini [2] - Silvia Sabbioni [3] - Giandomenico Russo [2] - Massimo Negrini [1]

Dipartimento di Morfologia, Chirurgia e Medicina Sperimentale, Università di Ferrara, FERRARA, Italy [1] - Laboratorio di Oncologia Molecolare, IDI-IRCCS, ROME, Italy [2] - Dipartimento di Scienze della Vita e Biotecnologie, Università di Ferrara, FERRARA, Italy [3]

Introduction: Chronic lymphocytic leukemia (CLL) is a malignancy of B cells with immunophenotype of memory cells. CLL patients show different clinical course: CLL clones with no IGHV gene mutations, CD38+ or ZAP-70+ B cells and high expression of TCL1 exhibit an aggressive, usually fatal course, whereas patients with mutated clones, CD38- or ZAP-70- B cells and low TCL1 expression show an indolent course. Various studies identified microRNA signatures associated with differential prognosis and progression. In particular, miR-181b is down-regulated in aggressive forms of CLL and its expression is inversely correlated to TCL1 levels. An increasing number of studies are considering microRNAs as potential therapeutic agents. Given that MCL-1, BCL-2 and TCL1 are proved targets for miR-181b, that Akt activation is enhanced by TCL1 and a crosstalk between Akt and ERK or NFkB exists, we performed a preclinical evaluation of miR-181b therapeutic efficacy in a CLL mouse model.

Materials and methods: We used the well characterized Eμ-TCL1FL-tg mouse model for CLL to perform *in vitro* and *in vivo* experiments. Isolated tumor cells from spleen of TCL1-tg mice were transfected with miR181b. Modulation of miR181b targets and other factors was assessed by Western blot analysis, while apoptosis and cell survival were evaluated by flow cytometry following Annexin/PI staining. Human CLL-derived cell-line EHEB was used for the same experimental strategy. Transgenic mice were treated with miR-181b and leukemic expansion was evaluated through immunophenotyping of peripheral blood by flow cytometry. Lifetime data were used for survival analysis of treated mice vs controls.

Results: Our data indicate that miR-181b is able to modulate TCL1, MCL1, BCL2 and other factors important in the pathogenesis of CLL such as Akt, ERK and NFkB; following transfection with miR181b, we measured a decrease in tumor cell viability of both tg-mice lymphocytes and human cell-line. Finally, we observed a slowdown of leukemic cell expansion and increased survival of TCL1-tg mice treated with miR-181b.

Conclusions: miR-181b may represent a potential novel therapeutic tool for CLL treatment.

R 5
MIRNA AND GENE REGULATORY PATHWAY OF STAGE I EPITHELIAL OVARIAN CANCER: RECONSTRUCTING CANCER CIRCUITS

Enrica Calura [1] - Gabriele Sales [1] - Paolo Martini [2] - Robert Fruscio [3] - Eliana Bignotti [4] - Antonella Ravaggi [4] - Lara Paracchini [5] - Mariacristina Di Marino [5] - Laura Zanotti [4] - Dionyssios Katsaros [6] - Germana Tognon [7] - Enrico Sartori [7] - Sergio Pecorelli [8] - Maurizio D'incalci [5] - Sergio Marchini [5] - Chiara Romualdi [1]

Department of Biology, University of Padova, Padova, Italy [1] - Department of Biomedical Sciences, University of Padova, Padova, Italy [2] - Clinic of Obstetrics and Gynecology, University of Milano-Bicocca, San Gerardo Hospital, Milano, Italy [3] - Division of Gynecologic Oncology, "Angelo Nocivelli" Institute of Molecular Medicine, University of Brescia, Brescia, Italy [4] - Department of Oncology, IRCCS - "Mario Negri" Institute, Milano, Italy [5] - Department of Gynecology/Oncology, S. Anna Hospital, University of Torino, Torino, Italy [6] - Department of Obstetrics and Gynecology, University of Brescia, Brescia, Italy [7] - Division of Gynecologic Oncology of "Angelo Nocivelli" Institute of Molecular Medicine and Department of Obstetrics and Gynecology, University of Brescia, Brescia, Italy [8]

Introduction: It is becoming increasingly evident that the current difficulties in improving the clinical management of epithelial ovarian cancer (EOC) reside within its complex nature that hampers the possibility to identify the critical circuits driving tumor growth and therapy response.

Stage I EOC is an infrequent disease characterized by a good prognosis since more than 80% patients survive five years the end of chemotherapy. However, despite the therapy results, we are currently unable to predict, at diagnosis, who will relapse or not, and neither evaluate with efficacy the severity of the disease.

Studies performed so far by our laboratory demonstrated that altered expression of miR-200c is an independent prognostic factor (Marchini *et al,* Lancet Onc, 2011), suggesting that defects in the mechanisms of transcription regulation are associated with patient outcome.

Materials and methods: Following this idea, we decide to analyse miRNA expression integrated in gene circuit developing a dedicated new system able to integrate miRNAs in gene pathways and identify circuits of functionally related genes and

miRNAs showing coordinated expression changes (Calura *et al*, NAR 2014). We used this new approach to study EOC progression within the stage I EOC. 257 snap-frozen stage I EOC biopsies have been collected from three independent tumour tissue collections. A subset of samples have been profiled for gene and miRNA expression and used for the integrated analyses of circuits identifying a pathway composed of 44 functionally related elements. The expression values of all the circuit elements have been assessed in all the 257 collected biopsies and expression levels have been used to perform univariate and multivariate survival analyses.

Results: We identified 15 miRNAs and 9 genes with prognostic value that compose a barcode specific for each patient, useful to evaluate the patient-specific level of risk. Specifically, we elaborated an index representing the activation state of the studied circuit in each patient. The index has been used to efficiently stratify patients in high, medium or low risk classes resulting in a prediction of patients outcome with a sensitivity=88% and specificity=91%. Finally, the same criteria have been applied to evaluate the risk in an external and independent validation set of 50 patients confirming the powerful predictive value of risk assessment of the identified circuit.

Conclusions: Our results present the first pathway, entirely composed of elements with prognostic value, described in stage I EOC.

R 6
DOWNREGULATION OF MIR-30A INDUCES CHEMORESISTANCE IN OVARIAN CANCER CELLS THROUGH INCREASE OF ENDOTHELIN A RECEPTOR

Roberta Cianfrocca [1] - Rosanna Sestito [2] - Laura Rosanò [2] - Elisa Semprucci [2] - Valeriana Di Castro [3] - Gabriella Ferrandina [4] - Anna Bagnato [3]

Molecular Pathology Laboratory, Regina Elena National Cancer Institute, Rome, Italy [1] - Molecular Pathology Laboratory, Regina Elena National Cancer Institute Via Elio Chianesi, 53, 00144 Rome, Italy. [2] - Molecular Pathology Laboratory, Regina Elena National Cancer Institute Via Elio Chianesi, 53, 00144 Rome, Italy [3] - Gynecologic Oncology Unit, Catholic University of Rome [4]

Chemotherapy is the preferred therapeutic approach for the therapy of epithelial ovarian carcinoma (EOC), but a successful long-term treatment is prevented by the development of drug resistance. Interference with endothelin-1 (ET-1)/ET_A receptor (ET_AR) signaling pathway has emerged as a promising strategy for cancer therapy. ET-1/ET_AR axis is upregulated in chemoresistant EOC cell lines and associated with acquisition of chemoresistance and epithelial-mesenchymal transition (EMT) phenotype and poor prognosis. Because recent works have underlined the involvement of microRNAs (miRNAs) in cancer development and drug-resistence, in this study we determine whether in 2008 and A2780 sensitive and resistant EOC cell lines is present an aberrant expression of miRNAs involved in the control of ET_AR expression, which may explain the overexpression of this receptor and its mediated pathways in chemoresistant EOC. Based on the bioinformatics tools, we selected putative miRNAs able to recognize the 3'UTR of ET_AR. Among these, only miR-30a resulted downregulated in chemoresistant EOC cells compared with parental cells. In addition, the ectopic expression of miR-30a suppressed the protein expression of ET_AR. Importantly, through the mutation of the two miR-30a active sites on ET_AR mRNA, we ensured that this sequence-specific binding was responsible for the ET_AR downregulation. Moreover, miR-30a overexpression was able to restore drug sensitivity by enhancing the susceptibility of these cells to cis-platinum-induced apoptosis, as supported by the reduced cell viability and increased of PARP cleavage. In addition, miR-30a re-introduction in chemoresistant EOC cells, impaired their capacity to promote the invasive process and reverted EMT phenotype. Similarly, we demonstrated that the treatment with macitentan, a potent ET_AR antagonist with significant affinity for ET_BR, counteracted the effects elicited by ET_AR pathway in chemoresistant EOC cells restoring drug-sensitivity and in resistant EOC xenografts sensitized to chemotherapy. Finally, in platinum-resistant specimens from EOC patients we observed a decreased miR-30a expression compared to sensitive ones, indicating that lower levels of miR-30a and higher expression of ETAR in human EOC tissues were significantly correlated with chemoresistance. This study for the first time demonstrated miR-30a- dependent regulation of ETAR in human chemoresistant EOC, further supporting that the regulation of ET_AR represents a valid therapeutic opportunity for circumventing chemoresistance in EOC patients. Supported by AIRC.

R 7
EVIDENCE OF A CORRELATION BETWEEN BCL-2 PROTEIN AND MIR-211 EXPRESSION IN MELANOMA CELL LINES

Teresa De Luca [1] - Andrea Pelosi [1] - Daniela Trisciuoglio [1] - Simona D'aguanno [1] - Armando Felsani [2] - Andrea Urbani [3] - Maria Giulia Rizzo [1] - Donatella Del Bufalo [1]

Regina Elena National Cancer Institute, IRCCS, Rome, Italy [1] - Institute of Cell Biology and Neurobiology, Consiglio Nazionale delle Ricerche, Rome, Italy [2] - University of Tor Vergata, University, Rome, Italy [3]

Introduction: Bcl-2 is a proto-oncogene often associated with poor prognosis in melanoma. In this context, we previously demonstrated the ability of bcl-2 protein to increase tumorigenic and metastatic potential in melanoma cells. Recent studies implicate microRNAs (miRNAs) as important agents in the progression of cancer including melanoma. In particular, miRNAs regulate several molecular pathways, such as invasion and metastasis, by targeting various oncogenes and tumour suppressors. Among these, miR-211, that is located within the melastatin-1 (TRPM1) gene, is prevalently expressed in the melanocyte lineage and acts as oncosuppressor in melanoma. Both miR-211 and TRPM1 expression are co-ordinately regulated by the transcription factor MITF.

Materials And Methods: Whole Transcriptome Analysis was performed to analyze change in gene expression in M14 human melanoma cell line and its derivative bcl-2 overexpressing clone. To identify new bcl-2-related gene networks, analysis of gene expression followed by Ingenuity Pathway Analysis (IPA) was performed. MiR-211 and its target genes expression were assessed by quantitative Real-time PCR (RT-qPCR). Chromatine Immunoprecipitation (ChIP) assay was performed to analyze MITF consensus binding elements at the TRPM1 promoter.

Results: The top functional network identified by IPA was cellular movement, with 50 molecules signed. Notably, we found that most significative nodes of this network were miR-211 predicted targets, and some of them were upregulated in bcl-2 overexpressing cells respect to the control ones. These results lead us to investigate if there is a correlation between the expression of bcl-2 and miR-211. We found that expression of miR-211 in M14 parental cells is higher than in bcl-2 overexpressing cells, suggesting that miR-211 is modulated by bcl-2. mRNA levels of IGF2R and TGFBR2, two known mir-211 target genes, were evaluated. While TGFBR2 mRNA was found to be upregulated in bcl-2 overexpressing cells when compared to control cells, no modulation of IGF2R was observed. Analysis of pri-miR-211 and TRPM1 levels in M14 and bcl-2 overexpressing cells indicates that bcl-2 regulates miR-211 at transcriptional level. ChIP experiments revealed that MITF is mainly recruited at TRPM1 promoter in M14 cells compared to its derivative bcl-2 clone.

Conclusions: Our results demonstrated a correlation between the expression of bcl-2 protein and miR-211 in melanoma cell lines through a MITF-dependent mechanism.

R 8
REGULATION OF MIR30A-5P AND MIR30A-3P AND THEIR ROLE IN THE ZEB2-MIR200 AXIS IN TRIPLE-NEGATIVE BREAST CANCER

Alessandra Di Gennaro [1] - Giulia Brisotto [1] - Valentina Damiano [1] - Michela Armellin [1] - Andrea Gini [2] - Tiziana Perin [3] - Antonella Zucchetto [2] - Paolo Carli [4] - Herman Spaink [5] - Ewa Snaar-jagalska [5] - Roberta Maestro [1] - Manuela Santarosa [1]

CRO, Aviano National Cancer Institute, Experimental Oncology 1 Unit, Aviano (PN), Italy [1] - CRO, Aviano National Cancer Institute, Epidemiology and Biostatistics Unit, Aviano (PN), Italy [2] - CRO, Aviano National Cancer Institute, Pathology Unit, Aviano (PN), Italy [3] - CRO, Aviano National Cancer Institute, Clinical Oncology Unit, Aviano (PN), Italy [4] - Institute of Biology, Department of Molecular Cell Biology, Leiden, Netherlands [5]

Introduction: Carcinoma cells may undergo a dedifferentiation program that provides them with increased cell plasticity due to the acquisition of mesenchymal and stem-like features. A level of regulation of this phenomenon involves the small non coding RNA called microRNAs (miRNAs) whose aberrant expression is frequently observed in breast cancer (BC). This study aimed to shed light on miR30a-5p and miR30a-3p role in the control of cell plasticity in triple-negative breast cancer (TNBC).

Materials and methods: We examined miR30a-5p/3p expression in BC and in TNBC using the series from TCGA and from our Institution. The association between miR30a-5p/3p level and clinicopathologic factors and prognosis were analyzed. *In vitro* and *in vivo* assays were conducted to identify the targets of miR30a-5p and -3p and the mechanism by which they control cell plasticity. Furthermore, using computational approaches we identified binding sites for several transcription factors like p53 and ZEBs proteins in the putative miR30a promoter. *In vitro* studies will be performed to verify the regulation of miR30a expression.

Results: Down-regulation of miR30a-5p/3p occurs in BC and in particular in TNBC. Low levels of these miRNAs were associated with lymph node positivity and shorter disease-free and overall survival in TNBC. Functional studies indicated that miR30a-5p and miR30a-3p directly targeted ZEB2 transcript and inhibited migration and invasion of breast cancer cell lines *in vitro* and in a zebrafish xeno-transplantation model. Reintroduction of miR30a restored epithelial features and triggered miR200c expression. Accordingly, miR30a-5p/-3p and miR200c levels positively correlated in TNBC. As preliminary data, we observed an increase of miR30a-5p/3p expression when ZEB2 was down-regulated and when wt-p53 was overexpressed. Moreover, the expression of both miRNAs was higher in wt-p53 compared to p53-mutated BC (TCGA series) supporting the hypothesis that miR30a is up-regulated by p53.

Conclusions: Our study indicates that both strands of miR30a, by intersecting the ZEB2-miR200c axis, act as tumor suppressors preventing breast cancer cell dissemination and aggressiveness. Importantly, their down-regulation identifies a subset of TNBC with worse prognosis. Finally, miR30a could be regulated by ZEBs factors and p53.

R 9
MIR-660 IS DOWNREGULATED IN LUNG CANCER PATIENTS AND ITS REPLACEMENT INHIBITS LUNG TUMORIGENESIS BY TARGETING MDM2-P53 INTERACTION

Orazio Fortunato [1] - Mattia Boeri [1] - Massimo Moro [1] - Carla Verri [1] - Mavis Mensah [1] - Davide Conte [1] - Luca Roz [1] - Ugo Pastorino [2] - Gabriella Sozzi [1]

Fondazione IRCCS Istituto Nazionale dei Tumori, Tumor Genomics Unit, Milan, - [1] - Fondazione IRCCS Istituto Nazionale dei Tumori, Thoracic Surgery Unit, Milan, - [2]

Lung cancer represents the leading cause of cancer-related death in developed countries. Despite the advances in diagnostic and therapeutic techniques, the 5-year survival rate remains very low. The research for novel therapies directed to biological targets has modified the therapeutic approach, but the frequent engagement of resistance mechanisms and the substantial costs, limit the ability to reduce lung cancer mortality. MicroRNAs (miRNAs) are small non-coding RNAs that have been shown to have a variety of regulatory functions and to play roles in controlling cancer initiation and progression. In this study we found that mir-660 expression is down-regulated in lung tumors compared with adjacent normal tissues and in plasma samples of lung cancer patients with poor prognosis, suggesting a potential functional role of this miRNA in lung tumorigenesis. Transient over-expression of mir-660 using miRNA mimics reduced migration, invasion, proliferation properties and increased apoptosis in NCI-H460, LT73, A549 p53 wild-type lung cancer cells. Furthermore, stable over-expression using lentiviral vectors in NCI-H460 and A549 cells, inhibited tumor xenograft growth in immunodeficient mice (95% and 50% reduction compared to control respectively). The effects of mir-660 over-expression were absent in H1299, a lung cancer cell line lacking p53 locus, both in *in vitro* and *in vivo* assays. We further identified and

validated mouse double minute 2 (MDM2), a key regulator of the level and function of p53 tumor suppressor protein, as a new direct target of mir-660.

Our finding supports that mir-660 may acts as a tumor suppressor miRNA and we suggest the replacement of mir-660 expression as a new therapeutic approach for p53 wild-type lung cancer treatment.

R 10
MELAMIRNOME: FROM MELANOCYTE CULTURE TO METASTATIC MELANOMA

Marco Galasso [1] - Maurizio Previati [1] - Linda Minotti [1] - Fabio Corrà [1] - Carlotta Zerbinati [1] - Carlo Maria Croce [1] - Stefano Volinia [1]

Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Ferrara, Italy [1]

Introduction: Malignant melanoma is the deadliest form of skin cancer and the incidence of melanoma continues to increase faster than any other type of malignancy. Many studies are now investigating non-coding microRNAs (miRNAs) in melanoma. miRNAs, deregulated in several cancers, are endogenous short RNAs that control proteins outputs.

Materials and methods: Test cohort: Eighty samples, including 15 pairs of matched primary/metastatic tumors, 12 normal epidermis, 11 cultured melanocytes, 10 cultured keratinocytes, 17 melanoma cell lines were analyzed on miRNA microarrays. Patients with melanoma were chemotherapy naïve. The primary melanocytes were derived from neonatal foreskin. Validation cohort: miRNA and mRNA expression data and corresponding clinical information for the skin cutaneous melanoma (SKCM) datasets were from The Cancer Genome Atlas (TCGA). From the SKCM dataset, we selected patients with miRNA and mRNA data. For a multivariable analysis, the Cox proportional hazard model was applied to identify miRNAs with independent prognostic value.

Results: We first studied melanoma cell lines and cultured melanocytes to investigate the melanoma miRnome. Epidermis specific miRNAs were removed, ensuring that we did not include over-expressed miRNAs from infiltrating cells, such as keratinocytes. miR-204 and miR-211 were specific of normal melanocytes. Initially we identified a miRNA signature for transformation. Then we investigated the metastatic tumors and cell lines, which displayed expression changes in a subset of miRNAs: miR-93, miR-222, miR-17-5p, miR-106a, miR-206, miR-204, miR-211 and miR-145. Later, we investigated the miRNAs involved in prognosis.

Conclusions: We confirmed a subset of miRNAs that exert their role in the progression of melanoma and described novel prognostic indicators in melanoma patients.

R 11
MIRNA A-TO-I RNA EDITING EVENTS IN HUMAN BRAIN AND GLIOBLASTOMA

Angela Gallo [1] - Sara Tomaselli [1] - Federica Galeano [1] - Vinicia Polito [1] - Shahar Alon [2] - Eli Eisenberg [2] - Franco Locatelli [1]

IRCCS, Ospedale Pediatrico Bambino Gesù, Roma, Italy [1] - Raymond and Beverly Sackler School of Physics and Astronomy, University of Tel-Aviv, Tel-Aviv, Israel [2]

[1]Oncohaematology Department, Ospedale PediatricoBambinoGesù, IRCCS, Rome 00165, Italy; [2] Raymond and Beverly Sackler School of Physics and Astronomy, Tel-Aviv University, Israel; [3]Università diPavia, Pavia 27100, Italy.

RNA editing is a widespread epigenetic mechanism that converts Adenosines to Inosines within a double-stranded RNA (dsRNA). In humans, A-to-I RNA editing is catalyzed by the adenosine deaminases that act on RNA (ADAR) enzymes, which recognize and bind dsRNA structures and deaminate at specific or random positions several coding and regulatory RNAs. Since dsRNAs are structures also present in miRNA precursors, it is expected that even this class of RNAs undergoes A-to-I editing. ADARs activity has the potential to affect both the maturation step and the miRNA-mRNA recognition.

Using next-generation sequencing (NGS) approaches supplemented by an extensive bioinformatic analysis, we identified A-to-I editing events in precursors and mature miRNAs derived from human brain tissues and astrocytoma cell lines in which we modulated the expression of ADAR enzymes. Of note, we observed that ADAR2 can alter a large number of miRNAs, while ADAR1 restricts its action over only few miRNAs. Furthermore, we observed that when the RNA editing event occurs within the miR seed, editing not only re-direct over novel target genes but sensibly reduces the total number of mRNA targets compared to their unedited versions. In this way, the editing generates a novel class of miRNAs not coded by the DNA, but by the ADAR enzymes.

Finally, as in GBMs the level of RNA editing is generally extremely low or undetectable, the pool of mature unedited and edited miRNA is reduced to the only wild-type, profoundly transforming the miR-mRNA networks in a cancer cell.

R 12
REGULATION OF MICRORNA PROCESSING BY MUTP53 IN COLON CANCER

Francesca Garibaldi [1] - Emmanuela Falcone [1] - Gianluca Bossi [1] - Daniela Trisciuoglio [1] - Giulia Piaggio [1] - Aymone Gurtner [1]

IFO-Istituti Fisioterapici Ospitalieri, Istituto Nazionale dei tumori Regina Elena, Roma, Italy [1]

Introduction: Human cancers are characterized by deregulated miRNA expression and defects in miRNA biogenesis promoting cellular transformation and tumorigenesis; still, the mechanisms through which miRNAs are regulated in cancer remain unclear.

Tumor suppressor p53, mutated in approximately 50% of human cancers, can acquire GOF activities favouring tumor induc-

tion, maintenance, spreading; miRNAs can be regulated by wtp53 at transcriptional and post-transcriptional level but few data about mutp53-dependent miRNA expression are available yet.

Results: Our results, coming from a genome wide analysis, reveal 34 miRNAs up-regulated after interference of mutp53 in human colon adenocarcinoma cells.

Validation of microarray's analysis for mature miRNAs, pri-miRNAs and pre-miRNAs shows that mutp53 regulates the majority of miRNAs at post-transcriptional level, thus suggesting a mut-p53-mediated miRNA processing inhibition.

The *in vivo* association between the target pri-miRNAs and Drosha is significantly attenuated in the presence of mut-p53. We found that the endogenous mut-p53 inhibits the interaction between Drosha and p68/p72 RNA helicases, regulatory factors of the microprocessor complex, by binding and sequestering them. Indeed, p68/p72 overexpression increases mutp53–dependent miRNA levels.

Moreover, we found that 5 of the post-transcriptional regulated miRNAs show tumor suppressive properties, playing a role in apoptosis, cell cycle arrest, EMT repression and migration inhibition.

Conclusions: These data support the idea that mutp53 is one of the key factors leading to the decreased expression of miRNAs in human colon cancers by interfering with Drosha-mediated miRNA processing.

Materials and methods : Experiments have been performed with human colon adenocarcinoma cells SW480, HT-29 and WiDr carrying the mutated p53 R273H. Lentiviral infection (constitutive p53 interference); microarray by tebu-bio (mature miRNAs); qRT-PCR by Applied Biosystems (mature, pri- and pre- miRNAs); Co-Immunoprecipitation assay and Western Blot (Drosha-p68/p72 interaction); RNA-ChIP analysis (Drosha-pri-miRNAs association); proliferation assay, FACS and wound healing assay (proliferation, cell cycle and migration analysis).

R 13
POTENTIAL MIRNA BIOMARKER CANDIDATES FOR THE ACCURATE DETECTION OF ENDOMETRIAL ATYPICAL HYPERPLASIA

Simona Giglio [1] - Viviana Annibali [2] - Roberto Cirombella [1] - Stefano Volinia [3] - Emidio Luciani [1] - Andrea Vecchione [1]

Department of Clinical and Molecular Medicine, Sapienza University, Rome, Italy [1] - Dept Neurosciences, Mental Health and Sensory Organs, Sapienza University, Rome, Italy [2] - Department of Morphology and Embryology, University of Ferrara, Ferrara, Italy [3]

Introduction:Endometrial carcinoma (EC) is the most common malignant tumor of the female genital organs in industrialized countries and comprises 4% of all cancers in women worldwide. Estrogen-dependent tumors (type I, endometrioid endometrial carcinomas) account for 80% to 85% of cases and non-estrogen-dependent (type II, nonendometrioid endometrial carcinomas) account for the rest. EC type I is generally thought to develop via precursor lesions along with the increasing accumulation of molecular genetic changes. Indeed, Endometrial hyperplasia with atipia (CAH) is the least common type of hyperplasia, but is the type most likely to progress to type 1 endometrial carcinoma which accounts for 97% of uterine cancers, whereas simple hyperplasia (SH) rarely progresses to carcinoma.MicroRNAs (miRNAs) are a class of small, non-coding, single-stranded RNAs that negatively regulate gene expression mainly binding to 3'-untranslated region (UTR) of target mRNAs at the post-transcriptional level. Recent studies have demonstrated that aberrant expressions of miRNAs are closely associated with the development, invasion, metastasis and prognosis of various cancers including EC.The aim of this study was to investigate the hypothesis that changes in miRNAs represent potentially useful biomarkers for the diagnosis of endometrial hyperplasia with atypia and prediction of outcome in endometrial cancer.

Methods:We compared the expression profiles of 723 human miRNAs from 19 cases of CAH and 18 SH using Affymetrix Gene Chip following RNA extraction from formalin-fixed paraffin-embedded tissue.

Results: Profiling of microRNAs by GeneChip miRNA Affymetrix microArray allowed us to identify a "signature" of 8 microRNAs (miR-379,miR-205,miR-146a,miR-200b,miR-200a,miR-542-5p,miR-192,miR-1260b) aberrant expressed in CAH samples. Interestingly, the results of our study confirm the higher expression of miR-200 family members which have been reported most frequently upregulated in endometrial cancers.

Conclusions:Aberrant expression of miRNAs expression are common in endometrial hyperplasia. They might serve to increase the diagnostic reproducibility and improve discrimination between atipia and simple hyperplasia. those expression profiles of biomarkers also might be used to predict the potential for progression from endometrial hyperplasia to invasive cancer.These preliminary observations suggest that miRNAs could represent promising biomarkers for diagnosis and prognosis in endometrial hyperplasia.

R 14
MICRORNA-193A NEGATIVELY REGULATES UROKINASE AND IN COMBINATION WITH SORAFENIB IMPAIRS THE AGGRESSIVE PROPERTIES OF HCC CELLS

Ilaria Grossi [1] - Alessandro Salvi [1] - Isabel Conde [1] - Edoardo Abeni [1] - Bruna Arici [1] - Nazzario Portolani [2] - Sergio Barlati [1] - Giuseppina De Petro [1]

Departemnt of Molecular and Translational Medicine, Division of Biology and Genetic, University of Brescia, Brescia, Italy [1] - Department of Clinic and Experimental Sciences, Surgical Clinic, University of Brescia, Brescia, Italy [2]

Introduction: Hepatocellular carcinoma (HCC) is the most common primary malignant tumor of the liver and the third most common cause of cancer-related death worldwide. Recently, the multikinase inhibitor Sorafenib has been used for the treatment of advanced HCC and it is the unique systemic drug proved to be effective in this disease. Nevertheless, some patients do not benefit from this therapy so the implementation of innovative molecular approaches is needed. microRNAs are short non coding RNAs that negatively regulate gene expression

in several physiological and pathological conditions, including HCC. Accumulated evidence describes miRNA as potential new tools for molecular targeted therapies in HCC. The purpose of this study was to identify a novel approaches to sensitize the HCC cells to Sorafenib.

Materials and methods: miR-193a was validated as negative regulator of uPA (urokinase-type plasminogen activator) in 2 undifferentiated HCC-derived cell lines (HA22T/VGH and SKHep1C3) by transient transfection of miR and anti-miR molecules. The molecular interaction between miR-193a and uPA mRNA target was verified by luciferase reporter assay. The miR-193a expression level was evaluated in 39 HCC biopsy specimens and corresponding peritumoral (PT) tissues, by stem-loop real time PCR. The HCC cells were transfected with miR-23b (SKHep1C3) and miR-193a (HA22T/VGH) and then treated with Sorafenib; the effects on cellular proliferation and apoptosis were tested. The effect of Sorafenib on c-met protein expression levels was texted by western blotting.

Results: The miR-193a negatively regulated uPA expression. In both cell lines, the ectopic expression of miR-193a determined cellular proliferation inhibition and apoptosis induction. Furthermore, the miR-193a was down-regulated in HCC respect to PT tissues, more in cirrhotic HCCs than in non-cirrhotic ones. Interestingly, the combined treatment with Sorafenib and miRNAs showed a significantly proliferation inhibition of HCC cells and a major effect on apoptosis induction. The c-met protein levels decreased in HCC cells treated with Sorafenib.

Conclusions: Our results outline new advances in post-transcriptionally miR-mediated regulation of uPA, in the validation of miRs that regulate HCC unfavourable prognostic markers (i.e. uPA) and they provide *in vitro* evidence for innovative therapeutic strategies based on Sorafenib in combination with miRNAs.

R 15
NF-Y REGULATES E-CADHERIN EXPRESSION BY MIRNA-200 FAMILY IN COLON CANCER.

Emmanuela Falcone [1] - Francesca Garibaldi [1] - Aymone Gurtner [1] - Giulia Piaggio [1]

Regina Elena National Cancer Institute, Hospital, Rome, Italy [1]

Introduction: miRNAs, small noncoding RNAs, have emerged as key post-transcriptional regulators of gene expression by causing transcript degradation or translational repression and a number of tools have been developed for predicting miRNA targets. Their activities are implicated in cancer progression or suppression by affecting growth, transformation, invasion, metastasis and response to treatment. A widespread deregulation of miRNAs is commonly observed in human cancers and promotes tumorigenesis. Thus, miRNAs may be potential targets for cancer therapy; still, the mechanisms through which miRNAs are regulated in cancer remain unclear.

Results: Recently, we catalogued, from literature, a set of common miRNAs deregulated in colon cancer. This search retrieved 118 deregulated miRNAs. Through a computational analysis on 5,5 kb around the TSS, we identified conserved NF-Y consensus motif in 39 (corresponding to 55 miRNAs) of the 118 miRNA's promoters, among them the two promoters of miR-200 family. Being potent inhibitors of epithelial-mesenchymal transition (EMT), the members of miR-200 family are down-regulated in aggressive human cancers. By ChIP experiments performed in human colon cancer cells we observed that NF-Y directly binds the CCAAT-box of miR-200 family promoters and this binding correlates with the appearance of open chromatin marks. Consistent with this, NF-Y silencing and overexpression experiments indicate that NF-Y sustains E-cadherin protein expression regulating miRNA-200 family expression at transcriptional level. Moreover, we find that the over-expression of the regulatory subunit of the trimer, NF-YA, inhibits cell migration. Finally, through a computational analysis we identified NF-Y as putative target of miR-200 family members.

Conclusions: These data strongly suggest a main role for NF-Y in the regulation of miR-200 family members.

Materials and methods: All experiments were performed on colon adenocarcinoma cell lines (sw480, ht29, lovo, rko). NF-Y overexpression was obtain by transient transfection. NF-Y depletion was obtain by lentiviral infection. The transcriptional role of NF-Y on miRNA-200 was demonstrated by *in vivo* ChIP experiments, luciferase assays, pri-miRNAs and mature miRNAs level analysis (Real-Time PCR using the $2^{-\Delta Ct}$ method). Migration assays were perform with Boyden chambers. All experiments were performed several time. P values were determined using t test.

R 16
DOWN-REGULATION OF JUNCTIONAL ADHESION MOLECULE-A (JAM-A) IS CONTROLLED BY MIR-21 DURING COLORECTAL CANCER PROGRESSION

Andrea Lampis [1] - Jens Hahne [1] - Claudio Murgia [2] - Viola Paulushock [3] - Matteo Fassan [4] - Joanne Edwards [3] - Paul Horgan [3] - Owen Sansom [2] - Luigi Terracciano [5] - Michael Karin [6] - Carlo Croce [7] - Chiara Braconi [1] - Nicola Valeri [1]

Institute of Cancer Research, University of London, London, United Kingdom [1] - The Beatson Institute for Cancer Research, University of Glasgow, Glasgow, United Kingdom [2] - Institute of Cancer Sciences, University of Glasgow, Glasgow, United Kingdom [3] - ARC-NET Research Centre, University of Verona, Verona, Italy [4] - Institute of Pathology, University Hospital Basel, Basel, Switzerland [5] - University of California, University of California, San Diego, United States [6] - The Ohio State University, The Ohio State University, Columbus, United States [7]

Introduction: MicroRNAs are small non-coding-RNAs that control cell homeostasis and are deregulated in human colorectal cancer (CRC). MiR-21 up-regulation is frequent in CRC and represents a driver for tumour initiation and progression. Junctional Adhesion Molecule A (JAM-A) is a tight junction protein involved in regulation of para-cellular permeability in epithelial and endothelial cells. JAM-A forms homodimers and regulates cell adhesion, polarity and proliferation. Monoclonal antibodies against JAM-A are currently tested in the preclinical setting as potential targeted therapies. JAM-A loss appears to be an early event in CRC initiation but very little is known about the role of JAM-A in human sporadic CRC. The aim of our study was to investigate the pattern of expression of JAM-A in human CRC

and to define whether its deregulation is a consequence of aberrant microRNA expression.

Materials and methods : JAM-A expression was analysed by immunohistochemistry in 883 cases of human sporadic CRC. MiR-21 and JAM-A expression was analysed in 10 human CRC cell lines by qPCR and Western-Blotting. MiR-21 silencing and over-expression were performed using Exiqon LNAs and Ambion Pre-miR respectively. MiR-21 binding site in JAM-A 3'UTR was predicted using RNA Hybrid and the area encompassing the predicted seed region was cloned downstream of the Firefly Luciferase gene for Luciferase Reporter Assay.

Results: JAM-A down-regulation was observed in cancer compared to normal adjacent tissues. JAM-A was progressively down-regulated in the progression from normal epithelium, dysplasia, intraepithelial and invasive cancer. Poorly differentiated cancers showed total loss of JAM-A. Shifted staining from apical to baso-lateral compartment was observed in 25% of all cancers. Silencing and re-expression of miR-21 in CRC cell lines resulted in increase and down-regulation of JAM-A protein expression respectively. Luciferase reporter assay experiments were able to define the potential seed region by which miR-21 interacts with JAM-A 3'UTR.

Conclusions: Our data suggest that JAM-A expression is deregulated during human colorectal cancer progression as a consequence of miR-21 over-expression. These results highlight how miR-21 could potentially exert its' actions on para-cellular permeability and cell polarity acting on junctional adhesion molecules. Understanding the mechanisms of JAM-A loss or relocalization may help to stratify patient's treatment and identify patients who might benefit from anti-JAM-A monoclonal antibodies.

R 17
THE TRANSCRIPTOME OF PROSTATE CANCER: A NON CODING RNA POINT OF VIEW.

Maurizia Mello-Grand [1] - Paola Ostano [1] - Lidia Sacchetto [1] - Ilaria Gregnanin [1] - Giulia Bottoni [1] - Francesco Ilardi [1] - Giuseppina M. Carbone [2] - Giovanna Chiorino [1]

Laboratory of Cancer Genomics, Fondazione Edo ed Elvo Tempia, Biella, Italy [1] - Institute of Oncology Research, Oncology Institute of Southern Switzerland, Bellinzona, Switzerland [2]

Introduction: Prostate cancer (PCa) is the leading cancer diagnosis and the second most common cause of cancer-related death in men in Europe. It is multifocal and highly heterogeneous and current diagnostic methods are still unable to distinguish indolent from aggressive lesions. Furthermore, for recurrent castration resistant prostate cancer no long lasting therapies still exist.

Many genomic alterations in PCa have been described by high-throughput techniques. Recently, non-coding RNAs have emerged as important players in many physiological and pathological processes including development, differentiation and cancer, but their role and relevance to clinical practice and PCa management is still far from being well understood.

Materials and Methods. We performed transcriptional profiling of 65 localized PCas and 20 normal prostate tissues of age-matched healthy men by means of microarrays. We focused on non coding RNA (ncRNA) and selected long intergenic ncRNAs (lincRNAs) and small nuclear/nucleolar RNAs (snRNAs) significantly upregulated in tumours versus normal samples and those significantly associated with biochemical recurrence (median follow-up: 9 years). In order to begin to understand their biological role, we performed functional annotation analysis on the transcripts most (anti)correlated with the selected ncRNAs. Furthermore, altered expression of specific ncRNAs was validated by qPCR in tumor tissues. *In situ* hybridization was also applied to analyze their expression patterns within cell types and cellular compartments. Interestingly, some of the selected ncRNAs could be also evaluated in the plasma samples of an independent cohort of PCa patients and healthy men.

Results: Whole transcript analysis allowed us to uncover novel ncRNAs whose expression is altered in PCa *vs* normal prostate tissue. Finally, we associated their expression with unique transcriptional signatures and potential functional role in tumorigenesis. Some of these ncRNAs could also be detected in patients' plasma underlining their potential diagnostic power. The long patients' follow-up allowed detection of interesting ncRNAs associated with biochemical recurrence.

Conclusions: Integration of transcriptomic data at different levels lead to the identification of novel ncRNAs with a potential diagnostic and prognostic role in prostate cancer. Their association with a unique transcriptional signature suggests a potential functional role in prostate cancer progression.

R 18
NOTCH AND NF-KB SIGNALING PATHWAYS REGULATE MIR-223/FBXW7 AXIS IN T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA

Rocco Palermo [1] - Vivek Kumar [2] - Saula Checquolo [3] - Luca Tottone [2] - Gioia Testa [2] - Stefano Indraccolo [4] - Alessandra Vacca [5] - Isabella Screpanti [2]

Center for Life Nano Science@Sapienza, Istituto Italiano di Tecnologia, Rome, Italy [1] - Department of Molecular Medicine, Sapienza University, Rome, Italy [2] - Department of Medico-Surgical Sciences and Biotechnology, Sapienza University, Latina, Italy [3] - Istituto Oncologico Veneto, IRCCS-Padova, Padua, Italy [4] - Department of Experimental Medicine, Sapienza University, Rome, Italy [5]

Introduction: Deregulated Notch signaling is linked to T-cell acute lymphoblastic leukemia (T-ALL) and increasing evidence suggests NFkB signaling as one of the major mediators of Notch-induced tumorigenesis. Recent studies have highlighted the key role of altered expression of miRNAs in several tumors including human leukemias and several miRNAs have been shown to cross-talk with Notch pathway. However, the transcriptional regulators of miRNAs, as well as the relationships between Notch signaling and miRNA deregulation, are poorly understood.

Material and methods: Cell sorting: $CD4^+CD8^+$ DP thymocytes from Notch3 transgenic mice were isolated by FACSAria cell sorter. RT-PCR: The expression levels were determined by Taqman Primers for coding genes and by TaqMan MicroRNA Assay Kits (Applied B.). miRNA profiling: miRNAs were profiled using stem–loop RT–PCR based TLDA cards (Applied

B.). Statistical Analysis: Raw data files from TLDA were processed by RealTime StatMiner (Integromics). Trasductions: The Notch3 silencing in Molt3 cells was carried out with pLKO-puro-IPTG-LacO lentivirus expressing sh RNA for Notch3 (Sigma). Transfections: Mimic or Anti-miR (Thermoscientific) were transfected by by using Neon®Transfection System (Invitrogen).

Results: Among seven miRNAs, consistently regulated by overexpressing or silencing Notch3, we focused our attention on miR-223, whose putative promoter analysis revealed a conserved RBPjk/NFkB nested binding site. Luciferase and CHIP assays on the promoter region of miR-223 show that both Notch and NF-kB are novel coregulatory signals of miR-223 expression. Notably, the Notch-mediated activation of miR-223 represses the tumor suppressor FBXW7 in T-ALL cell lines. Moreover, we observed the inverse correlation of miR-223 and FBXW7 expression in a panel of T-ALL patient-derived xenografts. Finally, we show that miR-223 inhibition prevents T-ALL resistance to γ-secretase inhibitor (GSI) treatment.

Conclusions: Overall our data demonstrate the existence of a cooperative cross-talk between Notch and NFkB in miR-223 regulation. Moreover, we elucidate the oncogenic role of miR-223 in T-ALL through the repression of the tumor suppressor FBXW7. Finally, we observed that ectopic modulation of miR-223 expression in human T-ALL cell lines affects their ability to respond to GSI treatment, suggesting that miR-223 could be involved in GSI sensitivity and its inhibition may be exploited in target therapy protocols.

R 19
LINC-RNAS LANDSCAPE ANALYSIS IN STAGE I EOC AND CLINICAL IMPLICATIONS

Lara Paracchini [1] - Paolo Martini [2] - Stefano Cagnin [2] - Mariacristina Di Marino [3] - Luca Beltrame [3] - Robert Fruscio [4] - Tommaso Grassi [3] - Antonella Ravaggi [5] - Eliana Bignotti [5] - Germana Tognon [5] - Giovanna Chiorino [6] - Chiara Ghimenti [6] - Maurizio D'incalci [3] - Chiara Romualdi [2] - Sergio Marchini [3]

Department of Oncology, IRCCS Istituto di Ricerche Farmacologiche "Mario Negri", Milano, Italy [1] - Department of Biology, University of Padova, via U. Bassi 58/B, 35121 Padova, Italy [2] - Department of Oncology, IRCCS Istituto di Ricerche Farmacologiche "Mario Negri", via La Masa 19, 20145 Milan, Italy [3] - Clinic of Obstetrics and Gynecology, University of Milano-Bicocca, San Gerardo Hospital, 20900 Monza, Italy [4] - Angelo Nocivelli Institute of Molecular Medicine, Division of Gynecologic Oncology, University of Brescia, Viale Europa 11, 25123 Brescia, Italy. [5] - Cancer Genomics Laboratory, Fondazione Edo ed Elvo Tempia Valenta, via Malta 3, 13900 Biella, Italy [6]

Introduction. Epithelial Ovarian Cancer (EOC) is the sixth most common cancer in women. Stage I represents about 10% of EOC and is generally characterized by good prognosis in fact less than 20% of patients relapse. Experimental evidences from our laboratory showed that Stage I EOC patients with poor prognosis have transcriptional alterations, therefore we decided to investigate a new class of RNA with transcriptional regulation functions, the long non coding-RNAs (linc-RNAs). The aim of our current work is to identify a correlation between linc-RNAs expression and clinical outcome of Stage I EOC patients.

Materials and Methods. 203 snap-frozen stage I EOC tumor biopsies, 45 of which relapsed, were gathered together from three independent Italian tumor tissue collections. Median follow up was 9 years, with the three grades and the four cellular histotypes homogeneously represented. Gene Expression analysis and qRT-PCR were used to identify, analyze and validate linc-RNAs expression. Clinical parameters were correlated in multivariate analysis with OS and PFS. *In vitro* models were exploited to investigate the localization of selected linc-RNAs. Digital droplet PCR (Bio-Rad) was used for copy number variation analysis of PVT locus gene.

Results. A lincRNA based signature associated to relapse was identified by microarray analysis. Signature validation by qRT-PCR revealed that three out of 14 selected lincRNAs were independently associated with poor outcome (p-value < 0.05 in OS and/or in PFS in multivariate analysis) in multivariate analysis: linc-SERTAD2, linc-SOX4 and linc-MYC (PVT1). Patients with increased expression of selected linc-RNAs were associated with poor outcome ($p<0.05$). *In vitro* studies showed that the three linc-RNAs have predominantly a nuclear localization in all examined six ovarian carcinoma cell lines, thus suggesting they have a key role in transcriptional regulation of gene expression.

Conclusion. Our results represent the first evidence that the expression levels of specific linc-RNAs are strongly associated with prognosis of stage I EOC patients. In line with our previous analysis, these data confirm that within stage I EOC, mechanisms controlling transcription regulation are deregulated in those patients who relapse. If these data are confirmed in further studies they will provide the basis to improve patients stratification at time of diagnosis.

R 20
ABNORMAL EXPRESSION OF WT1-AS, MEG3 AND ANRIL LONG NON-CODING RNAS IN PRIMARY MYELOFIBROSIS AND ITS CLINICAL CORRELATIONS.

Valentina Pennucci [1] - Roberta Zini [1] - Ruggiero Norfo [1] - Paola Guglielmelli [2] - Elisa Bianchi [1] - Simona Salati [1] - Giorgia Sacchi [1] - Zelia Prudente [1] - Elena Tenedini [3] - Samantha Ruberti [1] - Sebastiano Rontauroli [1] - Chiara Paoli [2] - Tiziana Fanelli [2] - Carmela Mannarelli [2] - Enrico Tagliafico [3] - Alessandro Maria Vannucchi [2] - Sergio Ferrari [3] - Rossella Manfredini [1]

Centre for Regenerative Medicine, University of Modena and Reggio Emilia, Modena, Italy [1] - Department of Experimental and Clinical Medicine, University of Florence, Florence, Italy [2] - Center for Genome Research, University of Modena and Reggio Emilia, Modena, Italy [3]

Introduction. Long non-coding RNAs (lncRNAs) are emerging as key regulators of gene expression in normal and cancer cells by recruiting chromatin remodeling complexes. Despite lncRNAs characterization in several tumor types, little is known about their role in malignant hematopoiesis. In particular, lncRNAs expression has never been investigated in cells from primary myelofibrosis (PMF) patients. PMF is a Philadelphia negative chronic Myeloproliferative Neoplasm (MPN) that originates from deregulated clonal proliferation of hematopoietic stem cell associated with overproduction of mature blood cells. Molecular basis underlying MPNs pathogenesis were partially

unraveled in 2005-2006 with the identification of somatic mutations of *JAK2* and *MPL*, after which many other mutations were identified. Recently, several new molecular pathogenetic mechanisms were proposed, such as the aberrant expression of coding and non-coding RNAs.

Materials and methods. In order to identify other molecular abnormalities held by PMF patients, we investigated the expression of *CDKN2B-antisense (ANRIL)*, *MEG3* and *WT1-antisense* lncRNAs, previously described as involved in hematological malignancies, in CD34+ cells from PMF patients.

Results. The results evidence that the majority of PMF samples displays a co-upregulation of *WT1* and its antisense RNA compared to controls. These samples also show an increased *MEG3* expression. In these patients, we found a correlation with high Dynamic International Prognostic Scoring System (DIPPS) plus score and elevated number of circulating CD34+ cells. Moreover, the expression pattern of *CDKN2B/ANRIL* distinguishes a group of patients characterized by an upregulation of *CDKN2B*, and, among these, a subgroup with downregulated *ANRIL*. Of note, this group of patients was characterized by a higher grade of bone marrow fibrosis and by the presence of *JAK2V617F* mutation.

Conclusions. To our knowledge, this is the first study that describes the expression profiles of human lncRNAs in CD34+ cells of PMF patients. Our data demonstrate that *WT1-as*, *MEG3* and *ANRIL* lncRNAs are aberrantly expressed in PMF CD34+ cells compared to healthy controls. Moreover, different patterns of expression are correlated with some patient' clinical characteristics. Our results suggest that a deregulated expression of these lncRNAs could play a role in PMF pathogenesis and progression.

R 21
A NOVEL PROGNOSTIC MICRORNA SIGNATURE IN MALIGNANT PLEURAL MESOTHELIOMA

Anna Truini [1] - Simona Coco [1] - Ernest Nadal [2] - Carlo Genova [1] - Maria Giovanna Dal Bello [1] - Irene Vanni [1] - Angela Alama [1] - David George Beer [2] - Francesco Grossi [1]

Lung Cancer Unit, IRCCS AOU San Martino-IST, Genova, Italy [1] - Department of Surgery, University of Michigan, Ann Arbor, United States [2]

Introduction: Malignant pleural mesothelioma (MPM) is an aggressive tumor predominantly associated with asbestos exposure. Patients diagnosed with MPM have a poor outcome (median survival 10 months) and novel diagnostic and therapeutic approaches are needed. MicroRNAs (miRNAs) play a role in tumorigenesis and progression in MPM. The aim of this study was to identify miRNAs associated with poor prognosis in MPM which may be potentially actionable targets in the future.

Materials and methods: We identified 11 MPM long survivors (LS,>36 months) and 15 MPM short survivors (SS,<12 months) diagnosed at the IRCCS AOU San Martino-IST (Genova) from 1998-2008 that did not undergo tumor resection. Sections from FFPE biopsy blocks were macrodissected and total RNA was extracted. Twenty-six MPM and 3 additional normal pleura samples were miRNA profiled using the Agilent platform Human miRNA Microarray 8x60K including 2006 miRNAs. Expression data were normalized using GeneSpring software (v.12.6). Class-comparison analysis between MPM/pleura and SS/LS was performed using a t-test adjusted for multiple comparisons using Benjamini-Hochberg. Overall survival (OS) curves were estimated using the Kaplan-Meier method and compared with the log-rank test.

Results: Patients' characteristics: median age, 67 years (53-77); males (81%), females (19%). The most frequent histotype was epithelioid (69%), followed by sarcomatoid (12%), biphasic (4%) and unknown (15%). No differences in age, gender and histotype were observed among LS and SS. By class-comparison analysis, 30 miRNAs were significantly up-regulated and 11 down-regulated in MPM versus normal pleura (adjusted p-value <0.05). Fourteen miRNAs were significantly associated with outcome in the univariate survival analysis and differentially expressed in MPM. A miRNA signature was calculated based on the top 5 prognostic miRNAs (unfavorable, miR-1224; favorable, miR-99a, let-7b, let-7c and let-7i) and patients were classified into low or high-risk. High-risk MPM patients had a significantly shorter median OS (4.1 months, 95% CI 2.2-5.9) as compared with low-risk patients (median not reached, Log-rank p<0.001). Relevant pathways, such as PI3K/AKT, WNT, p53 and MAPK, were associated with these top 5 miRNAs by pathway analysis based on predicted targeted genes.

Conclusions: A prognostic miRNA signature was identified by profiling a cohort of unresected MPM. Further validation is warranted using an independent cohort of MPM.

R 22
CELLULAR ACTIVITY OF MICRORNAS DYSREGULATED IN BREAST CANCER

Carlotta Zerbinati [1] - Fabio Corrà [1] - Linda Minotti [1] - Marco Galasso [1] - Maurizio Previati [1] - Stefano Volinia [1]

Department of Morphology, Surgery and Experimental Medicine, University, Ferrara, Italy [1]

Introduction: Breast cancer (BC) is one of the major health problems worldwide and it is the second cause of cancer-related death in women. Patients often develop resistance to the current therapies. For this reason, the identification of new specific clinical molecular markers and pharmacologic targets in cancer research is an ongoing challenge. By regulating the expression of target genes, microRNAs can have a tumor suppressor or oncogenic role.

Materials and methods: We studied several human BC-derived cell lines: MCF-7, MDA-MB-231, MDA-MB-468, MDA-MB-361, SKBR3, T47D, BT474, ZR75.1, MDA-MB-453, HBL-100, and the breast non-tumor cell lines: 184A1, MCF10A.

All the cell lines were transfected with either one of 38 miRNAs (miR-21, 26b, 28-5p, 33b, 99a, 126, 126*, 130b, 138, 142-5p, 143, 181a, 202, 203, 206, 210, 218, 222, 145, 301a, 302a, 320c, 326, 484, let-7d*, 93, 103, 1307, 148, 328, 874, 151, 10a, 25, 30a, 615, 27a, 9), identified as important in BC (Volinia et al. Genome Res. 2010; Volinia S. and Croce CM. PNAS 2013).

Cell proliferation was determined by means of the xCELLigence RTCA System and the analysis of cell viability was carried out by performing MTS and PMS. The miRNAs were further investigated for their capacity to affect cell migration, cell invasion, and RNA profiles.

Results: The main outcome of our studies has been the identification from such wide list of a few miRNAs involved in the regulation of cell growth and invasion. In a first not exhaustive screening, we have identified some genes, which negatively correlated with those key miRNAs.

Conclusions: In this work, we investigated the possible causal role of microRNAs associated to breast cancer. We can conclude that we could dissect and prioritize *in vitro* the functional role of miRNAs in breast cancer.

R 23
EARLY TARGETS OF MIR-34A IN NEUROBLASTOMA

Pasqualino De Antonellis [1] - Marianeve Carotenuto [1] - Jonathan Vandenbussche [2] - Gennaro De Vita [3] - Veronica Ferrucci [3] - Chiara Medaglia [3] - Iolanda Boffa [3] - Alessandra Galiero [3] - Daniela Spano [3] - Lucia Liguori [1] - Antonio Verrico [1] - Johannes H. Schulte [4] - Pieter Mestdagh [5] - Jo Vandesompele [5] - Kris Gevaert [2] - Massimo Zollo [6]

Centro di Ingegneria Genetica e Biotecnologie Avanzate ,, CEINGE, napoli, Italy [1] - Department of Medical Protein Research,, VIB, B-9000, Ghent,, Belgium [2] - Centro di Ingegneria Genetica e Biotecnologie Avanzate ,, CEINGE, NAPOLI, Italy [3] - University Children's Hospital Essen, University Children's Hospital Essen,, Essen, Germany [4] - Center for Medical Genetics, Ghent University Hospital, B-9000 Ghent, Belgium., Ghent University Hospital, B-9000, Ghent, Belgium [5] - Dipartimento di Medicina Molecolare e Biotecnologie Mediche,, Università 'Federico II', Napoli, Italy [6]

Introduction: MiRNAs are non-coding, single-stranded RNAs of ca. 22 nucleotides involved in translational control mechanism. In general they negatively regulate their targets through the bind with canonical 8-mer complementary sites, called "seeds", within the 3′ untranslated regions (UTRs) of their mRNA targets.

Pediatric neuroblastoma (NBL) is the most common extracranial solid childhood tumor. It is responsible for approximately 15% of all childhood cancer mortality with an incidence of 1 to 5 per million children per year. The vast majority of NBLs arise sporadically and the most of the tumors are found in the abdomen (65%), often in the adrenal medulla, or elsewhere in the human body where sympathetic nervous system components are present. The clinically heterogeneous nature of NBL partly arises from its biological and genetic heterogeneity. Moreover, genetic and molecular findings are now routinely incorporated into therapeutic decision making.

Materials and methods: In our study, we used proteomics to screen for targets of miR-34a in neuroblastoma (NBL). We examined the effect of miR-34a overexpression using a tetracycline inducible system in two NBL cell lines (SHEP and SH-SY5Y) at early time points of its expression (6, 12 and 24 hours). Proteome analysis using post-metabolic labeling led to the identification of more then 2,000 proteins, and among these 186 were found regulated (112 proteins down- and 74 up-regulated).

Results: Prediction of miR-34a targets by bioinformatics showed that 32 transcripts hold miR-34a seed sequences in their 3' UTR. By combining the proteomics data with the Kaplan Meier gene-expression studies, 7 new gene products were identified that correlated with worse clinical outcome. These were further validated in vitro by 3' UTR seed sequence regulation.

In addition generation of "interactome" by using Michigan Molecular Interactions (MiMI), indicated that altogether these proteins affect signaling pathways that are able to regulate cell cycle and proliferation, focal adhesions and other cellular properties which overall enhance tumor progression (including signaling pathways such as TGF-β, WNT, MAPK and FAK).

Conclusions: In conclusion, proteome analysis has here identified early targets of miR-34a with relevance to neuroblastoma tumorigenesis. Along with previous studies, our data strongly suggest miR-34a as useful tool for a better chance in therapeutic success of NBL.

Signal Transduction And Intracellular Trafficking

S 1
EPHA2 IS A NOVEL PLAYER OF THYROID TUMORIGENESIS

Chiara Allocca [1] - Alessia Parascandolo [1] - Maria Domenica Castellone [2] - Massimo Santoro [3]

Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Università Federico II, Napoli, Italy [1] - Istituto di Endocrinologia ed Oncologia Sperimentale, Consiglio Nazionale delle Ricerche, Napoli, Italy [2] - Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Università Federico II, napoli, Italy [3]

Introduction: Malignant thyroid lesions are the most common endocrine cancers with an incidence rate stably increasing over the past few decades. To identify novel molecular mediators of thyroid tumorigenesis we performed a loss-of-function genetic screening in thyroid cancer cell line with a library of synthetic small interfering RNA (*si*RNA) targeting the human kinome and identified EPHA2 (*Erythropoietin-Producing Hepatocellular* carcinoma cell line A2) tyrosine kinase receptor as a positive regulator of thyroid cancer cell viability.

Materials and methods: Thyroid cancer cells were silenced with siRNAs targeting all human protein kinases. EPHA2 expression in thyroid cancer cell lines and tissue samples was tested by RT-PCR and Western blotting upon treatment with BRAF and RET inhibitors. Proliferation, invasion and migration were tested in silenced and control cells. *In vivo* xenograft experiments with stably EPHA2 silenced cells were also performed.

Results: EPHA2 expression levels were analyzed in a panel of thyroid cancer and normal cell lines, indicating over-expression of the gene at the mRNA and at the protein levels, as well as increased phosphorylation in cancer compared to normal control. 8505C thyroid cancer cells stably silenced with shRNA targeting EPHA2 showed phenotypical changes as well as reduced ability to grow and to form foci *in vitro*. We demonstrated that silenced cells had reduced ability to close the wound, to invade reconstituted matrigel and to perform invasive growth in 3D matrigel, when compared to parental cells. EPH silenced cells featured also impaired ability to form tumors when injected into the right dorsal portion of BALB/c nude mice. To study the signaling pathways regulating EPHA2 expression and phosphorylation, we treated thyroid cancer cells with small compounds inhibiting BRAF or RET/PTC and demonstrated that both treatments blunted EPHA2 phosphorylation.

Conclusions: Here we show that EPHA2 is over-expressed and constitutively phosphorylated in most of the thyroid neoplastic cell lines as compared to normal controls. We also provide mechanistic insights on EPHA2 activation in thyroid cancer cells. Finally, we show evidences that EPHA2 has a protumorigenic role in thyroid cancer since its knock-down reduced proliferation, motility and invasiveness, suggesting its potential use as marker of thyroid malignancy and as target for novel therapeutic strategies.

S 2
DIRECT TRANSDUCTION OF RECEPTOR-TYPE PROTEIN TYROSINE-PHOSPHATASE GAMMA (PTPRG) INTRACELLULAR CATALYTIC DOMAINS IN K562 CELLS

Giuseppe Bellisola [(1)] - Luisa Tomasello [(2)] - Zeno Fiorini [(2)] - Giovannino Silvestri [(3)] - Marzia Vezzalini [(2)] - Claudio Sorio [(2)]

Department of Pathology and Diagnostics, Azienda Ospedaliera Universitaria Integrata Verona, Verona, Italy [(1)] - Department of Pathology and Diagnostics, University of Verona, Verona, Italy [(2)] - University of Maryland School of Medicine, Marlene and Stuart Greenebaum Cancer Center, Baltimore, United States [(3)]

Introduction: Protein tyrosine phosphorylation/dephosphorylation is subverted in Chronic Myeloid Leukemia (CML) stem cell where the chimeric BCR/ABL fusion gene codifies for protein products (e.g. p210) with a constitutively increased protein TK activity which blocks cell differentiation and induces cell proliferation. However, when exposed to tyrosine kinase inhibitors (TKIs), most CML cells can regain the sensitivity to pro-apoptotic stimuli[1] suggesting that endogenous regulation of BCR/ABL can represent a valuable strategy to control its oncogenic activity. We have previously shown that the expression/activity of receptor-type tyrosine-protein phosphatase gamma (PTPRG) is down regulated in CML blasts[1].

Materials and methods: The intra-cytoplasmic catalytic domains (ICDs) of human PTPRG fused to HIV-1 Tat penetratin peptide[2] (Tat) was transduced into K562 cells that were analyzed by synchrotron radiation (SR) Fourier transform (FT) infrared (IR) microspectroscopy (microFTIR)[3]. Supervised methods and unsupervised multivariate data analysis such as principal component analysis (PCA) and hierarchical cluster analysis (HCA) were applied to dataset of IR spectra.

The uptake of rICD-Tat proteins in K562 cells was studied by confocal fluorescence microscopy. The levels of phosphotyrosine were studied in the proteins cell lysates by immunoblotting with specific antibodies. The percentages of viable and apoptotic cells were determined by MTT assay and by flow cytometric analysis of fluorescence in cells stained for Annexin-V and Propidium Iodide, respectively.

Results: Decreased levels of protein phosphotyrosine and increased apoptosis were observed in samples exposed to IMA or to the PTP-active rICD-Tat, respectively, as compared to untreated CTRLs and CML cells transduced with the rD1028A-ICD-Tat protein Accurate classification was obtained by hierarchical cluster analysis (HCA) applied to dataset of SR FTIR absorbance spectra.[4]

Conclusions: In conclusion, an active oncosuppressive protein tyrosine phosphatase was efficiently transduced by HIV-1 Tat technology into leukemic cells. PTP-active rICD-Tat protein was able to oppose the abnormal BCR/ABL TK activity and induce cell apoptosis in CML cells.

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S 3
THE DOUBLE-STRANDED RNA ADENOSINE DEAMINASE, ADAR1, REPRESENTS A NOVEL SUBSTRATE FOR AKT

William Blalock [(1)] - Mirco Raffini [(2)] - Manuela Piazzi [(3)] - Irene Faenza [(3)] - Alberto Bavelloni [(4)]

Institute of Molecular Genetics-National Research Council of Italy (IGM-CNR), Rizzoli Orthopedic Institute, Bologna, Italy [(1)] - RAMSES Laboratory, Rizzoli Orthopedic Institute, Bologna, Italy [(2)] - Cell Signalling Laboratory, Department of Biomedical Sciences, University of Bologna, Bologna, Italy [(3)] - Laboratory of Musculoskeletal Cell Biology, Rizzoli Orthopedic Institute, Bologna, Italy [(4)]

Introduction: AKT kinases are important for the survival proliferation and differentiation of multiple cell lineages and represent one of two signal transduction pathways that greatly influence translation initiation (the other being PKR). Interestingly, aberrant AKT signaling also happens to represent one of the more frequent alterations in cancer and drug-resistance. The effects of AKT are generally mediated through the phosphorylation of downstream substrates, which may lead to activation or inactivation of diverse target proteins. While many known AKT substrates are considered to be mainly cytoplasmic proteins (Bad, mTOR complex, GSK-3), AKT also has roles in nuclear signaling. To better characterize the role of aberrant AKT in the nucleus, we sought to identify novel nuclear AKT substrates.

Materials and methods: Affinity purification-tandem mass spectrometry (AP-MS/MS) employing a phospho-AKT substrate antibody was used to identify potential AKT substrates in

isolated nuclei from a cell line containing constitutively-active AKT. AP-MS/MS led to the identification of multiple proteins; some known to be substrates of AKT, while others represented potentially novel AKT substrates. One protein among the later group, the adenosine deaminase acting on dsRNA (ADAR)-1 was chosen to further validate its relationship to AKT1, 2 and 3, using co-immunoprecipitation and in vitro kinase assays.

Results: ADAR1 p110, a mainly nuclear isoform of ADAR1, was found to interact with both AKT and PKR in the nucleus. In addition, both endogenous AKT or the individual recombinant AKT1, 2 and 3, but not PKR, were found to phosphorylate ADAR1 p110 in vitro with the following kinetics AKT1=AKT3>AKT2.

Conclusions: ADAR1 likely represents the most active post-transcriptional RNA editing enzyme in the cell. Enzymatic activity of ADAR1 in regions of complex RNA structure results in the deamination of adenosine to inosine, which is interpreted as guanine. ADAR1-dependent editing occurs in both coding and non-coding regions of RNAs; thus ADAR1 can lead to variability in the amino acid coding of proteins, alteration of the target sequence of cell derived miRNA, or alternate mRNA splicing, a process influencing multiple forms of cancer. In metastatic melanoma, ADAR1 activity was recently shown to influence the balance between miRNA that have been shown to target AKT1 and AKT2. Here we demonstrate that ADAR1 is directly modified by the AKT kinases.

S 4
INTERACTION BETWEEN SONIC HEDGEHOG AND BOMBESIN NEUROPEPTIDE RECEPTOR PATHWAYS IN SMALL CELL LUNG CARCINOMA

Maria Domenica Castellone [1] - Mikko Olavi Laukkanen [2] - Silvio Gutkind [3]

Istituto di Endocrinologia ed Oncologia Sperimentale, Consiglio Nazionale delle Ricerche, Napoli, Italy [1] - SDN Foundation, SDN Foundation, Napoli, Italy [2] - Oral and Pharyngeal Cancer Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, United States [3]

Introduction: Small cell lung carcinoma (SCLC) is a neuroendocrine, very aggressive, highly metastasizing and lethal lung cancer type and it accounts for about 15% of all lung cancers. Our data identify a novel molecular network acting in SCLC linking autocrine BBS and Shh circuitries, and suggest Shh inhibitors as novel therapeutic strategies against this aggressive cancer type.

Materials and methods: SCLC lung tissue samples and cells lines were analyzed by Western blotting, Immunohistochemistry and RT-PCR for expression of molecules of Shh and BBS/GRPR pathways. Interaction between the two signaling pathways was tested by Gli-luciferase reporter assay, nuclear fractionation and Immunofluorescence upon BBS stimulation. Thymidine incorporation, Cell Count and Cell Titre Blue assay were used to test effect of Shh inhibitors on SCLC cells proliferation in response to BBS. Soft agar colony formation assay and growth in 3D matrigel of Shh silenced versus control cells were also performed.

Results: We show that SCLC tumour samples feature co-expression of Shh and BBS-cognate receptor (Gastrin-Releasing Peptide Receptor: GRPR). We also demonstrate that BBS activates Gli in SCLC cells, which is crucial for BBS-mediated SCLC proliferation because cyclopamine, an inhibitor of the Shh pathway, hampered the BBS-mediated effects. BBS binding to GRPR stimulated Gli through its downstream Gαq and Gα12/13 GTPases, and consistently, other Gαq and Gα13 coupled receptors (such as muscarinic receptor, m1, and thrombin receptor, PAR-1) and constitutively active GαqQL and Gα12/13QL mutants stimulated Gli. By using cells null for Gαq and Gα12/13, we demonstrate that these G proteins are strictly necessary for Gli activation by BBS. Moreover, by using constitutively active Rho small G-protein (Rho QL) as well as its inhibitor, C3 toxin, we show that Rho mediates GPCR-, Gαq- and Gα12/13- dependent Gli stimulation. At the molecular level, BBS caused a significant increase in Shh gene transcription and protein secretion that was dependent on BBS-induced GPCR/Gαq-12/13/Rho mediated activation of NFκB, which can stimulate a NFκB response element in the Shh gene promoter.

Conclusions: Our findings shed light on the mechanism underlying Shh pathway activation in SCLC and identify a novel molecular network acting in SCLC linking autocrine BBS and Shh circuitries, suggesting Shh inhibitors as novel therapeutic strategies against this aggressive cancer type.

S 5
INTRACELLULAR SIGNALLING IN TE85 OSTEOSARCOMA CELLS EXPRESSING P2X7R ISOFORMS A AND B

Anna Lisa Giuliani [1] - Elena Adinolfi [1] - Carlotta Roncato [1] - Davide Colognesi [1] - Francesca Amoroso [1] - Tommaso Zordan [1] - Elena De Marchi [1] - Marina Capece [1] - Alison Gartland [2] - Francesco Di Virgilio [1]

Dept of Morphology, Surgery and Experimental Medicine, University of Ferrara, Ferrara, Italy [1] - The Mellanby Centre for Bone Research, Dept of Human Metabolism, The University of Sheffield, Sheffield, United Kingdom [2]

Background. Osteosarcoma is the most frequent primary malignant bone cancer. The natural history of osteosarcoma is sill mostly unknown and genetic complexity is a hallmark of high grade osteosarcoma so that, currently, no ideal target has been identified. Several efforts are thus in progress to identify new biomarkers as possible targets of efficacious therapies, especially in counteracting pathologic bone remodelling and alleviating associated pain. The P2X7 receptor (P2X7R), a receptor for extracellular ATP widely distributed in human tissues, has recently been shown to have a central role in carcinogenesis, as it enhances *in vivo* tumor cell growth and cancer-associated angiogenesis (Adinolfi E et al, Cancer Res. 2012) as well as *in vitro* cancer cell invasiveness (Adinolfi E et al, FASEB J. 2010). P2X7R activity is tightly regulated by extracellular ATP. Indeed, low ATP levels induce the ion channel formation, whereas higher concentrations (nM) provoke opening of a pore permeable to high molecular mass molecules. Among the nine different naturally occurring splice variants known, P2X7RA is the

full-length and full-functioning isoform, whereas P2X7RB is a shorter isoform which lacks the pore forming ability while retaining channel properties. Co-expression of the A and B isoforms in HEK293 cells has been demonstrated to potentiate receptor functions (Adinolfi E et al, FASEB J. 2010). P2X7R-related intracellular signalling includes various events such as activation of NFATc1 and PKC/MAPK pathways, as well as activation of PI3K/Akt signalling (Amoroso F et al, Cell Death Dis, 2012; Grol MW et al, Am J Physiol, 2012).

Aim of the study. Investigate the role of A and B isoforms of the P2X7R in intracellular signaling linked to receptor activation in Te85 human osteosarcoma cells.

Methods. Te85 osteosarcoma cells, which lack endogenous P2X7R expression, were transfected with P2X7RA and P2X7RB, separate or in combination. Proliferation was assessed as a kinetic up to 72 hours in serum starvation conditions. Intracellular calcium levels following stimulation with P2X7R agonists were measured by fluorimetry. NFATc1 levels were assessed by ELISA on nuclear extracts. Total and phosporylated Akt were detected by WB.

Results and conclusion. P2X7R expression conferred a proliferative advantage in absence of serum as shown by increased cell growth rate of all Te85 transfectants compared to Te85 wt. All transfected clones showed increased Ca^{2+} mobilization, the highest response found in Te85 co-transfected with both P2X7RA and B. Nuclear levels of NFATc1 were increased in all transfectants. Finally, higher Akt phosphorylation levels were found in Te85-P2X7RA clones. In conclusion, P2X7R expression in osteosarcoma cells induce augmented proliferation and activation of specific intracellular signalling pathways susceptible of targeting for cancer therapy.

S 6
CXCR4 AND CXCR7 TRANSDUCE THROUGH MTOR IN HUMAN RENAL CANCER CELLS

Caterina Ieranò [1] - Maria Napolitano [1] - Sara Santagata [1] - Francesca Guardia [1] - Giovanni Botti [1] - Anna Grimaldi [2] - Elena Antignani [1] - Claudia Consales [1] - Anna Riccio [1] - Merlin Nanayakkara [3] - Maria Vittoria Barone [3] - Michele Caraglia [2] - Stefania Scala [1]

Oncological Immunology, National Cancer Institute "Fondazione G.Pascale", Naples, Italy [1] - Department of Biochemistry, Biophysics and General Pathology, Second University of Naples, Naples, Italy [2] - Department of Traslational Medical Science and European Laboratory for the Investigation of Food Induced Disease(ELFID), University of Naples "Federico II", Naples, Italy [3]

Introduction: Treatment of metastatic renal cell carcinoma (mRCC) has improved significantly with the advent of agents targeting the mTOR pathway, such as temsirolimus and everolimus. However, their efficacy is thought to be limited by feedback loops and cross talk with other pathways leading to the development of drug resistance. Since CXCR4-CXCL12-CXCR7 axis has been described to play a crucial role in renal cancer; the cross talk between mTOR pathway and CXCR4-CXCL12-CXCR7chemokine receptor axis has been investigated in human renal cancer cells.

Materials and methods: The SN12C and A498 human renal cancer cell lines were stimulated with CXCL11/CXCL12 with or without AMD3100 orCCX771 and pP70S6K and p4EBP1 expression were measured by Western blot analysis. Then, CXCL12/CXCL11 dependent-cell migration was examined in SN12C andA498 in presence of RAD001. Moreover, delay in wound healing was evaluated in SN12C andA498 in presence of RAD001 with CXCL12or CXCL11 and relative inhibitors AMD3100 and anti-CXCR7.

Results: In SN12C and A498 the common CXCR4-CXCR7 ligand, CXCL12, and the exclusive CXCR7 ligand, CXCL11 activated mTOR through P70S6K and 4EBP1 targets. The mTOR activation was specifically inhibited by CXCR4 antagonists (AMD3100, anti CXCR4-12G5 and Peptide R, a newly developed CXCR4 antagonist) and CXCR7 antagonists (anti CXCR7-12G8 and CCX771, CXCR7 inhibitor). To investigate the functional role of CXCR4, CXCR7 and mTOR in human renal cancer cells migration and wound healing were evaluated. SN12C and A498 cells migrated toward CXCL12 and CXCL11; CXCR4 and CXCR7 inhibitors impaired migration and the treatment with mTOR inhibitor, RAD001, further inhibited it. Moreover, CXCL12 and CXCL11 induced the wound healing which was impaired by the AMD3100, the anti CXCR7 and RAD001. In SN12C and A498 cells, CXCL12 and CXCL11 promoted actin reorganization characterized by thin spikes at the cell periphery, while AMD3100 and anti CXCR7 impaired CXCL12/CXCL11-induced actin polymerization and RAD001 treatment further reduced it. In addition when cell growth was evaluated in the presence of CXCL12, CXCL11 and mTOR inhibitor an additive effect was detected by the CXCR4, CXCR7 antagonists and RAD001.

Conclusions: In conclusion the entire axis CXCR4-CXCL12-CXCR7 regulate mTOR signaling in renal cancer cells offering new therapeutic opportunities and targets to overcome resistance to mTOR inhibitors.

S 7
EXTRACELLULAR SUPEROXIDE DISMUTASE REGULATES THE PRIMARY AND CANCER CELL GROWTH BY INFLUENCING THE EXPRESSION OF RAS SUPERFAMILY GTPASE REGULATORY PROTEINS GEFS, GAPS, AND GDIS

Mikko Laukkanen [1] - Francesca Cammarota [1] - Tiziana Esposito [1] - Francesco Fiscardi [1]

SDN Foundation, IRCSS SDN, Naples, Italy [1]

Introduction: RAS-induced tumorigenesis has been suggested to follow a three-stage model consisting of an initial RAS activation, senescence induction, and evasion of p53-dependent senescence checkpoints. While reactive oxygen species (ROS) act as second messengers in RAS-induced senescence, they are also involved in oncogenic transformation by inducing proliferation and promoting mutations. We studied the impact of extracellular superoxide dismutase (SOD3), which dismutases hydrogen peroxide to superoxide anion at cell membranes, on mesenchymal and epithelial cell proliferation to dissect the SOD3 –driven mechanisms in tumorigenesis.

Materials and methods: We studied the effect of SOD3 *in vitro* and *in vivo* using mouse embryonic fibroblast (MEF) pri-

mary cells, TPC1 papillary thyroid cancer and 8505c anaplastic thyroid cancer cells. The expression studies were performed using qRT-PCR, Western blotting, microarray, and protoarrays. phenotypic and functional assays included, senescence, DNA damage, focus, apoptosis, comet, soft agar, and matrigel migration assays.

Results: Based on our data, *sod3* RNA interference in *H-RasV12*-transduced cells markedly inhibited cell growth, while *sod3* over-expression in MEFs initially caused a proliferative burst followed by the activation of DNA damage checkpoints, induction of p53-p21 signal transduction, and senescence. Subsequently, *sod3*-transduced MEF cells developed co-operative *p21-p16* down-regulation and acquired transformed cell characteristics such as increased telomerase activity, loss of contact inhibition, growth in low-nutrient conditions, and *in vivo* tumorigenesis.

Interestingly, as reported previously with RAS, we showed a dose-dependent response to SOD3 *in vitro* and *in vivo*: low level expression induced MEF immortalization with consequent transformation and cancer cell proliferation while high SOD3 expression reduced the growth. In anaplastic cancer cells high SOD3 expression reduced cell proliferation independently of growth arrest pathway by regulating RAS superfamily GTPase regulatory gene (GEF, GAP, and GDI) expression causing neutralization of RAS GTP loading and consequent mitogen pathway signal transduction.

Conclusions: Our results indicate that SOD3 influences the RAS superfamily GTPase regulatory gene expression. The identified GEFs, GAPs, and GDIs could serve as a therapy target at an early phase of tumorigenesis.

S 8
277 - PEPTIDE-GUIDED TARGETING OF GPR55 FOR NEW THERAPEUTIC STRATEGIES OF CANCER

Maria Giovanna Mosca [(1)] - Enrico Iaccino [(2)] - Selena Mimmi [(2)] - Ileana Quinto [(2)] - Giuseppe Scala [(2)] - Daniela Corda [(1)] - Stefania Mariggiò [(1)]

Institute of Protein Biochemistry, National Research Council, Napoli, Italy [(1)] - Department of Clinical and Experimental Medicine, University "Magna Graecia" of Catanzaro, Catanzaro, Italy [(2)]

Introduction: Increasing evidence indicates that G-protein-coupled receptors are 'druggable' targets in cancer treatment and prevention[1]. GPR55 has been recently identified as the lysophosphatidylinositol receptor and several aspects of its physiology are still under investigation[2]. GPR55 is involved in cancer progression since its expression level correlates with the invasive potential of metastatic cells[2]. This study is aimed to investigate GPR55 function in development of osteoclast precursors toward mature osteoclasts, and to define the physiological role of GPR55 in bone resorption as well as bone metastasis formation. By taking advantage of phage display peptide libraries, we will develop peptide binders of GPR55 in order to modulate receptor signalling, which could represent useful tools for novel therapeutic approaches of bone diseases related to GPR55 disregulation.

Results: Whole-cell-based phage display screening was performed by heterologous expression of full-length GPR55 receptor in HEK293 cells as bait. Based on selected mimotope binders of GPR55, we designed a number of candidate peptides that have been tested for specific binding to GPR55, as determined by FACS and confocal microscopy. Peptides showing the highest specificity and affinity to GPR55 will be further tested in functional assays of GPR55 signalling. To this end, we have characterized the lysphosphatidyinositol-triggered GPR55 signalling in a macrophage cell line. In this cell system, agonist stimulation of GPR55 increased the intracellular calcium level and AKT phosphorylation, and modulated the actin cytoskeleton with filopodia appearence. These events were blocked by GPR55 down-regulation by RNA interference as well as by addition of GPR55 antagonists, such as cannabidiol and ML191. Moreover, RANK-dependent differentiation of macrophages into mature osteoclasts resulted in a strong up-regulation of GPR55 mRNA levels, while GPR55 antagonists affected the completion of this process, suggesting an active role of GPR55 in osteoclast differentiation.

Conclusions: We are defining the role of GPR55 in macrophage differentiation into mature osteoclasts, which represent a suitable model to test the efficacy of peptide binders of GPR55. These peptides have potential applications for tumor diagnosis and therapy by *in vivo* targeting GPR55-positive cancer cells.

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S 9
TWO DISTINCT ANTITUMOR PATHWAYS ACTIVATED BY TRANSFECTED POLY(I:C) IN ANDROGEN-INDEPENDENT PROSTATE CANCER CELLS

Sara Palchetti [(1)] - Donatella Starace [(1)] - Antonio Filippini [(1)] - Elio Ziparo [(1)] - Anna Riccioli [(1)]

DAHFMO - Section of Histology and Medical Embryology, Sapienza University of Rome, Rome, Italy [(1)]

Introduction: Prostate cancer (PCa) represents the second leading cause of cancer death in men and develops as a result of the accumulation of genetic and epigenetic alterations. Data from literature suggest that new therapeutic targets are emerging and in particular, it is known that the activation of Toll-like Receptors 3 (TLR3) expressed by cancer cells has a pro-apoptotic and thus anti-tumoral effect in different tumors (Cheng & Xu, 2010). We previously demonstrated that the synthetic analogue of dsRNA poly(I:C), specific TLR3-ligand, induces apoptosis in the androgen-dependent prostate cancer cell line LNCaP in a TLR3-dependent fashion, whereas a weaker apoptotic effect is observed in more aggressive and androgen-independent prostate cancer cell lines PC3 (Paone et al, 2008) and DU145 (Galli et al, 2013). In this regard, we have recently demonstrated that the encapsulation of poly(I:C) with three different formulations of cationic liposomes was up to 10 times more efficient than the free drug in eliminating both PC3 and DU145 cells (Palchetti et al, 2013). These data suggest that transfected poly(I:C) could raise apoptotic rate by stimulating cytosolic dsRNA receptors. In the present paper we analyzed the receptors and signalling

pathways involved in apoptosis induced by poly(I:C) transfected by lipofectamine (the most common transfection agent) compared with free poly(I:C) in PC3 and DU145 cells.

Materials and Methods: We evaluated cell viability by MTT assay and apoptosis by cell cycle analysis by FACS and caspase activity. SiRNA approach and Western Blot analysis were performed to determine the receptors and signal transduction molecules involved in transfected poly(I:C)-induced effects.

Results: Poly(I:C) transfected by lipofectamine [in-poly(I:C)] inhibits cell viability in PC3 and DU145 cells in a dose dependent manner with the highest efficiency at 2μg/ml of poly(I:C) compared to twelve-fold higher poly(I:C) concentration (25μg/ml) and induces caspase 8 and 9-dependent apoptosis.

By using genetic inhibition of different poly(I:C) receptors we demonstrated the crucial role of TLR3 and Src in in-poly(I:C) induced apoptosis. On the other hand, we show that IRF3-mediated signaling causes the upregulation of TLR3, cytosolic receptors (RLH) and interferon-beta expression. Our data highlight the multiple signaling triggered by in-poly(I:C) leading to antitumor responses.

Conclusions: We can conclude that the treatment of PC3 and DU145 cells with in-poly(I:C) activates two distinct anti-tumor pathways: one mediated by TLR3, dependent on Src, leading to a remarkable apoptosis and the other one mediated by RLH, dependent on IRF-3, leading to their up-regulation and interferon-beta expression.

S 10
IFN?? ENHANCES THE EXPRESSION OF CXCL13 AND SDF-1 CHEMOKINES AND CXCR4 AND CCR7 CHEMOKINE RECEPTORS IN CUTANEOUS T CELL LYMPHOMA

Maria Cristina Picchio [1] - Cristina Lazzeri [2] - Debora Pomponi [1] - Enrico Scala [1] - Gianluca Ragone [1] - Cristina Cristofoletti [1] - Giuseppe Alfonso Lombardo [1] - Alessandro Monopoli [1] - Giandomenico Russo [1] - Maria Grazia Narducci [1]

IDI-IRCCS, IDI-IRCCS Hospital, Roma, Italy [1] - Department Clinical Immunology, La Sapienza University, Roma, Italy [2]

Introduction: Cutaneous T-cell lymphoma (CTCL) represents a malignant expansion of CD4+CD45RO+ memory lymphocytes that show a strong propensity for the skin. Mycosis fungoides (MF) and the more aggressive variant Sezary syndrome (SS) are the major clinical variants of CTCLs (Olsen E, 2005). SS cells trafficking is largely regulated by chemokines, particularly by CXCR4-SDF-1 and CXCL13-CXCL19/21-CCR7, two axes recently demonstrated as novel therapeutic target. Roferon-A (Interferon a2a) therapy is commonly used in the treatment of CTCL. As IFN-a is responsible for the transcriptional regulation of hundreds of genes also through STATs activation, a gene found hyperactivated also in SS, in this study we investigated the INFa -induced expression of chemokines in SS/MF with the aim to clarify the INFa therapeutic mechanism in CTCLs.

Materials & Methods CXCL13 and SDF-1 plasmatic and expression levels were analyzed by ELISA (R&D) and qRT-PCR in 22 SS and 73 MF untreated (UNT) and INF-a treated (T) patients. STAT3 and phospho-STAT3 expression (Cell Signalling Technology) was evaluated by Western Blotting (WB) and ImmunoFluorescence analysis (IF) in Hut78, a CTCL cell line. STAT3 siRNA (Ambion) was transfected (siPORT) in Hut78 cells and STAT3 knockdown was verified by WB.

Results: CXCL13 and SDF-1 plasmatic levels measured by ELISA resulted significantly increased in T respect UNT-SS with 640±470 pg/ml versus 44±46 pg/ml (p=0.0002) and with 1757±259 pg/ml versus 1132±323 pg/ml (p=0.04) respectively. These data were also confirmed at mRNA levels by qRT-PCR performed on sorted neoplastic lymphocytes INFa-stimulated *in vitro* (n=5) demonstrating an increase of CXCL13 of 3 Fold Change (FC) (p=0.03), SDF-1 4.5 FC, CXCR4 2,3 FC (p=0.05), CCR7 1,8 FC compared to UNT samples.

IF and WB analyses performed in Hut78 revealed enhanced phosphorylation of STAT3 following INF-a stimulation. STAT3 siRNA knockdown in T and UNT Hut78 followed by RT-PCR (n=2) showed a decreased expression of CXCR4 and CCR7 respect controls.

Conclusion: INFa treatment strongly enhances, both *in vivo* and *in vitro*, the chemokine expression in CTCL potentially perturbing the SS/MF cell trafficking ability. In addition, STAT3 siRNA knockdown reduces the expression of CXCR4 and CCR7 receptors in Hut78 cell line.

S 11
NF-KB SIGNALING INHIBITION BY CD99 FAVORS NEURAL DIFFERENTIATION IN EWING SARCOMA

Selena Ventura [1] - Alessandra De Feo [2] - Gianfranco Mattia [2] - Carolina Coscia [2] - Dave Nt Aryee [3] - Piero Picci [1] - Heinrich Kovar [3] - Alessandra Carè [2] - Katia Scotlandi [1]

Laboratorio di Oncologia Sperimentale, CRS Sviluppo di Terapie Biomolecolari, Istituto Ortopedico Rizzoli, Bologna, Italy [1] - Dipartimento di Ematologia, Oncologia e Medicina Molecolare, Istituto Superiore di Sanità, Roma, Italy [2] - Laboratory of Molecular Biology, Children's Cancer Research Institute, St. Anna Kinderkrebsforshung, Vienna, Austria [3]

Introduction Ewing sarcoma (ES), a relatively rare malignant bone tumor in children and adolescents, is characterized by EWS-FLI1 fusion protein and high expression of CD99, a cell surface glycoprotein. Both EWS-FLI1 and CD99 were reported to regulate cell malignancy and differentiation but their hierarchical relationships have never been defined. In this study we focused on investigating their impact on the regulation of the nuclear factor-kappa B (NF-kB) signaling and clarify its role as mediator of ES cell differentiation. In addition we evaluated the ES-released exosomes as a new way of intercellular communication and their possible involvement in NF-kB-dependent ES differentiation.

Materials and Methods The activity of several transcription factors (AP1, SRE, CREB, E2F and NF-kB) was evaluated by gene reporter assay. In three ES cell lines (TC-71, IOR/CAR and ASP14) we silenced CD99, EWS-FLI1 and NF-kB itself, alone or in combination. NF-kBp65 was also transiently overexpressed in TCshCD99 and CARshCD99 clones. Neural differentiation was detected evaluating β-III tubulin by immunofluorescence. Exosomes, purified from cell supernatants accord-

ing to standard procedures, were characterized for some exosome markers (Cav1, Rab5b, LAMP2) and for the expression of CD99 and NF-kB. The functional effects deriving from their fusion with recipient target cells were evaluated.

Results NF-kB was found heterogeneously expressed in a panel of ES cell lines. Stable CD99 silencing was found to decrease NF-kB activity ($p < 0.05$) without differences at RNA and protein levels. On the contrary, EWS-FLI1 knockdown increased NF-kB transactivation ($p < 0.01$)**.** The effects of CD99 on NF-kBp65 activity are dominant with respect to EWS-FLI1. Cells silenced for CD99 but not for EWS-FLI1 showed significant induction of neural differentiation, which was lost when cells were induced to re-express NF-kB. Interestingly, this CD99-dependent differentiation was confirmed by an exosome-based transport as, after fusion with sh-CD99-exosomes, we observed a significant NF-kB downregulation associated with increased differentiation capabilities of recipient control ES cells.

Conclusion Our findings support the concept that inhibition of NF-kB is able to induce terminal neural differentiation of ES cells. NF-kB signaling appeared to be dominantly regulated by CD99 in ES and this regulation is maintained in and efficiently delivered by exosomes. Funded by AIRC to KS (IG14049) and AC (IG13247).

Targeted Therapy

T 1
TARGETING P70S6 KINASE ACTIVITY IMPAIRS BREAST CANCER CELL SURVIVAL AND SUPPRESSES LOCAL RELAPSE AFTER SURGERY

Barbara Belletti [1] - Ilenia Segatto [1] - Stefania Berton [1] - Maura Sonego [1] - Tiziana Perin [2] - Samuele Massarut [3] - Gustavo Baldassarre [1]

CRO of Aviano, National Cancer Institute, Dept. Translational Research, Aviano, Italy [1] - CRO of Aviano, National Cancer Institute, Pathology Unit, Aviano, Italy [2] - CRO of Aviano, National Cancer Institute, Breast Unit, Aviano, Italy [3]

Introduction: For early breast cancer (EBC) patients, local relapse represents what mostly influences disease outcome. Notably, 90% of local recurrences occur at or close to the same quadrant of the primary cancer. Surgery itself and the consequent process of wound healing have been proposed to stimulate local recurrences via pathway(s) still to be clarified. The ribosomal protein $p70^{S6K}$ has been implicated in breast cancer cell response to post-surgical inflammation, supporting the hypothesis that it may be crucial also for breast cancer recurrence.

Materials and methods: Here, we used *in vitro* and *in vivo* models of breast cancer to investigate whether interfering with $p70^{S6K}$ activity, either genetically or pharmacologically, can suppress breast cancer cell survival and, eventually, disease relapse.

Results: Our experiments demonstrate that interfering with $p70^{S6K}$ activity strongly impaired breast cancer cell survival *in vitro* and local relapse *in vivo*. After *in vitro* characterization of the role of $p70^{S6K}$ in breast cancer cells, we generated a mouse model of EBC in which we recapitulated the disease course and the therapeutic approaches used in clinic. Using this *in vivo* model we have established the role of $p70^{S6K}$ in tumor growth and, more importantly, in local recurrence formation. Peri-operative treatment using specific pharmacological inhibition of $p70^{S6K1}$ was sufficient to reduce by 83% the rate of local recurrence. The significance of our results was confirmed in human specimens, collected from EBC patients undergone lumpectomy first and surgical widening to clear surgical margins in a second time, showing that $p70^{S6K}$ activity is induced by surgery, also in human disease. Finally, our data demonstrate that the molecular mechanism by which $p70^{S6K}$ is able to promote cell survival and, eventually, breast cancer relapse, relies on a $p70^{S6K}$/Gli1/Bcl2 signaling axis.

Conclusions: Taken together, our results provide a biological rationale for a novel peri-operative treatment targeting $p70^{S6K}$ pathway, directed to compensating the harmful consequences of surgery and to restraining local recurrence of breast cancer.

T 2
IDENTIFICATION AND CHARACTERIZATION OF NEW PIN1 INHIBITORS

Elena Campaner [1] - Alessandra Rustighi [1] - Alberto Cristiani [1] - Alessandro Zannini [1] - Gali Golan [2] - Ori Kalid [2] - Giannino Del Sal [1]

Laboratorio Nazionale CIB, Dipartimento di Scienze della Vita, Università degli Studi di Trieste, Trieste, Italy [1] - Karyopharm Therapeutics, Karyopharm Therapeutics, Natick, MA, United States [2]

Introduction: Cancer is a disease mainly characterized by uncontrolled cellular growth, due to the deregulation of signal transduction pathways that control proliferation, genomic stability and metabolism. Reversible phosphorylation of proteins represents one of the most important regulatory mechanism in the cell to regulate such pathways and their interconnections. Among them, Serine/Threonine-Proline motifs (Ser/Thr-Pro) are exclusive phosphorylation sites for a series of proline-directed kinases. Of note, many oncogenes and tumor suppressors are directly modulated by prolyl-directed phosphorylation. The prolyl-isomerase Pin1 was identified as a key peptidyl-prolyl cis/trans isomerase (PPIase) endowed with the ability to change the conformation of proteins at the level of phosphorylated Ser/Thr-Pro motifs. Indeed, we and others have shown that Pin1 is involved in the pathogenesis of cancer and in the acquisition of several aggressive phenotypes that are dampened by reduction of its levels or activity in tumor cells. For these reasons Pin1 was proposed as a molecular target for cancer therapy. Despite many Pin1 inhibitors have been isolated none of these has reached clinical trials so far due to insufficient pharmacological characteristics.

Materials and methods : The activity of this putative Pin1 inhibitor was tested by different experiments such as protease-coupled isomerization assay, Western Blot analysis, qRT-PCR, colony formation assay, mammospheres assay.

Results: In this work we have launched a validation process to identify novel potential Pin1 inhibitors derived from a screening of a synthetic compounds library. These compounds were designed with the intent of isolating a covalent Pin1 inhibitor in order to increase biochemical efficiency. To this aim we reproduced and optimized a Pin1-specific isomerization assay in order to measure impairment of Pin1 catalytic activity by the compounds. Positive hits of this assay were subsequently tested in cell-based assays. We demonstrated that one compound is able to impinge on several Pin1 functions in cancer cells.

Conclusions: We have found a novel promising Pin1 inhibitor able to interfere with Pin1 functions both *in vitro* and at the cellular level. Further developments regarding the specificity of the compound and the *in vivo* use of this drug are planned.

T 3
INHIBITION OF CHK1 AND WEE1 AS A NEW THERAPEUTIC APPROACH IN MANTLE CELL LYMPHOMA

Rosaria Chilà [1] - Laura Carrassa [1] - Alessandra Basana [1] - Francesca Ricci [1] - Federica Guffanti [1] - Monica Lupi [1] - Andrea Rinaldi [2] - Luciano Cascione [2] - Francesco Bertoni [2] - Giovanna Damia [1]

Istituto di Ricerche Farmacologiche Mario Negri, Istituto di Ricerche Farmacologiche Mario Negri, Milan, Italy [1] - Institute of Oncology Research, Institute of Oncology Research, Bellinzona, Switzerland [2]

Introduction: Mantle Cell Lymphoma (MCL) is an aggressive lymphoma for which there is not yet a standard therapy. Deregulation of cell cycle is its main pathogenic feature due to the t(11;14)(q13;q32) translocation, which causes cyclinD1 overexpression. Chk1 and Wee1 proteins regulate replication initiation, replication fork stability and mitotic entry; combined treatment of Chk1 and Wee1 inhibitors has a strong synergistic cytotoxic effect in solid tumors, but little is known on their effect in hematologic cancer such as lymphomas. The effects of a Chk1 inhibitor and a Wee1 inhibitor alone or in combination was investigated in MCL *in vitro* and *in vivo*.

Materials and methods: Hematologic cancer cell lines were treated with Chk1 inhibitor (PF-00477736) and Wee1 inhibitor (MK-1775). The quantification of single and combined treatments were evaluated by Calcusyn Software. Apoptosis was measured by Caspase-3 activity and TUNEL Assay. Western blot analysis was performed with standard techniques. *In vivo* experiments were performed by transplanting Jeko-1 MCL subcutaneously in nude mice.

Results: The PF-00477736 median IC50 value in MCL was 30 nM, lower than the one in 9 solid tumors (337 nM) and in 8 hematologic tumors (184 nM). The MK-1775 median IC50 value in MCL was 106 nM, lower than the one in solid tumors (615 nM) and in hematologic tumors (287 nM). A strong synergism of the combination was observed in all MCL cell lines. Molecular analysis showed that the combination caused a deregulation of cell cycle, an increased activity of CDK1/2 and activation of apoptosis. Inhibition of the CDK4/6-cyclinD1 complex neutralized the cytotoxic effect of PF-00477736 and MK-1775 in Jeko-1 MCL cells. In addition, the sensitivity to PF-00477736 and MK-1775 was higher in 2 multiple myeloma (MM) cell lines harbouring the t(11;14) than in 3 MM cell lines without the translocation. *In vivo* treatment of mice bearing Jeko-1 xenografts showed a remarkable antitumor effect with tumor regressions observed at non-toxic doses.

Conclusions: Our data provide a strong preclinical evidence for the role of Chk1 and Wee1 inhibitors as new therapeutic approach in MCL and warrant investigation in clinical setting.

T 4
IN VITRO AND IN VIVO EFFICACY OF TRASTUZUMAB-DM1 IN HER-2 OVEREXPRESSING NSCLC CELL LINES

Daniele Cretella [1] - Francesca Saccani [1] - Federico Quaini [1] - Caterina Frati [1] - Costanza Lagrasta [2] - Pier Giorgio Petronini [1] - Roberta Alfieri [1]

Department of Clinical and Experimental Medicine, University of Parma, parma, Italy [1] - Department of Biomedical, Biotechnological and Translational Sciences, University of Parma, parma, Italy [2]

Background: A number of molecular aberrations have been identified in NSCLC. Epidermal growth factor receptor (EGFR) has been successfully targeted in NSCLC patients harbouring activating-EGFR mutations by tyrosin kinase inhibitors (gefitinib, erlotinib). HER-2 represents a relatively new therapeutic target for NSCLC. The potential clinical relevance of HER-2 expression in NSCLC is currently under evaluation, however, the recent role of HER-2 amplification in the acquisition of resistance to TKI, reported in 12-13% of patients, may render HER-2 a potential target not only in breast cancer but also in NSCLC. Trastuzumab emtansine (T-DM1) is a new specific antibody-drug conjugate incorporating trastuzumab with the microtubule-inhibitor agent DM1. Based on these considerations, we analyzed the effects of T-DM1 in a panel of NSCLC cell lines both *in vitro* and *in vivo*.

Results: In a panel of NSCLC cell lines, we demonstrated that T-DM1 inhibited cell viability in cell lines overexpressing HER-2 more efficiently than trastuzumab. We compared trastuzumab and T-DM1 in antibody-dependent cellular cytotoxicity (ADCC) assay showing that trastuzumab and T-DM1 induced ADCC in a similar way. In gefitinib resistant HER-2 overexpressing PC9/HER2cl1 cell clone, we observed that T-DM1 treatment inhibited both cell viability and reduced AKT and p70S6K phosphorylation compared to parental cell line. HER-2 protein membrane expression is post-transcriptionally regulated by cell density and we observed that cells cultured at high density showed a reduction of HER-2 protein membrane levels as well as a reduction of T-DM1 effects. Finally, we performed an *in vivo* experiment showing that the efficacy of T-DM1 is regulated by tumour mass and by HER-2 expression. After T-DM1 treatment, a 73% reduction of neoplastic epithelial cells in small tumors compared to 36% in large tumors was observed. Immunohistochemical analysis showed a stricking difference in the surface expression of HER-2: neoplastic cells composing small tumors showed higher extent and intensity of HER-2 immunolabeling than large tumors.

Conclusions: Based on these data, T-DM1 shows promising anti tumor effects in NSCLC cell lines with HER-2 overexpression and our investigation strongly supports the contention that HER-2 expression in NSCLC is regulated by the tumor mass and its structural organization which in turn condition the efficacy of T-DM1.

T 5
TARGETED THERAPY OF ADULT T CELL LEUKEMIA/LYMPHOMA USING THE ORALLY BIOAVAILABLE SYNTHETIC RETINOID ST1926

Nadine Darwiche [1] - Hiba El Hajj [1] - Bariaa Khalil [2] - Botheina Ghandour [1] - Rihab Nasr [1] - Rana Abdel-samad [1] - Ghassan Dbaibo [1] - Claudio Pisano [3] - Ali Bazarbachi [1]

American University of Beirut, Faculty of Medicine, Beirut, Lebanon [1] - American University of Beirut, Biology Department, Beirut, Lebanon [2] - Biogem, Research Institute, Biogem, Research Institute, Ariano Irpino (AV), Italy [3]

Adult T cell leukemia/lymphoma (ATL) is an aggressive neoplasm caused by the human T cell leukemia virus type 1 (HTLV-1). The HTLV-1 oncoprotein Tax plays an important role in ATL pathogenesis. ATL carries a poor prognosis due to chemotherapy resistance and immunosuppression stressing the need for alternative therapies. Retinoids are major regulators of cellular proliferation and differentiation. Natural retinoids such as all-*trans* retinoic acid are used as chemopreventive and therapeutic agents in cancer, particularly in acute promyelocytic leukemia (APL). Unfortunately, the use of natural retinoids is limited by side effects and acquired resistance. Therefore, synthetic retinoid analogs that couple increased specificity and reduced toxicity were developed. ST1926 is a novel orally bioavailable synthetic retinoid that has shown potent anticancer and antileukemic properties, and has reached phase I clinical trials.

We investigated the preclinical efficacy of ST1926 in ATL and peripheral T cell lymphomas. Clinically achievable concentrations of ST1926 induced a dramatic inhibition of cell proliferation in malignant human T cell lines and primary ATL cells, with minimal effect on resting or activated normal lymphocytes. ST1926 induced massive apoptosis, DNA damage, and upregulation of p53 proteins in malignant T cells, while it caused an early downregulation of Tax proteins in HTLV-1 positive cells. In murine ATL, oral treatment with ST1926 prolonged survival and reduced leukemia cell infiltration, white blood cell counts, and spleen mass. In spleens of ST1926-treated animals, p53 and p21 proteins were upregulated, PARP was cleaved while Tax transcripts were reduced. To our knowledge, this is the first study that shows a promising effect of a retinoid in leukemia translational research beyond APL.

T 6
THE HAT INHIBITOR CPTH6 PREFERENTIALLY INHIBITS IN VITRO AND IN VIVO PROLIFERATION ON PATIENT-DERIVED LUNG CANCER STEM-LIKE CELLS

Marta Di Martile [1] - Marianna Desideri [1] - Teresa De Luca [1] - Chiara Gabellini [1] - Adriana Eramo [2] - Michele Milella [3] - Simone Corradori [4] - Daniela Secci [4] - Donatella Del Bufalo [1] - Daniela Trisciuoglio [1]

Experimental Chemotherapy Laboratory, Regina Elena National Cancer Institute, Rome, Italy [1] - Department of Hematology, Oncology and Molecular Medicine, Istituto Superiore di Sanità, Rome, Italy [2] - Division of Medical Oncology A, Regina Elena National Cancer Institute, Rome, Italy [3] - Drug Chemistry and Technologies, "Sapienza" University, Rome, Italy [4]

Introduction: Epigenetic enzymes targeting is emerging as promising approach for cancer therapy. In this context, we recently identified the thiazole derivative 3-methylcyclopentylidene-[4-(4'-chlorophenyl)thiazol-2-yl]hydrazone (CPTH6) as a novel pCAF and GCN5 histone acetyltransferases inhibitor (HATi). CPTH6 exerts *in vitro* antitumoral effect in a panel of human tumor cell lines derived from different histotypes. In this study, we evaluated the efficacy of CPTH6 alone or in combinatorial regimes with chemotherapic agents, using *in vitro* and *in vivo* models of Non Small Cell Lung Cancer (NSCLC).

Materials And Methods: The biological effect of CPTH6 was analyzed in a panel of NSCLC cell lines (H1299, H460, A549, CALU1, CALU3) and in patient-derived Lung Cancer Stem Cell (LCSC) models (LCSC 34, LCSC 36, LCSC 136). Cell viability was tested by MTT and CellTiterGlo. Apoptosis induction and cell cycle perturbation were assessed by flow cytometry. DNA damage induction was revealed by Western Blot and Immunofluorescence analyses. Pharmacological interaction between CPTH6 and cytotoxic drugs was assessed by conservative isobologram analysis. Nude and NOD/SCID mice were used for *in vivo* experiments.

Results And Discussion: The HATi CPTH6 reduced cell viability of both NSCLC and LCSC lines *in vitro*. Interestingly, it was more efficient in LCSC (IC50 values ranging from 10 to25μM) than NSCLC lines (IC50 values ranging from 60 to 300μM). Furthermore, the growth inhibitory effect seemed to be tightly related to baseline expression of acetylated alpha-tubulin, which was particularly prominent in sensitive LCSC. Unlike LCSC models, in which CPTH6 treatment induced cell cycle perturbation and apoptosis even at low doses, in NSCLC lines CPTH6 triggered a cell cycle perturbation associated to DNA damage. When CPTH6 was used in combination with Pemetrexed or Cisplatin, it showed a strikingly synergistic reduction in cell viability both in NSCLC and LCSC lines. *In vivo* experiments confirmed the antitumor efficacy of CPTH6, particularly in LCSC-derived models. In fact, while CPTH6 induced a pronounced growth inhibitory effect on LCSC136 derived tumors, it only produced a little impact on H1299 tumor growth.

Conclusions: Overall, CPTH6 could be a valuable agent for the treatment of NSCLC and it should be further studied as a possible antineoplastic agent.

T 7
EFFECTS OF HEDGEHOG SIGNALING INHIBITION ON EPITHELIAL-STROMAL INTERACTIONS IN TRIPLE NEGATIVE BREAST CANCER CELLS.

Concetta Di Mauro [1] - Roberta Rosa [1] - Valentina D'amato [1] - Luigi Formisano [1] - Roberta Marciano [1] - Lucia Raimondo [1] - Alberto Servetto [1] - Bianca Maria Veneziani [2] - Sabino De Placido [1] - Roberto Bianco [1]

Department of molecular medicine and surgery, University of Naples Federico II, Naples, Italy [1] - Department of molecular medicine and medical biotechnologies, University of Naples Federico II, Naples, Italy [2]

Introduction: Triple-negative breast cancer (TNBC) is a group of tumors that do not express HER2, estrogen and progesterone receptors. Due to reduced response to conventional antitumor therapies and poor prognosis, new targeted agents are needed for such aggressive sub-type of breast cancer. Multiple lines of evidence support the idea that deregulation of Hedgehog (Hh) signaling has a role in the pathogenesis of breast cancer, in part through the promotion of epithelial-stromal interactions. Therefore, the inhibition of the Hh pathway has been proposed as an interesting therapeutic approach.

Methods: The main objective of this study is to investigate the role of Hh signaling pathway in TNBC. To this aim, we used a panel of human breast cancer cell lines, including five cancer cells lines positive for ER, PR and HER2 expression (nTNBC) and five Triple Negative Breast Cancer cell lines (TNBC). The effects induced by the Smo-inhibitor NVP-LDE225 on proliferation, angiogenesis and signal transduction of breast cancer cells were investigated.

Results: GLI1, one of the major transcription factors induced by Hh signaling activation, is consistently more expressed in TNBC than in nTNBC cell lines. Consistently, NVP-LDE225 treatment induced a more pronounced inhibitory effect on TNBC, in terms of tumor growth: while nTNBC cells display an IC_{50} of ~ 5mM, TNBC cell lines are more sensitive, with an average IC_{50} of ~ 2mM. In addition, Hh inhibition caused a robust impairment of TNBC cells invasion capabilities. These effects are coupled with a strong inhibition of VEGFA production by both tumor and stromal cells (human fibroblasts and HUVECs). Accordingly, NVP-LDE225 treatment interfered with HUVEC capillary tube formation, an effect even more evident than that observed with bevacizumab, the only targeted agent approved to date for TNBC patients.

Conclusions: Our results suggest that Hh has a specific role in breast epithelial-stromal interactions by regulation of angiogenesis. An orthotopic in vivo experiment in nude mice xenografted with TNBC cells, testing the combination of NVP-LDE225 with bevacizumab, is ongoing.

T 8
RADIOIODINATES VERSUS RADIOMETAL SCFVD2B AS BETTER TOOLS FOR PROSTATE CANCER IMAGING

Barbara Frigerio [1] - Fabio Benigni [2] - Elena Luison [1] - Francesco Caroli [1] - Giulio Fracasso [3] - Marco Colombatti [3] - Silvana Canevari [4] - Claudio Pascali [5] - Anna Bogni [5] - Mariangela Figini [1]

Molecular Therapies Unit, Molecular Medicine and Experimental Oncology Department, Fondazione IRCCS Istituto Nazionale Tumori, Milano, Italy [1] - Department of Urology, San Raffaele Scientific Institute, Milano, Italy [2] - Department of Pathology and Diagnostic, Section of Immunology, University of Verona, Verona, Italy [3] - Functional Genomics Unit, Molecular Medicine and Experimental Oncology Department, Fondazione IRCCS Istituto Nazionale dei Tumori, Milano, Italy [4] - Department of Nuclear Medicine, Fondazione IRCCS Istituto Nazionale dei Tumori, Milano, Italy [5]

Prostate cancer (PCa) is the most common cancer in elderly men in developed countries and represents the second-leading cause of death by tumor. PCa has a low response to conventional therapies in its castration-resistant phase. Thus, it is necessary to develop new therapeutic strategies. Antibody-based reagents represent a promising strategy to provide physicians with potentially effective diagnostic and therapeutic tools. One of the most suitable targets for antibody-mediated approaches is Prostate Specific Membrane Antigen (PSMA), a type II transmembrane protein, which is an already known tumor-associated antigen, particularly in metastatic and androgen-independent cancer. It has been demonstrated that PSMA expression and enzymatic activity are increased in prostate cancer and they have been also correlated with disease aggressiveness.

A monoclonal antibody named D2B directed against the h-PSMA extracellular domain was obtained by conventional hybridoma technology. However, because of their molecular size, whole antibodies often display a poor tumor penetration, which is indeed a critical parameter for effective radioimmunotherapy. We re-shaped the murine monoclonal antibody D2B into scFv format, a polypeptide formed by the variable regions of the heavy and light chain joined by a short peptide linker. Due to its smaller size, scFv represents the ideal candidate as diagnostic reagent, giving two main advantages: a faster blood clearance and a better penetrability in the tissue. ScFvD2B has been characterized and showed an optimal stability and good cell-interaction features despite its monovalent binding. Aim of this study is to evaluate *in vivo* the target specificity of scFvD2B when labeled with different radioisotopes for molecular imaging purposes.

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T 9
DEVELOPMENT OF 3-OXO-CHOLENIC DERIVATIVES AS EPH ANTAGONISTS ABLE TO BLOCK IN VITRO ANGIOGENESIS

Iftiin Hassan Mohamed [1] - Carmine Giorgio [1] - Riccardo Castelli [1] - Alessio Lodola [1] - Elisabetta Barocelli [1] - Marco Mor [1] - Simonetta Russo [1] - Matteo Incerti [1] - Daniele Pala [1] - Massimiliano Tognolini [1]

Department of Pharmacy, University of Parma, Parma, Italy [1]

Introduction: Eph receptor tyrosine kinases and their membrane-bound ephrin ligands are involved in many biological processes as cell migration and morphology, synaptic plasticity and angiogenesis both during embryogenesis and adult tissues. Alterations of this system have been found in human cancers and in particular EphA2 overexpression is often correlated with aggressive tumor phenotypes and poor prognosis. Therefore, Eph-ephrin system represents a promising target in cancer field. Through an ELISA-based binding screening, we recently identified lithocholic acid (LCA), a secondary bile acid able to modulate Eph-ephrin activity. LCA resulted a novel Eph receptor antagonist but it is also a known ligand of other receptors including TGR5 or FXR. For these reasons, the development of specific off-targets-free Eph antagonists using LCA scaffold could be challenging. However, we identified the 3-oxo-cholenic acid, which offers an activity similar to LCA without affecting TGR5 or FXR. Based on this evidence, we used its scaffold to design and synthesize two new 3-oxo-cholenic derivatives, both presenting amino-acid residues on the lateral acidic chain.

Material and methods: The 3-oxo-cholenic acid and derivatives were tested for their ability to inhibit the interaction between EphA2 receptor and ephrin-A1 ligand using an ELISA binding assay. Moreover, the activity on the EphA2 receptor was analyzed through the evaluation of the receptor phosphorylation levels in prostate adenocarcinoma cell line (PC3) naturally presenting EphA2 receptor. Finally, the compounds were tested for their anti-angiogenic effect in human umbilical vein endothelial cells through the *in vitro* tube formation assay.

Results: The 3-oxo-cholenic acid and its derivatives competitively inhibited ephrin-A1 binding to the EphA2 receptor in the ELISA-binding assay and they were also able to inhibit the EphA2 phosphorylation induced by the physiological ligand in PC3 cell line. Moreover the compounds showed interesting anti-angiogenic properties in the tube formation assay, without cytotoxic effects.

Conclusions: The identification of the 3-oxo-cholenic derivatives could be a starting point for the development of novel specific anti-angiogenic Eph antagonists endowed with better affinity for the Eph-ephrin system.

T 10
SRC INHIBITION BLOCKS D16HER2-DRIVEN CARCINOGENESIS

Cristina Kalogris [1] - Chiara Garulli [1] - Lucia Pietrella [1] - Caterina Bartolacci [1] - Cristina Andreani [1] - Valentina Gambini [1] - Martina Tilio [1] - Maria Elexpuru Zabaleta [1] - Manuela Iezzi [2] - Barbara Belletti [3] - Cristina Marchini [4] - Augusto Amici [4]

Department of Bioscience and Biotechnology, University of Camerino, Camerino, Italy [1] - Aging Research Centre, University of Chieti, Italy. [2] - Division of Experimental Oncology 2, CRO, Aviano, Italy. [3] - Department of Bioscience and Biotechnology, University of Camerino, Camerino, Italy. [4]

The human epidermal growth factor receptor 2 (HER2) is a validated therapeutic target in breast cancer therapy. However, increasing evidence points to a major role for the Δ16HER2 splice variant, which has been identified as a clinically important and tumor-specific HER2 molecular alteration promoting aggressive metastatic breast cancer and conferring resistance to anti-HER2 therapies. Δ16HER2 has an increased transforming potency compared to wild type HER2 receptor, since it promotes constitutive dimerization of the receptor and activation of Src-mediated oncogenic signaling pathways. Consistently, mice transgenic for the human Δ16HER2 isoform (Δ16HER2 mice) develop invasive mammary carcinomas with early onset and 100% penetrance. From these mice, an epithelial cancer cell line (CAM6 cells) was established and characterized. When injected in syngeneic mice, CAM6 cells give rise to tumours growing with a short latency time and able to metastasize to lung. Δ16HER2 mice and CAM6 cells represent, respectively, *in vivo* and *in vitro* models to investigate Δ16HER2 behavior in terms of oncogenicity and response to therapies.

In this study, once confirmed the cooperation between Δ16HER2 and Src tyrosine kinase to promote breast tumorigenesis, we evaluated the ability of Saracatinib, a potent Src inhibitor, to suppress Δ16HER2-driven breast carcinogenesis. Saracatinib treatment resulted in a strong reduction of CAM6 cell migration, cell viability and in the induction of CAM6 cell death by apoptosis. *In vivo* experiments demonstrated that oral administration of Saracatinib reduced the development and growth of CAM6 transplantable tumors in FVB syngeneic mice and autochthonous mammary carcinogenesis in Δ16HER2 mice. Western blotting analysis revealed that suppression of Δ16HER2-positive breast cancer growth by Saracatinib was correlated exclusively with the inhibition Src activation, while the phosphorylation level of Δ16HER2 resulted unchanged.

These results provided the rationale for targeting Src in Δ16HER2-positive breast cancer.

T 11
COMBINATION OF NAB-PACLITAXEL/PACLITAXEL WITH BEVACIZUMAB: IN VITRO STUDY

Cristiana Lo Nigro [1] - Ornella Garrone [1] - Federica Tonissi [1] - Laura Lattanzio [1] - Annalisa Ghiglia [1] - Daniela Vivenza [1] - Martino Monteverde [1] - Marco Merlano [1]

A.O.Santa Croce e Carle, Ospedale Universitario Santa Croce e Carle, Cuneo, Italia [1]

Introduction: Taxanes are the treatment of choice of metastatic breast cancer. The combination of bevacizumab and taxanes improved response rate (RR) and progression-free survival (PFS) over chemotherapy alone. Starting from this point, we decided to study *in vitro* the effect on cell survival of the combination of paclitaxel and nab-paclitaxel with bevacizumab and related biological factors, including SPARC (vehicle protein involved in nab-paclitaxel uptake) VEGF/R at basal level and after treatments.

Material And Methods: We used 2 different breast cancer cell lines, MCF7 (ER+/HER2-) and MM231 (ER-/HER2-) together with HUVEC endothelial cells (Human Umbilical Vein Endothelial Cell), seeded in transwell plates to better mimic the in vivo microenvironment. We analysed proliferation and cell survival by MTT test, VEGF secretion by ELISA assay and selected proteins expression by western blot (VEGFR, SPARC).

Results: Seeding MCF7 with HUVEC, only the combination bevacizumab+nab-paclitaxel showed a higher effect than nab-paclitaxel alone ($p<0.01$), while the same effect of paclitaxel was obtained when given either alone or in combination with bevacizumab. In MM231 seeded with HUVEC both the combinations showed a significant reduction of survival compared to the two taxanes alone ($p<0.05$). We also detected an induction of VEGF secretion when MM231 were treated with nab-paclitaxel or paclitaxel (respectively $p<0.001$ and $p<0.01$), while, as expected, the addition of bevacizumab totally removed VEGF from the supernatant. This effect was not seen in MCF7. Analysis on MCF7 by western blot showed an induction of SPARC protein expression only when cells were treated with both combinations ($p<0.05$). In MM231 the upregolation of SPARC protein expression was seen only when cells were treated with bevacizumab+nab-paclitaxel ($p<0.05$), but not with bavacizumab+paclitaxel.

Conclusions: Our results confirmed that nab-paclitaxel could play an important role in inhibiting tumour proliferation through albumin-SPARC bound when administered with bevacizumab compared to the taxane alone. Moreover in ER+ cells bevacizumab+nab-paclitaxel demonstrated a higher effect than bevacizumab+paclitaxel in respect to the corresponding taxanes alone.

All together these results suggested that further clinical trials could be proposed to deeply investigate the combination of nab-paclitaxel and anti-angiogenic therapy in a specific group of ER+ breast cancer patients.

T 12
TARGETING GSK3-CALCINEURIN AXIS IN T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA (T-ALL)

Erich Piovan [1] - Valeria Tosello [2] - Fulvio Bordin [1] - Stefano Indraccolo [2] - Valentina Agnusdei [1] - Sonia Minuzzo [1] - Alberto Amadori [1] - Erich Piovan [2]

Department of Surgery, Oncology and Gastroenterology, University of Padova, Padova, Italy [1] - UOC Immunologia e Diagnostica Molecolare Oncologica, Istituto Oncologico Veneto—IRCCS, Padova, Italy [2]

T-cell acute lymphoblastic leukemia (T-ALL) accounts for approximately 15% of pediatric and 25% of adult cases of ALL. Progress in T-ALL therapy has been impressive, with cure rates approaching 80% for children and 50% for adults. T-ALL is classified into subgroups based on exclusive genetic alterations and/or deregulated expression of specific transcription factors. Besides cell autonomous alterations, stromal-derived signals also contribute to leukemia progression and resistance to therapy. Calcineurin (CnA) is a calcium-activated serine/threonine phosphatase critical to a number of developmental processes in the cardiovascular, nervous and immune system. CnA has the ability to dephosphorylate a broad range of proteins, with the most studied being NFAT (nuclear factor of activated T cells). Although critically involved in many aspects of normal T cell survival, proliferation and activation, the direct implication of CnA and/or its downstream NFAT targets in lymphomagenesis and cancer in general has only recently been reported. So far, available evidence shows that NFAT transcription factors are mediators of CnA action in different cancers. However, it is possible that NFAT factors are not the only targets of CnA in leukemogenesis as CnA can dephosphorylate other factors possibly relevant to its oncogenic properties. With this perspective in mind we used tandem affinity chromatography, followed by mass spectrometry to purify novel CnA interacting partners in human T-ALL cells. We found a large set of novel proteins associated with CnA, including transcriptional regulators and protein modifiers. Glycogen synthase kinase-3b (GSK3b) emerged as an important CnA interacting partner. As little is currently known about the significance of GSK3 to T-ALL cell survival and pathobiology we pursued this aspect further. GST-pulldown assays demonstrated the interaction between CnA and GSK3b to be direct and *in vitro* kinase assays demonstrated altered phosphorylation of GSK3b in the presence of CnA which resulted in modification of GSK-3b enzymatic activity. Pharmacological inhibition of GSK-3 activity was able to sensitize human T-ALL cell lines and primografts to the action of CnA inhibitors *in vitro* and *in vivo*. In summary, we establish GSK3 as direct target of CnA and establish a synergistic interaction between GSK3 and CnA inhibition in the treatment of T-ALL.

T 13
MODULATION OF ERBB RECEPTORS EXPRESSION BY HISTONE DEACETYLASE INHIBITORS INCREASED THE ANTITUMOR ACTIVITY OF AN ANTI-ERBB3 MONOCLONAL ANTIBODY IN PRIMARY CULTURES FROM NSCLC PATIENTS

Maria Serena Roca [1] - Chiara Ciardiello [1] - Alessia Noto [2] - Claudia De Vitis [1] - Luigi Aurisicchio [3] - Francesca Bruzzese [1] - Elena Di Gennaro [1] - Gennaro Ciliberto [1] - Rita Mancini [2] - Alfredo Budillon [1] - Alessandra Leone [1]

Istituto Nazionale per lo Studio e la Cura dei Tumori, "Fondazione Giovanni Pascale"- IRCCS – Italia, Naples, Italy [1] - "La Sapienza", University of Rome, Rome, Italy [2] - Takis, srl, Rome, Italy [3]

In the last years several evidences suggested that ErbB3, a member of the HER family receptors, has a key role in the development and progression of several cancers including non-small cell lung cancer (NSCLC), and above all in the establishment of resistance to therapies, leading to major efforts towards the development of anti-ErbB3 approaches. We recently demonstrated in head and neck cancer cells that, depending on the ErbB3 expression level and on the tumor cell phenotype (epithelial *vs* mesenchymal), vorinostat, one of the two clinically approved histone deacetylase inhibitors (HDACi), differentially regulates HER receptors expression at the transcriptional level and/or by modulating protein degradation (Bruzzese F. et al. 2011). Our group has developed a monoclonal antibody against ErbB3 called A3 that induces receptor internalization and degradation, inhibits growth and induces apoptosis only in cells overexpressing surface ErbB3 and potentiates the efficacy of EGFR TKIs (Noto A. et al. 2013).

In this study we first show, by using a set of malignant pleural effusion derived cell cultures from NSCLC patients (Mancini R. et al. 2011), that the combination of the anti-ErbB3 antibody A3 with HDACi such as vorinostat or valproic acid (VPA), synergistically affects cell proliferation and induces apoptosis and DNA damage. Interestingly, synergistic interaction was observed in both fully epithelial cells expressing all HER receptors including ErbB3, as well as in NSCLC cells that had undergone epithelial to mesenchymal transition and expressed very low levels of ErbB3. We provide evidences suggesting that, in epithelial cells, the observed synergism is due to time- and dose-dependent down-regulation of all HER receptors expression and signaling by either vorinostat or VPA. On the contrary, in two A3-resistant mesenchymal cells expressing undetectable levels of ErbB3, we observe time- and dose-dependent increase of mRNA and protein levels as well as surface expression of ErbB3, paralleled by down-regulation of EGFR, ErbB2. Interestingly, ErbB3 induction was achieved also at low doses of both vorinostat and VPA, corresponding to a plasma level easily reached in patients treated with these agents. Our results suggest that the combination treatment of antibodies against ErbB3 and HDACi represents an attractive strategy that warrant further evaluation, even in combination with other agents, for the treatment of NSCLC patients.

T 14
THE MITOGEN-ACTIVATED PROTEIN KINASE ERK5 AS A NEW TARGET FOR THE TREATMENT OF HEPATOCELLULAR CARCINOMA

Elisabetta Rovida [1] - Giovanni Di Maira [2] - Ignazia Tusa [1] - Stefania Cannito [3] - Claudia Paternostro [3] - Xianming Deng [4] - Nathanael Gray [4] - Atanasio Pandiella [5] - Persio Dello Sbarba [1] - Maurizio Parola [3] - Fabio Marra [2]

Dipartimento di Scienze Biomediche Sperimentali e Cliniche, Università di Firenze, Firenze, Italia [1] - Dipartimento di Medicina Sperimentale e Clinica, Università di Firenze, Firenze, Italia [2] - Dipartimento di Medicina e Oncologia Sperimentali, Università di Torino, Torino, Italia [3] - Dana-Farber Cancer Institute, Harvard Medical School, Boston, Stati Uniti D' America [4] - Instituto de Biología Molecular y Celular del Cáncer, CSIC, Universidad de Salamanca, salamanca, Spagna [5]

Introduction: The Extracellular Signal-Regulated kinase 5 (ERK5 or BMK1) is involved in tumour development. The ERK5 gene may be amplified in hepatocellular carcinoma (HCC) but its biological role has not been clarified. In this study we explored the role of ERK5 expression and activity in HCC in vitro and in vivo.

Materials and methods : ERK5 expression was evaluated in human liver tissue. Cultured HepG2 and Huh-7 were studied after ERK5 knockdown by siRNA or in the presence of the specific pharmacologic inhibitor, XMD8-92. The role of ERK5 in vivo was assessed using mouse Huh-7 xenografts.

Results: In tissue specimens from patients with HCC, a higher percentage of cells with nuclear ERK5 expression was found both in HCC and in the surrounding cirrhotic tissue, compared to normal liver tissue. Treatment of Huh-7 or HepG2 cells with XMD8-92 or with ERK5 siRNA decreased HCC cell proliferation, increased the proportion of cells in G0/G1 phase, and augmented the expression of p27Kip. Genetic or pharmacologic inhibition of ERK5 prevented cell migration induced by EGF or hypoxia, and caused cytoskeletal remodelling and rearrangement of focal adhesions. In mouse xenografts, the rate of tumour appearance and the size of tumours were significantly lower when Huh-7 were silenced for ERK5. Moreover, systemic treatment with XMD8-92 of mice with established HCC xenografts markedly reduced tumour growth and decreased the expression of the proto-oncogene c-Rel.

Conclusions: ERK5 regulates the biology of HCC cells and modulates tumour development and growth in vivo. This pathway should be investigated as a possible therapeutic target in HCC.

T 15
CYTOTOXIC ACTIVITY OF THE NOVEL MTOR INHIBITOR TORIN-2 IN B-PRECURSOR ACUTE LYMPHOBLASTIC LEUKEMIA AS A NEW THERAPEUTIC OPTION TO PREVENT AKT REACTIVATION

Carolina Simioni [1] - Alice Cani [1] - Alberto Martelli [2] - Ayman Ali Mohammed Alameen [1] - Giorgio Zauli [3] - Silvano Capitani [1] - Luca Maria Neri [1]

Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Ferrara, Italy [1] - Department of Biomedical and Neuromotor Sciences, University of Bologna, Bologna, Italy [2] - Institute for Maternal and Child Health, IRCCS "Burlo Garofolo", Trieste, Italy [3]

B-precursor acute lymphoblastic leukemia (B-pre-ALL), is a malignant disorder characterized by abundance of B-cell lymphoblasts in blood and bone marrow. Dysregulation of the PI3K kinase/AKT/mTOR pathway is involved in pathogenesis of many human malignancies, including leukemias. Thus, targeting the PI3K/AKT /mTOR pathway is an attractive therapeutic strategy and different small molecule inhibitors are under clinical investigation. RAD001, a mTORC1 inhibitor, has been shown to affect cell cycle progression and survival with a potentially relevant therapeutic efficacy. However, prolonged inhibition of mTORC1 often leads to AKT re-phosphorylation at Ser^{473}which may counteract RAD001 activity.

We therefore hypothesized that dual inhibition of mTORC1 and mTORC2 would provide a superior outcome in ALL as compared to inhibition of mTORC1 alone.

We tested the activity of the novel second generation ATP-competitive mTOR inhibitor Torin-2 and its capability to prevent AKT reactivation after mTORC1 inhibition with RAD001 in B-pre-ALL. Furthermore we explored if dual targeting of mTORC1 and AKT may achieve results similar to those obtained with Torin-2 alone.

We used a panel of B-pre-ALL to test the drugs by MTT Assay and Western Blotting after different time exposures of the cells. Cell cycle, apoptosis and autophagy were analyzed by flow cytometry, Western Blotting and fluorescent staining.

In all the B-pre-ALL cell lines Torin-2 showed a powerful cytotoxicity, inhibiting the growth of each cell line in a dose-dependent manner, with an IC_{50} in the nanomolar range as assessed by MTT assays. The major proteins along the

PI3K/AKT/mTOR signaling pathway were heavily dephosphorylated after 2 hrs of drug exposure.

This inhibition lasted up to at least 48 hrs at variance to RAD001, that already after 24 hrs was unable to prevent AKT reactivation. However the association of RAD001 with MK-2206, an allosteric AKT inhibitor, prevented AKT reactivation and reached a significant cytotoxicity.

Our data suggest an interesting cytotoxic activity of Torin-2 in B-pre-ALL acting on both mTORC1 and mTORC2 as assessed by their substrate inhibition.

Torin-2 alone suppresses feedback activation of PI3K/AKT, whereas RAD001 requires the addition of MK-2206 to achieve the same efficacy. These two pharmacological options targeting PI3K/Akt/mTOR at different points of the signaling pathway cascade might represent a new promising therapeutic strategy for treatment of adult B-pre-ALL patients.

T 16
3-PHOSPHOINOSITIDE–DEPENDENT KINASE 1 IS REQUIRED FOR ANCHORAGE INDEPENDENT GROWTH OF KRAS MUTATED CELLS

Desiana Somale [1] - Paolo Armando Gagliardi [1] - Laura Di Blasio [1] - Giorgio Seano [1] - Roberto Sessa [1] - Alberto Puliafito [1] - Giulia Chiaverina [1] - Federico Bussolino [1] - Luca Primo [1]

Institute for Cancer Research and Treatment, Candiolo, 10060, Italy, Department of Oncology, University of Torino, Turin, 10060, Italy, Candiolo, Italy [1]

Introduction: 3-phosphoinositide-dependent protein kinase 1 (PDK1) is a pivotal element of the PI3K signaling pathway since it phosphorylates Akt/PKB through the interaction with phosphatidylinositol (3,4,5)-trisphosphate. Recent data indicate that PDK1 is overexpressed in multiple tumor types and that alterations of PDK1 are critical in the context of oncogenic PI3K activation. However, the role of PDK1 in tumor progression is still controversial. Here, we show that PDK1 is required for anchorage-independent growth and tumor growth of cells harboring *KRAS* mutations.

Materials and Methods: MDAMB231, HMEC and HCT116 were infected with lentiviral vectors silencing PDK1 or overexpressing it and they were tested in soft agar. MDAMB231 were also tested for the tumor growth.

Results: PDK1 silencing leads to increased anoikis, reduced soft-agar growth and pronounced apoptosis inside tumors of MDAMB231 breast cancer cells. Interestingly, these phenotypes are reverted by PDK1 wild-type but not kinase-dead mutant suggesting a relevant role of PDK1 kinase activity.

Complementary, PDK1 overexpression is able to increase anchorage independent growth and tumor growth of MDAMB231 cells. Furthermore, in non-tumorigenic HMEC mammary cells PDK1 silencing prevents the anchorage independent growth that is obtained by KRAS G13D expression. In HCT116 colon cancer cells, which harbor both *PIK3CA* and *KRAS* mutations, PDK1 silencing impairs anchorage independent growth. Interestingly, the isogenic cells derived from HCT116 cells in which PI3KCA mutant allele was removed are still sensitive to PDK1 silencing as parental cells, indicating that PDK1 silencing effects are not due to a role in the signalling of mutated PI3KCA.

The effects of PDK1 silencing or overexpression are not mediated by the PDK1 most studied effector Akt, since the expression of constitutively active forms of Akt in PDK1-knockdown MDAMB231 cells is unable to rescue the anchorage-independent growth. In addition, Akt down-regulation and pharmacological inhibition do not inhibit PDK1 overexpression effects.

Conclusion: In summary this results suggest that PDK1 contributes to anchorage independent growth and tumor growth of different tumor cells harboring KRAS mutation.

T 17
DISCOVERY AND DEVELOPMENT OF LOW MOLECULAR WEIGHT EPH-EPHRIN PROTEIN-PROTEIN INHIBITORS ENDOWED WITH IN VITRO ANTIANGIOGENIC ACTIVITY

Massimiliano Tognolini [1] - Iftiin Hassan-mohamed [1] - Carmine Giorgio [1] - Matteo Incerti [1] - Elisabetta Barocelli [1] - Simona Bertoni [1] - Daniele Pala [1] - Marco Mor [1] - Simonetta Russo [1] - Alessio Lodola [1]

Dipartimento di Farmacia, Università di Parma, Parma, Italy [1]

Introduction: The EPH-ephrin system plays a key role in tumorigenesis and its de-regulation correlates with a poor clinical prognosis in many solid tumors. Agents targeting the EPH-ephrin system could be potentially useful for the inhibition of different facets of cancer progression. However, even if some classes of small molecules targeting EPH-ephrin interactions have been reported, their use is hampered by poor chemical stability and low potency. Stable and potent ligands are essential to obtain strong pharmacological data.

Materials and methods: We recently identified lithocholic acid (LCA) as a competitive and reversible -compound able to disrupt EPH-ephrin interaction showing a Ki value of 49 μM. Using LCA scaffold as a reference structure and starting from a docking model of the EPHA2-LCA complex we designed and synthetized a series of amino acid conjugates of lithocholic acid and the new compounds were characterized for their ability to disrupt the EPHA2-ephrin-A1 interaction by means of an ELISA assay developed in our lab.

Potent antagonists of the EPHA2 receptor were tested for their ability to interact with physiological-targets of LCA (FXR, TGR5) to check for the overall selectivity of the new compounds.

Results: The L-homo-Trp-conjugated of LCA (UniPR129) emerged as a potent and selective antagonist of EPH receptors, inactive on the FXR and TGR5 receptors. UniPR129 reversibly and competitively disrupted EPHA2-ephrin-A1 interaction with Ki = 370 nM in an ELISA binding assay and it showed low micromolar potency in cellular functional assays, including inhibition of EPHA2 activation, cell migration and cell rounding and disruption of *in vitro* angiogenesis without cytotoxic effects.

Structure of UniPR129 was modified, with the aim to improve its pharmacokinetic properties, leading to the identification of the orally bioavailable compound UniPR500.

Conclusions and implications: The discovery of UniPR129 represents not only a major advance in potency compared to the existing EPH-ephrin antagonists but also an improvement in terms of cytotoxicity, making this molecule a useful pharmacological tool and a promising lead compound. Moreover, steroid scaffold could be modified in order to obtain potent, selective, and soluble bioavailable compounds.

Tumor Immunology And Immunotherapy

U 1
THE ROLE PLAYED BY CD73 IN A NATURAL HUMAN CD73 KNOCKOUT

Chillemi Antonella [1] - Valeria Quarona [1] - Gianluca Zaccarello [1] - Nicola Lo Buono [1] - Andrea Zito [1] - Valentina Mariani [1] - Carlo Arduino [2] - Franco Fais [3] - Massimo Geuna [4] - Ornella Azzolino [5] - Tiziana Musso [6] - Alberto Horenstein [1] - Fabio Malavasi [1]

Lab of Immunogenetics, Department of Medical Sciences, University of Torino, Torino, Italy [1] - Medical Genetics, "Città della Salute e della Scienza" Hospital, Torino, Italy [2] - Human Anatomy Section, Department of Experimental Medicine, University of Genova, Genova, Italy [3] - Department of Pathology, Ordine Mauriziano Hospital, Torino, Italy [4] - Department of Molecular Biotechnology and Health Science, University of Torino, Torino, Italy [5] - Department of Public Health and Pediatric Sciences, University of Torino, Torino, Italy [6]

The ectoenzyme CD73 controls the terminal phosphohydrolysis of AMP, which is then converted to adenosine (ADO). CD73 is expressed by normal cell and ectopically by different tumors, which exploit the production from of ADO as a way to escape immune defenses.

We analyzed two sisters (age 77 and 78) with inactivating mutations in both copies of the *CD73* gene, with a non-functional cell surface CD73. The clinical manifestations shared by the 9 cases reported in the world are referred as secondary to an accumulation of crystals of calcium phosphate in the arterial walls, prevalently in the lower limbs (St. Hilaire C. et al, 2011).

The aim of this work was an analysis of the immune conditions of the patients, not yet considered in these natural CD73 knockout. Leukocytes from the two $CD73^-$ siblings were analyzed for phenotype and for the composition of T, B, NK and myeloid populations, which were within normality ranges. Similar results were obtained with Tregs and MDSCs.

EBV-immortalized B lymphocytes (also $CD73^-$) were analyzed for Ig class switch recombination (CSR) described as dependent upon the presence of functional CD73 (Schena et al, 2012). Molecular IgHV rearrangements in B lymphocytes showed the presence of Ig switched transcripts (IgG and IgA). Same results were observed on EBV lines. Somatic hypermutation detected in IgM and IgG transcripts and mutated rearrangements identified in IgM and IgG transcripts were similar to age-matched controls. These results indicate that the patients have the ability to diversify the BCR repertoire.

One $CD73^-$ EBV line was characterized for the ability to secrete cytokines, notably those with tolerogenic potential. The same line was transfected with a human *CD73* gene, obtaining a correction of the surface phenotype. The $CD73^+$ line was analyzed for viability and cell growth, Ig rearrangements and for the production of a panel of selected cytokines and ADO. The lines were also comparatively tested for signaling and migratory potentials.

The enzymatic activity of CD73, using AMP as exogenous substrate and evaluated by HPLC UV assay, was nearly absent in both PBMC and $CD73^-$ lines. A minute residual activity was reduced to background in the presence of levamisole, an inhibitor of TNAP. Currently, secretion of endogenous ADO generated from cytoplasmic pathways are under evaluation as a surrogate source of the ADO.

U 2
THE COMPLEMENT SYSTEM IN THE IMMUNOSURVEILLANCE OF HER-2 POSITIVE MAMMARY CANCER

Silvio Bandini [1] - Marco Macagno [1] - Albana Hisi [2] - Laura Conti [1] - Stefania Lanzardo [1] - Amanda Bello [1] - Manuela Iezzi [2] - Federica Cavallo [1]

Molecular Biotechnology Center, University of Turin, Turin, Italy [1] - Aging Research Center, G. D'Annunzio University Foundation, Chieti, Italy [2]

Introduction: Complement system consists of a number of plasma and membrane-bound proteins acting at the interface of innate and adaptive immunity. Beside its role against infections, complement is an important component of tumor microenvironment. To evade complement-mediated inhibition cancer cells express membrane-bound and fluid-phase complement regulators. However, the importance of complement as microenvironment regulator of tumor growth is not yet elucidated. Currently available data are contradictory, with a few studies pointing to a protective function for the complement in the host response against malignant cells while others highlight potential tumor-promoting activities of the complement system. This discordance is not surprising in view of the fact that the complement functionally contributes to a plethora of distinct immunological and inflammatory processes, each of them having unique context-dependent outcomes in different tumor models.

With a 100% penetrance all inbred BALB/c female mice transgenic for the rat ErbB-2 (Her-2/neu) oncogene (BALB-neuT mice) provides a unique opportunity to evaluate the impact of complement system on a model of Her-2 positive breast cancer, a very aggressive tumor that is denoted by rapid development, high metastatization and worst prognosis. The step-wise progression of these autochthonous tumors recapitulates several molecular and genetic features of human cancer, among which a slow progression, the natural occurrence of invasion and metastasis, and the presence of a long-lasting interaction between the evolving lesion and its microenvironment.

Materials and methods: BALB-neuT/C1qaKO were generated by crossing BALB-neuT male mice with BALB-C1qaKO and females.

Results: By crossing BALB-neuT with BALB/c mice deficient for C1qa , we observed that the onset of the first palpable tumor is accelerated with a dramatic increase both in the number of mammary glands with tumor and in the tumor growth rate. Whole mounts of the mammary gland show that at the same week of age a higher number of focal lesions is spread all over mammary glands in BALB-neuT/C1qaKO compared to BALB-neuT mice.

Conclusions: These data show, probably for the first time, that complement proteins can hamper even the onset of a mammary cancer aggressively driven by the overexpression of the ErbB2 oncogene that results markedly accelerated by the absence C1qa in the tumor microenvironment.

U 3
TH17-TYPE CYTOKINES, IL-6 AND TNF-ALPHA SYNERGISTICALLY ACTIVATE STAT3 AND NF-KB TO PROMOTE COLORECTAL CANCER CELL GROWTH

Veronica De Simone [1] - Eleonora Franzè [2] - Giulia Ronchetti [2] - Alfredo Colantoni [2] - Massimo Claudio Fantini [2] - Davide Di Fusco [2] - Giuseppe S. Sica Sica [3] - Pierpaolo Sileri [3] - Thomas T Macdonald [4] - Francesco Pallone [2] - Giovanni Monteleone [2] - Carmine Stolfi [2]

Department of Systems Medicine, University of Rome "Tor Vergata', Rome, Italy [1] - Department of Systems Medicine, University of Rome "Tor Vergata", Rome, Italy [2] - Department of Surgery, University of Rome "Tor Vergata", Rome, Italy [3] - Centre for Immunology and Infectious Disease, Blizard Institute of Cell and Molecular Science, Barts and the London School of Medicine and Dentistry, London, UK [4]

Colorectal cancers (CRC) often show a dense infiltrate of cytokine-producing immune/inflammatory cells. The exact contribution of each cytokine in the activation of the intracellular pathways sustaining CRC cell growth is not understood. Here, we show that the supernatants of immune/inflammatory cells infiltrating human sporadic CRC potently enhance the growth of human CRC cells lines *in vitro*, in a STAT3 and NF-kB-dependent fashion. *In vitro* CD3+CD8- T cells produce large amounts of Th17-related cytokines (i.e., IL-17A, IL-17F, IL-21 and IL-22), TNF-α and IL-6. Simultaneous neutralization of both IL-17A and TNF-α, which abrogates NF-kB signalling, and IL-22 and IL-6, which abrogates STAT3 signalling, reduces the mitogenic effect of supernatants in CRC cells. Th17-related cytokines, TNF-α and IL-6 are also produced in excess in the early colonic lesions of $Apc^{min/+}$ mice, associated with enhanced STAT3/NF-kB activation. Mice given an oral STAT3/NF-kB inhibitor exhibit reduced colon tumorigenesis and diminished expression of IL-17A, IL-21, IL-22, TNF-α and IL-6 in the neoplastic areas. These observations suggest that dual inhibition of STAT3/NF-kB may be therapeutically useful in CRC patients.

U 4
SURVIVIN VACCINE FOR PANCREATIC ADENOCARCINOMA

Daniela Liberati [1] - Erica Dugnani [1] - Valentina Pasquale [1] - Francesca Bosè [2] - Vito Lampasona [3] - Cristina Brigatti [3] - Elisa Cantarelli [1] - Paola Cappello [4] - Maddalena Panigada [2] - Elisa Soprana [2] - Antonio Siccardi [2] - Lorenzo Piemonti [1]

Division of Immunology, Transplantation, and Infectious Diseases - DRI - Diabetes Research Institute, beta-cell biology, San Raffaele Scientific Institute, Milan, Italy [1] - Division of Genetics and Cell biology, Molecular immunology, San Raffaele Scientific Institute, Milan, Italy [2] - Center for Translational Genomics and BioInformatics , Genomic Unit for the diagnosis of human pathologies, San Raffaele Scientific Institute, Milan, Italy [3] - CeRMS, AO City of Health and Sciences, Dept molecular Biotechnology and Health Sciences, University of Turin, Turin, Italy [4]

Introduction: Survivin was ranked 21 among 75 prioritized TAAs in a pilot project and specific survivin immune response was described in different cancers. Survivin was found expressed in 80% of pancreatic ductal adenocarcinoma (PDC). So it could be an attractive target for pancreatic cancer immunotherapy. Our aim is to investigate, in a prophylactic setting, if prime/boost vaccination strategy using recombinant Survivin-expressing Poxviral vaccines could be effective in a murine model of PDC.

Materials and methods: Modified Vaccinia Ankara (rMVA) and FowlPox (rFPV) viruses expressing murine Survivin (mSurv) were generated by the "Red-to-Green gene swapping" method. Vaccination schedule: 8 weeks-old C57BL/6 wt mice were immunized with plasmid PVJ-tetOh-CMV-mSurv (prime), rMVA-mSurv (1° boost at 9 weeks-old) and rFPV-mSurv (2° boost at 11 weeks-old) or Empty-vectors as control. Post last boost, different groups of mSurv and empty mice were used for immunomonitoring or tumor challenge (an orthotopic implantation, 1 week post vaccination, of syngeneic mSurv-expressing cell line, Panc02).

Results: Overall immunological response to immunization per se: i)vaccines generated specific antibodies against rMVA or rFPV and the presence of the mSurv-transgene didn't determine differences in humoral responses towards the viral vectors. No anti-Survivin antibodies were detected in the sera; ii)there was no evidence of the presence of mSurv specific CD8+ T cells in immunized mice both ex-vivo (MHC-I pentamer-based flow cytometry) and post 24 h stimulation with different m-Surv peptides (intracellular INF-g staining). Survivin-vaccination prolonged the post-implant median survival compared to control (77 vs 69 days, p=0.006 Log-Rank analysis). 50 days post-implant, splenocytes and tumor-infiltrating lymphocytes (TIL) were characterized in flow cytometry: mSurv-vaccinated showed an increase of CD4+ and CD8+ effector T cells (CD44+ CD62L-) in TIL, but not in spleen.

Conclusions: Survivin vaccination improved survival in our murine PDC model. This suggests that vaccination might induce an immune reaction against Survivin itself or other Survivin-induced tumor antigens. Despite the evidence of a biological effect, no mSurv-specific B or T response were observed. However, the increase of effector T cells in TIL suggests that at tumoral site our vaccination is able to break tolerance toward self-antigen survivin triggering the immune response against cancer.

U 5
CD38 AND MYELOMA CAMOUFLAGE: A VIEW FROM THE BONE MARROW NICHE

Fabio Malavasi [1] - Alberto L. Horenstein [1] - Valeria Quarona [1] - Valentina Ferri [2] - Antonella Chillemi [1] - Gianluca Zaccarello [1] - Fabio Morandi [3] - Vito Pistoia [3] - Nicola Giuliani [2]

Department of Medical Sciences, University of Torino, Torino, Italy [1] - Hematology and Blood and Marrow Transplantation (BMT) Center, University of Parma, Parma, Italy [2] - Department of Experimental and Laboratory Medicine, Istituto Giannina Gaslini, Genova, Italy [3]

Introduction: The bone marrow (BM) provides a protected environment for generating a vast array of cell types and of soluble factors used locally or at a distance from their production site. The working hypothesis is that the myeloma cells subvert the cellular components of the BM niche (where they grow and expand), by using products generated *in situ* by the concerted action of ectoenzymes, which lead to the production of adenosine (ADO), a regulator of local immunological tolerance.

Materials and methods: CD38 and CD157, both NAD+-consuming enzymes, lead in the BM niche a chain of ectoenzymes, whose final product is exploited by the myeloma as part of its local camouflage and survival strategies. This hypothesis was tested on cells derived from BM aspirates from 25 myeloma patients. Myeloma cells, mesenchymal stem cells, osteoblasts and osteoclasts were used to identify the presence of ectoenzymes by means of immunohistochemistry and of cytofluography. Furthermore, the BM plasma was used to assay the presence of ADO using a sensitive *ad hoc* designed HPLC assay.

Results: The results obtained indicate that myeloma cells usurp the normal BM organization, replacing it with a niche that maximizes local growth and protection from immune defenses. Myeloma and surrounding cells are endowed with an ectoenzymatic pathway leading to ADO production in a discontinuous way, where the components do not need to be expressed by the same cell but should operate in a closed system. The CD38 (CD157)/CD203a/CD73 pathway is highly efficient in the myeloma niche and superior to the canonical CD39/CD73.

Conclusions: The proposed molecular circuit relies upon proteins which do not need to be ectopically expressed, a major difference from other models of immune evasion observed in breast or colon cancers, which are dependent on *de novo* expression of CD73. The myeloma niche represents a closed system, which consents an exchange of substrates and products for the ectoenzymes. For translational medicine, ADO levels in the myeloma niche might be an early prognostic indicator of an aggressive form of disease. Lastly, the presence of ADO lends support to the use of therapeutical anti-CD38 mAbs: the anti-CD38 mAb would exert cytotoxic functions on cells expressing the molecule, simultaneously depressing the enzymatic activity of CD38, which leads to the production of ADO.

U 6
NATURAL KILLER CELLS FROM MALIGNANT PLEURAL EFFUSIONS ARE RESPONSIVE TO INTERLEUKIN-2 BUT ARE ENDOWED WITH A POTENT PRO-ANGIOGENIC FEATURE

Silvia Zanellato [1] - Antonino Bruno [2] - Andrea Imperatori [3] - Maria Cattoni [3] - Cecilia Sampietro [3] - Nicola Rotolo [3] - Lorenzo Dominioni [3] - Adriana Albini [4] - Douglas M. Noonan [1] - Lorenzo Mortara [1]

Department of Biotechnology and Life Sciences, University of Insubria, Varese, Italy [1] - Fondazione MultiMedica, Onlus, Milano, Italy [2] - Department of Surgical and Morphological Sciences, University of Insubria, Varese, Italy [3] - Department of Research and Statistics Infrastructure, IRCCS Arcispedale Santa Maria Nuova, Reggio Emilia, Italy [4]

Introduction: Natural killer (NKs) cells are crucial effector cells of innate immunity able to kill tumor or virus-infected cells and able to regulate adaptive immune cells by cytokine release. Their activities depend on the fine balance between activating and inhibitory surface receptors. However, in tumor-bearing hosts the anti-tumor function of NKs are largely impaired. Recently we found that tumor infiltrating NKs in non-small cell lung cancer (NSCLC) are enriched in the $CD56^{bright}CD16^{-}$ NK cell subset characterized by a pro-angiogenic phenotype and function. Here we characterize fresh and 3 day in vitro-cultured NK cells derived from peripheral blood (PB) and pleural effusions (PE) of patients with primary or metastatic tumors of different origin, including mesothelioma, lung, breast and hepatocarcinoma.

Materials and methods: We investigated several surface markers, including: NKGD2, NKG2A, NKp30, NKp44, pan-KIR, and two decidual-like NK markers: CD9 and CD49a by FACS analysis. We also performed functional assays testing cytokine production upon PMA and ionomycin stimulation by intracellular staining, and a degranulation assay against the classic MHC class I^{-} K562 cells.

Results: Preliminary data show that percentage of NKs from PB and PE are similar but that the $CD56^{bright}CD16^{-}$ NK cell subset predominates in PE. Moreover, ex vivo NK cells from PE are partly positive for CD9 and CD49a markers. NK cells from PEs are able to generate a complex pro-angiogenic cytokine response, much higher than their counterparts in the PB, producing VEGF, IL-8, SDF-1, Osteopontin (OPN), and moderate/low levels of IFNg. Further, in vitro degranulation assays of NK cells from patients indicate that the cytotoxic capacity of these effector cells are very low (10-15%) for both ex vivo NK cells from PE and PB, but this activity is enhanced after 3 day culture with IL-2 and only partly enhanced with IL-2 plus TGFb. Finally, the IL-2 or IL-2 plus TGFb 3-day in vitro culture of NKs reinforced their pro-angiogenic potential preferentially in PE, but also in PB to a lesser extent.

Conclusions: In conclusion, our findings highlight the fact that NK cells from malignant pleural effusions are not anergic but are poorly cytotoxic and skewed toward a pro-angiogenic feature. Culture with IL-2 enhanced cytokine release, but the presence of TGFb further potentiated pro-angiogenic cytokine production.

U 7
TARGETING TGFBETA1 TO BREAK TUMOR-INDUCED IMMUNETOLERANCE: THE RATIONALE TO DESIGN COMBINED THERAPY

Sergio Occhipinti [1] - Costanza Angelini [2] - Laura Sponton [2] - Daniela Pierobon [2] - Augusto Amici [3] - Cristina Marchini [3] - Sara Bustreo [4] - Michela Donadio [4] - Franco Novelli [2] - Mirella Giovarelli [2]

Univirsity of Turin, Dep of Molecular biotechinology and health sciences, Turin, Italy [1] - Department of Medicine and Experimental Oncology, University of Turin, Torino, Italy [2] - Department of Molecular Cellular and Animal Biology, University of Camerino, Camerino, Italy [3] - C.O.E.S. Subalpine OncoHematology Cancer Center, San Giovanni Battista Hospital, Turin, Italy [4]

Introduction: Many cancer-related changes in alternative splicing have been identified to distinguish splicing patterns in normal breast compared to cancer samples. Cancer-specific events can result in proteins with "procancer" properties which may promote malignant transformation or confer a survival advantage on cancer cells, such as resistance to treatment.

To date, one naturally occurring HER2 spliced variants in breast cancer, namely Δ16HER2, arises from the in-frame deletion of exon 16 display a causal role in cancer development [Mitra et al., 2009]. From the immunologic perspective, mutations may be particularly potent vaccination targets as they can create neoantigens that are not subject to central immune tolerance. Whereas for other tumor antigen categories there is the likelihood of downregulation in metastases, mutations that occur in early tumor development are sustained in advanced disease. Due to these characteristics, Δ16HER2 represent a suitable target for immunotherapy in breast cancer.

Materials and methods: CD14+ monocytes were separated from PBMC of patients with primary or metastatic breast cancer overexpressing HER2, to generate dendritic cells (DCs). This DCs were transfected with plasmids coding for extracellular and transmembrane domains of human wild type (wt) HER2 or the splice variant Δ16HER2, then used to activate autologous T cells.

Results: Preliminary data show that wtHER2 is completely tolerated in patients with primary HER2+ breast tumor, while Δ16HER2-vaccine is able to elicit antitumor immuneresponse in vitro. When we test the same approach in patients with recurrent/metastatic breast cancer both wtHER2 and Δ16HER2-vaccine fail to mount T cell response. In both cases anti-HER2 immune response was rescued by TGFβ1 neutralization, suggesting a key role of this cytokine in tumor-induced immunetolerance.

Conclusions: These data suggest that TGFβ1 could represent an ideal target to counteract tumor-dependent immunosuppressive mechanisms in cancer patients increasing HER2-vaccine efficacy and provide the rationale to design combined therapy.

U 8
INCREASED EXPANSION AND FUNCTION OF CD4+CD25+FOXP3+ T REGULATORY CELLS IN RENAL CELL CARCINOMA PATIENT SAMPLES.

Sara Santagata [1] - Maria Napolitano [1] - Serena Zanotta [1] - Crescenzo D'alterio [1] - Sabrina Cecere [2] - Renato De Domenico [2] - Renato Franco [3] - Sisto Perdonà [2] - Sandro Pignata [2] - Stefania Scala [1]

Oncological Immunology, National Cancer Institute "Fondazione G.Pascale", Naples, Italy [1] - Department of Urology, National Cancer Institute "Fondazione G.Pascale", Naples, Italy [2] - Department of Pathology, National Cancer Institute "Fondazione G.Pascale", Naples, Italy [3]

Introduction: Renal cell carcinoma (RCC) is considered an immunogenic cancer, with pathological specimens frequently containing a large number of tumor infiltrating lymphocytes. In RCC patients the neoplasm acquires the ability to escape from the immune response, which has been assumed to depend on a direct interaction between the cancer and the immune effectors, with local recruitment and induction of immune-regulatory T cell $CD4^+CD25^+Foxp3^+$ (Treg). To evaluate the specific function of Treg in primary renal cancer. To this aim Treg were isolated from renal cancer primary tissues and compared to Treg from peripheral blood and healthy tissue of the the same patient.

Materials and methods: Autologous peripheral blood mononuclear cells (PBMCs) were isolated from 25 RCC primary cancer patients and from 15 healthy donors (HD) through density-gradient centrifugation. Single cells suspensions from tumor and healthy tissue were obtained via tissue digestion. Treg in peripheral blood (pb), healthy and tumor tissue was evaluated by flow cytometer (CD4/CD25/Foxp3). Treg and Teff cells were separated using the magnetic isolation cell kit. Treg inhibitory activity was evaluated in 13 patients by an in vitro suppression assay performed with a co-colture of purified Treg from pb, healthy and tumor tissue versus CFSE labeled autologous Teff cells at different ratio in presence of anti-CD3/CD28 antibody.

Results: Increase in Treg was detected in RCC patients, both in the pb and tumor tissues compared to controls ($p<0,001$). Moreover Treg from tumor tissues were significantly higher compared to peripheral blood ($p<0,01$). Functional analysis displayed that both circulating and tumor Treg RCC comparably suppressed the proliferation of Teff cells. Although Treg cells from HD were functionally active, their capacity to suppress the proliferation of Teff was reduced compared to Treg from patients.

Conclusions: RCC patients showed higher number of Treg cells both in peripheral blood and in tumor tissues as compared to HD. Moreover patients derived Treg showed a strong suppressive ability against autologous Teff cells. Thus antagonists of the Treg immune suppressive activity become desiderable in RCC.

U 9
TARGETING SURFACE MOLECULES WITH ENZYMATIC AND SIGNALING PROPRIETIES AS MAB-IMMUNOTHERAPY FOR HUMAN PROSTATE CANCER

Gianluca Zaccarello [1] - Andrea Zitella [2] - Valeria Quarona [1] - Antonella Chillemi [1] - Giovanna Chiorino [3] - Marilena Bellò [4] - Alessandro Tizzani [2] - Alberto L. Horenstein [1] - Gianni Bisi [4] - Fabio Malavasi [1]

Lab of Immunogenetics, Department of Medical Sciences, University of Torino, Torino, Italy [1] - Department of Urology, Città della Salute e della Scienza Hospital, University of Torino, Torino, Italy [2] - Cancer Genomics Lab, Fondo Edo Tempia, Biella, Italy [3] - Department of Nuclear Medicine, Città della Salute e della Scienza Hospital, Torino, Italy [4]

Introduction: New diagnostic and therapeutic tools differentiating tumor from normal prostate tissues and to characterize the biological features of the neoplastic cells are a clinical and social need. We have generated a panel of murine monoclonal antibodies (mAbs), selected for their ability to give signals to prostate cells or to trace in vivo primary and secondary tumors.

Materials and methods: Fresh and cancer prostate cells were used as immunizer. The tracking efficiency of the selected mAbs was tested by means of radio-immunoscintigraphy in mice transplanted with the human LNCaP line. The effects on post-trascriptional regulation induced by mAb ligation were tested by mRNA microarray on LNCaP cells.

Results: We selected mAb specific for receptors or ectoenzymes, or with the ability to modulate the transcriptome of the LNCaP line. One of these is specific for PSMA, a glycoprotein acting as N-acetylated α-linked acidic dipeptidase (NAALADase) and also of pteroyl poly-γ-glutamyl carboxypeptitdase (i.e., folate hydrolase). The molecule is homologous of the transferrin-1 and 2 receptors. The anti-PSMA mAb was used for radio-immunoscintigraphy in a prostate cancer murine model. mAb ligation is not followed in vitro by significant variations in the transcriptome of LNCaP.

Other mAbs displayed agonistic proprieties, regulating gene activation. GF/3E8 mAb modulated the expression of genes involved in carcinogenesis, metastatic spreading and transcription of cancer biomarkers. Other mAbs (GF/3D3 and C5), bind a 60 kDa heat-shock protein.

In vitro ligation by GF/3A3 mAb induces LNCaP to increase the expression of miRNAs, especially miR-99a. The members of the miR-99 family were downregulated in prostate tumors as compared with normal tissues. On the contrary, the overexpression of miR-99a negatively influences growth of prostate cancer cells. This suggest that miR-99a may play a role of tumor suppressor, anticipating its use as a cytostatic agent.

Conclusions: his study has enriched the armamentarium of mAbs specific for human prostate cancers. Ongoing efforts are now focused on using the inert reagents as carriers of radioactive tracers or toxins, to trace primary and secondary lesions. Next step will be the analysis of signals implemented after ligation of surface target molecules, including modulation of prostate cancer-specific miRNAs.

A third approach is to exploit the enzymatic features of PSMA as pro-drug, able to activate *in situ* anti-cancer drugs. More ambitious goal will be to use agonistic mAbs for differentiation therapies or to induce cell death.

Tumor Microenvironment: Inflammation, Hypoxia And Angiogenesis

V 1
THE MULTIFACETED ANTI-TUMOR ACTIVITY OF INTERLEUKIN-27 IN HUMAN LUNG CANCER

Irma Airoldi [1] - Silvia Esposito [2] - Maria Grazia Tupone [2] - Giulia Barbarito [1] - Marco Vincenzo Russo [2] - Serena Di Meo [2] - Carlo Sorrentino [2] - Emma Di Carlo [2]

Laboratory of Oncology, Istituto Giannina Gaslini, Genova, Italy [1] - Department of Medicine and Sciences of Aging, "G. d'Annunzio" University, Chieti, Italy [2]

Introduction: Despite advances in the clinical management, lung cancer mortality has remained largely unchanged over the past three decades, underlying the need for new treatment strategies. Interleukin (IL)-27 has revealed potent anti-tumor effects in various tumor models and, importantly, freedom from toxicity in preclinical trials. We thus investigated whether IL-27 may function as anti-tumor agent in lung cancer.

Materials and methods: Non small cell lung cancer cell lines Calu6 and SK-MES were selected, on the basis of their expression of IL-27R, to assess IL-27 anti-tumor activity both *in vitro* and *in vivo*. These cell lines were injected s.c. in immune-deficient mice, that were treated with hrIL-27, to investigate *in vivo* effects of the cytokine on tumor growth and development. Expression of IL-27/IL-27R was also assessed in surgical samples from lung cancer patients.

Results: *In vitro,* IL-27 treatment had no significant effects on proliferation and apoptosis of both cell lines. In SK-MES cells, typically endowed with spindle cell morphology, IL-27 significantly down-regulated expression of specific pluripotency and Epithelial-to-Mesenchimal-Transition (EMT) transcription factors. In both SK-MES and Calu-6 cell lines, IL-27 up-regulated the expression of the powerful granulocyte chemoattractant CXCL3. In SK-MES cells only, IL-27 also up-regulated IFN-g and down-regulated trombospondin-1 and laminin-a5 expression. *In vivo*, IL-27 reduced the growth of both Calu-6 and SK-MES tumors in association with areas of colliquative necrosis, a prominent infiltrate of granulocytes and macrophages, while a slight anti-angiogenic effect was detected particularly in SK-MES tumors. Myeloablative conditioning of tumor bearing mice greatly reduced the *in vivo* efficacy of IL-27, even if in the SK-MES tumors the phenotypic alterations associated with EMT down-regulation were still evident.

Finally, in patients' lung cancer tissues, IL-27R was mainly expressed by stromal infiltrating immune cells and often by tumor cells in both adenocarcinomas and squamous cell lung cancers.

Conclusions: Our results highlight novel aspects of IL-27 antitumor activity such as I. the capability to orchestrate myeloid cell re-education towards anti-cancer activities and, II. downregulate the expression of pluripotency and EMT related genes. These findings together with the evidence of IL-27R expression in lung cancer microenvironment strongly candidate this cytokine for its clinical use.

V 2
MULTIPLE MYELOMA-DERIVED EXOSOMES REGULATE OSTEOCLAST DIFFERENTIATION AND FUNCTION

Lavinia Raimondi [(1)] - Angela De Luca [(1)] - Daniele Bellavia [(1)] - Flores Naselli [(2)] - Mauro Manno [(3)] - Samuele Raccosta [(3)] - Nicola Amodio [(4)] - Pierfrancesco Tassone [(4)] - Giacomo De Leo [(2)] - Riccardo Alessandro [(2)]

Istituto Ortopedico Rizzoli; Università di Palermo, Istituto Ortopedico Rizzoli; Università di Palermo, Palermo, Italy [(1)] - DiBiMeF, Università di Palermo, Palermo, Italy [(2)] - Istituto di Biofisica, Consiglio Nazionale delle Ricerche, Palermo, Italy [(3)] - Dip.to di Medicina Clinica e Sperimentale, Università di Catanzaro, Catanzaro, Italy [(4)]

Introduction. Multiple myeloma (MM) is characterized by abnormal proliferation of malignant plasma cells (PCs) within the bone marrow (BM), which leads to osteolytic bone disease, anemia and renal failure. Despite important achievements in the understanding of MM pathogenesis have been obtained, MM is still an incurable malignancy. Bone metastases are the most frequent complication in MM resulting in pain, bone fractures, spinal cord compression and hypercalcemia. Normally, bone remodelling is a process in which the activity of bone-resorbing cells, osteoclasts, and bone-forming cells, osteoblasts, is well-balanced. In MM, this balance is impaired in favor of osteoclasts (OCL) thus damaging bone. Most of the osteoclastic activating factors are known to be released by tumor cells. Exosomes are small membrane vesicles of endocytic origin that are secreted by most cells; they contain microRNAs, mRNAs and proteins, which are actively transported from a donor cells to target cells. Exosomes have been thoroughly described for their functional relevance in cancer, contributing to immunomodulation, angiogenesis and metastasis. Here, we investigated whether MM derived exosomes may be involved in OCL differentiation and function.

Methods. MM-derived exosomes were isolated by differential centrifugation, followed by sucrose gradient and analyzed by Dynamic Light Scattering (DLS), western blotting and acetylcholinesterase assay. Internalization of MM derived exosomes by Raw264.7 cells was analysed by confocal microscopy. For *in vitro* study, Raw264.7 cells were treated with MM derived exosomes and then evaluated for the expression of osteoclast markers such as Cathepsin K, CalcR, MMP9 and TRAP. In vitro quantification of bone resorption was assessed by pit formation assay. MTT assay was used to evaluate the role of MM derived exosomes on Raw264.7 cell proliferation.

Results. *In vitro* study demonstrated the pro-differentiative effects induced by MM derived exosomes on a pre-osteoclast cell line model. MM-exosomes increased osteoclast markers expression and their function as evidenced by formation of typical lacunae on dentine slices. Cell proliferation assay showed that MM exosomes increased Raw264.6 proliferation.

Conclusion. Our results indicate that exosomes released by multiple myeloma cells modulate OCL proliferation and differentiation. Prospective studies are needed to identify the OCL activating factors transported by MM exosomes that are responsible for these events.

V 3
P2X7/PI3K/AKT/HIF1A PATHWAY: A NOVEL NETWORK INFLUENCING NEUROBLASTOMA GROWTH.

Francesca Saveria Amoroso [(1)] - Marina Capece [(1)] - Elena De Marchi [(1)] - Davide Cangelosi [(2)] - Alessia Franceschini [(1)] - Luigi Varesio [(2)] - Elena Adinolfi [(1)]

Department of Morphology, Surgery and Experimental Medicine, Section of Experimental Pathology, Oncology and Biology, University of Ferrara, Ferrara, Italy [(1)] - Laboratory of Molecular Biology, G. Gaslini Institute, Genova, Italy [(2)]

Introduction. P2X7 receptor for extracellular ATP has been focus of interest over the last years as a receptor promoting cell proliferation, tumor growth, engraftment, tumor vascularization and aerobic glycolysis.[1,2] In support of these findings, P2X7 expression was reported to be increased in several tumors, including breast and skin cancers, leukemia and neuroblastoma.[3,4,5] Neuroblastoma is a common and aggressive extra cranial solid tumor in childhood and still causes death in 40% of the cases. Here we elucidate the role of P2X7 receptor in neuroblastoma.

Materials and Methods. For *in vitro* and *in vivo* experiments we took advantage of two P2X7 expressing neuroblastoma cell lines: human ACN, that release VEGF in a P2X7 dependent fashion[1], and murine Neuro-2a. We injected them in *nude/nude* and Albino J mice to obtain an allogeneic and a syngeneic model, respectively.

Results. We show here that basal P2X7 receptor expression supported ACN and Neuro-2a tumor growth *in vivo*. P2X7 silencing and antagonism with AZ10606120 and A740003 caused cell growth arrest through the PI3K/Akt/HIF1α/GSK3β pathway modulation, both *in vitro* and *in vivo*. Pharmacological treatment of P2X7 also reduced mitochondrial potential in Neuro-2a cells. Moreover, we found a correlation between P2X7 mRNA expression and a decreased overall survival of stage IV neuroblastoma patients.

Conclusions. Altogether, these data show that P2X7 receptor plays an important role in neuroblastoma growth by modulating PI3K/Akt/HIF1α/GSK3β pathway. Thus, we suggest P2X7 receptor as a possible pharmacological target for neuroblastoma treatment.

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V 4

THE CROSS-TALK WITH B-CELL RECEPTOR (BCR) PATHWAY IS PART OF THE MICROENVIROMENT SIGNALS STIMULATING NOTCH1 ACTIVITY IN CHRONIC LYMPHOCYTIC LEUKEMIA (CLL) HARBORING NOTCH1 MUTATIONS

Francesca Arruga [1] - Branimir Gizdic [1] - Vittoria Tani [1] - Tiziana Vaisitti [1] - Davide Rossi [2] - Marta Coscia [3] - Luca Laurenti [4] - Giovanni D'arena [5] - Ozren Jaksic [6] - Gianluca Gaidano [2] - Silvia Deaglio [1]

Dept. Medical Sciences, University of Turin & Human Genetics Foundation, Turin, Italy [1] - Division of Haematology, Department of Translational Medicine, Amedeo Avogadro University of Eastern Piedmont, Novara, Italy [2] - Division of Hematology, University of Turin & A.O. Città della Salute e della Scienza, Turin, Italy [3] - Department of Hematology, Catholic University of Rome & A. Gemelli Hospital, Rome, Italy [4] - IRCCS-CROB, Centro di Riferimento Oncologico della Basilicata, IRCCS-CROB, Centro di Riferimento Oncologico della Basilicata, Rionero in Vulture (PZ), Italy [5] - Department of Hematology, Dubrava University Hospital, Zagreb, Croatia [6]

CLL is defined by the monoclonal expansion of mature B cells, accumulating in the peripheral blood (PB) and lymphoid organs. Given the heterogeneity of the clinical course, timely identification of high risk patients is a primary challenge.

NOTCH1 mutations are the most frequent somatic aberration in CLL, found in 5-10% at diagnosis, with frequency increasing to ~20% in progressive/relapsed cases. *NOTCH1* mutations identify patients with shorter overall survival, time to treatment and progression free survival, and represent an independent prognostic factor for the disease. Most mutations occur in exon 34, leading to PEST domain loss, resulting in NOTCH1 impaired degradation, stabilization of the intracellular domain (NICD) and deregulated signaling, both canonical and non-canonical.

Microenvironment interactions are critical to initiate and maintain signaling, even in the presence of mutations, otherwise NOTCH1 activity is rapidly switched off. This is in line with the view of CLL as a compartmentalized disease, with a quiescent fraction in PB and a proliferating tumor load in the lymph nodes and bone marrow. Here, microenvironment-driven pathways, such as the BCR pathway, provide signals that promote CLL cells proliferation and survival, likely leading to accumulation of genetic lesions or expansion of more aggressive subclones.

The aim of the study was to explore the cross-talk between the NOTCH1 and the BCR pathways and the role that *NOTCH1* mutations may play in this context. Effective BCR signaling was induced by stimulation with IgM independently of *NOTCH1* mutational status, using as read-outs the rescue from spontaneous apoptosis and the increased levels of *CCL3*. However, at a variance with *NOTCH1* WT cases, in *NOTCH1* M patients BCR activation resulted also in increased *NOTCH1* expression and activity. Of note, non-canonical pathway, as evaluated by expression of *DTX1*, was selectively interested. This is in line with previously published data, showing that culture of *NOTCH1* M CLL cells in conditions that mimic a lymphoid microenvironment *in vitro*, selectively activates non-canonical NOTCH1 pathway, and protects cells from drug-induced apoptosis. Finally, preliminary data revealed a more sustained response to ibrutinib in *NOTCH1* WT patients than in M, suggesting a role for NOTCH1 mutations in restraining ibrutinib activity, and paving the way to combine NOTCH1 and BCR inhibitors as a thera

V 5

INTERLEUKIN 6 DOWN-REGULATES P53 EXPRESSION AND ACTIVITY BY STIMULATING RIBOSOME BIOGENESIS: A NEW PATHWAY CONNECTING INFLAMMATION TO CANCER

Elisa Brighenti [1] - Carlo Calabrese [2] - Giuseppina Liguori [2] - Ferdinando Antonino Giannone [3] - Davide Trerè [1] - Lorenzo Montanaro [1] - Massimo Derenzini [1]

Department of Experimental, Diagnostic and Specialty Medicine, Bologna University, Bologna, Italy [1] - Department of Medical and Surgical Sciences, Bologna University, Bologna, - [2] - Biomedical and Applied Research Center CRBA, Azienda Ospedaliero-Universitaria di Bologna, Policlinico S. Orsola-Malpighi, Bologna, - [3]

Introduction: Chronic inflammation is an established risk factor for the development of many types of human cancers. The inflammatory substances released in the inflamed tissue can contribute to all the steps of carcinogenesis, favoring tumor initiation and promotion. Among the various substances present in the inflammatory milieu, interleukin (IL) 6 has been proven to play a fundamental role in tumorigenesis. We recently demonstrated that the up-regulation of rRNA transcription, by factors stimulating proliferation, down-regulates p53 expression and activity. We hypothesized that in chronic inflammation, in which cell proliferation stimulating factors are produced, a similar mechanism may be active, thus contributing to neoplastic transformation.

Materials and Methods: Using four human cell lines (HepG2, NCM460, LS174T, SW1990), we evaluated the effect of IL-6 treatment on rRNA transcription activity (by Real-time PCR analysis for 45s rRNA), on p53 expression and activity (by Real-Time PCR and Western Blot analysis) and on cellular phenotypic characteristics of epithelial-mesenchymal transition (by Immunofluorescence and Western Blot analysis). These parameters were also evaluated in the epithelial cells of colon biopsies from patients with ulcerative colitis.

Results: IL-6 treatment stimulated rRNA transcription in the human cell lines; this was the consequence of IL-6-dependent stimulation of *c-MYC* mRNA translation. The up-regulated ribosome biogenesis induced a reduction of p53 protein expression and function, as a consequence of increased p53-proteasomal degradation mediated by MDM2. The p53 down-regulation induced the acquisition of cellular phenotypic changes characteristic of epithelial-mesenchymal transition, such as a reduced level of E-cadherin expression, an increased cell invasiveness, and a decreased response to cytotoxic stresses. We found that these changes also occurred in colon epithelial cells of patients with ulcerative colitis and that disappeared after treatment with anti-inflammatory drugs.

Conclusions: The present results highlight a new mechanism that may link chronic inflammation to cancer, based on p53 down-regulation, activated by the enhancement of rRNA transcription upon IL-6 exposure. This mechanism could be active also in other pathological conditions, characterized by IL-6 release and increased risk of developing cancer, such as in type 2 diabetes and obesity.

V 6
TUMOR INFILTRATING NATURAL KILLER CELLS IN PATIENTS WITH COLORECTAL CANCER ARE SWITCHED TOWARDS THE DECIDUAL-LIKE PRO-ANGIOGENIC CD56BRIGHTCD16- NK CELL SUBSET

Antonino Bruno [1] - Sara Canali [2] - Silvia Zanellato [3] - Lorenzo Dominioni [4] - Luigi Boni [4] - Elisa Cassinotti [4] - Giulia David [4] - Lorenzo Mortara [3] - Douglas Noonan [3] - Adriana Albini [5]

Fondazione MultiMedica Onlus, Polo Scientifico e Tecnologico MultiMedica, Milan, Italy [1] - Fondazione MultiMedica Onlus, Fondazione MultiMedica Onlus, Milan, Italy [2] - Department of Biotechnology and Life Sciences, University of Insubria, Varese, Italy [3] - Department of Surgical and Morphological Sciences, University of Insubria, Varese, Italy [4] - IRCCS "Tecnologie Avanzate e Modelli Assistenziali in Oncologia", Arcispedale S. Maria Nuova, Reggio Emilia, Italy [5]

Introduction: Immune cells infiltrating tumors often show a polarized phenotype and attenuation of anti-tumor activity induced by several tumor microenvironment (TME) associated factors. Natural Killer (NK) cells are innate effector lymphocytes primarily involved in the immunosurveillance against tumors. Several NK cell subsets have been described; among these, the major subset, approximately 95% of peripheral blood NK cells, is $CD56^{dim}CD16^{+}$ and exerts strong cytotoxic activity. Approximately 5% of peripheral blood NK cells are $CD56^{bright}CD16^{-}$ and show cytotoxicity through strong cytokine production. In the developing decidua a third NK subset, termed decidual-NKs (dNKs) has been discovered. These dNKs, described as $CD56^{superbright}CD16^{-}$, display a cytokine-secreting, highly-angiogenic phenotype.

Since the contribution of NK cells to tumor angiogenesis represent a still unexpolored topic, we investigated whether NKs, as a key component of innate immunity, are directly involved in tumor progression, in the context of colo-rectal carcinoma (CRC).

Materials and methods: NK cells were isolated from resected tumor, adijacent tissues and peripheral blood of patients with CRC. A single cell suspension was obtained and subjected to flow cytometry for the assessment of surface antigen expression, including: CD45, CD3, CD56, CD16, NKGD, NKG2A, KIRs, and cytokine secretion (VEGF, PlGF, IL-8, IFNg).

Results: We found that the $CD56^{bright}CD16^{-}$ NK cell subset predominates over the cytotoxic $CD56^{dim}CD16^{+}$ subset in tissue samples. These cell are able to release high levels of VEGF, moderate levels of IL-8 and low amounts of IFNg. Our preliminary results investigating the expression of dNK surface markers including CD9 and CD49a indicated that only NK derived from patient tissue samples show increased expression of both markers. Moreover, we found out that conditioned media derived from CRC infiltrating NKs are able to promote capillary-like structure formation by human umbilical vein endothelial cells (HUVECs), supporting the hypothesis that tumor infiltrating NKs directly contribute to tumor angiogenesis.

Conclusions: taken together, our data clearly support the hypothesis that NKs may be considered as one of the key component of tumor microenvironment that significantly promote tumor progression.

V 7
CYTOKINOME PROFILES DURING HCV-RELATED CIRRHOSIS PROGRESSION TO LIVER CANCER

Francesca Capone [1] - Eliana Guerriero [1] - Patrizia Maio [2] - Alessandra Mangia [3] - Giovanni Colonna [4] - Giuseppe Castello [1] - Susan Costantini [1]

ISTITUTO NAZIONALE PER LO STUDIO E LA CURA DEI TUMORI "FONDAZIONE GIOVANNI PASCALE", IRCCS, Napoli, Italy [1] - Department of Infectious Diseases, Ospedale 'San Giuseppe Moscati', Avellino, Italy [2] - Liver Unit, Casa Sollievo della Sofferenza Hospital, San Giovanni Rotondo, Italy [3] - Dipartimento di Biochimica, Biofisica e Patologia Generale, Seconda Università degli Studi di Napoli, Napoli, Italy [4]

Introduction: The pathway leading to hepatocellular carcinoma (HCC) often begins with a viral infection, like viral hepatitis B virus (HBV) or C virus (HCV), that over decades can lead to severe complications such as chronic hepatitis, cirrhosis (LC), and cancer. Cytokines play an important role as informational molecules between cells in inflammation, regeneration, fibrosis, viral clearance and infection control, and also are implicated in the pathological processes occurring in the liver during viral infection. The type and role of immunological markers in cirrhosis compared to ones for HCC would be extremely useful for improving the prognosis through the process that from cirrhosis leads to HCC.

Materials and methods: We evaluated the serum levels of several cytokines, chemokines, adipokines and growth factors in 30 patients with HCV-related LC, in 34 patients with HCC and HCV-related LC and in 20 healthy controls by using a multiplex biometric ELISA-based immunoassay to identify those molecules that might be useful for defining and for monitoring the progression of liver cirrhosis to cancer. The nonparametric Mann-Whitney U test was used to evaluate differences between cytokine ratios in the patients and healthy controls. In particular $p<0.05$ is indicated with *, $p<0.01$ with **, and $p<0.0001$ with ***.

Results: Our data show that in HCC patients sHER-2/neu, sIL-6Ra, PECAM-1, PRL, IL-2R, IL-16, IL-18, CXCL12, HGF, and ghrelin are significantly up-regulated whereas adiponectin and adipsin are down-regulated in comparison to LC patients. Moreover, interactomic analysis shows that significant cytokines are strictly correlated and connected within hub nodes in the metabolic network involved in the development, progression and metastasis of HCC.

Conclusions: We suggest that serum levels of these biochemical markers have different significance during the disease progression; in fact, they characterize the degree of the carcinogenic state in the liver of patients with chronic inflammation and

LC. In future we will perform more detailed studies by collecting also LC and HCC patients with other risk factors to identify the possible markers of the different processes that can lead to cancer development also in absence of HCV- related LC.

V 8
NEW PLAYERS AND TARGETS IN TUMOR VASCULATURE

Elena Magrini [1] - Fabrizio Bianchi [1] - Andrea Doni [2] - Francesca Angiolini [1] - Alessandra Villa [1] - Massimiliano Mazzone [3] - Ugo Cavallaro [1]

IEO, Istituto Europeo di Oncologia, Milano, Italy [1] - Istituto Clinico Humanitas, Hospital, Milano, Italy [2] - VIB, KU Leuven, Leuven, Belgium [3]

Introduction: Anti-angiogenic therapy for tumor treatment, best exemplified by the anti-VEGF drug bevacizumab, has displayed a remarkable potential in certain cancer types. However, it is clear that novel vascular targets are needed to improve the efficacy of current anti-angiogenic strategies as well as to circumvent the resistance/evasion mechanisms that have emerged in different experimental models and in cancer patients.

The neural immunoglobulin-like cell adhesion molecule L1, that plays a crucial role in CNS development and plasticity, is aberrantly expressed in cancer-associated vessels, while it is not found in normal vasculature. However, the functional role of L1 in endothelial cells, and its contribution to tumor angiogenesis, remain elusive.

Materials and methods: To study the role of L1 in tumor vessels, we have combined a conditional knockout mouse model, in which the expression of L1 was specifically ablated in the endothelium, with an orthotopic model of pancreatic cancer.

In addition, to unravel the molecular mechanisms and pathways controlled by L1, gain and loss-of-function studies were conducted in cultured endothelial cells through ectopic overexpression or gene silencing, respectively.

Results: Our results implicated L1 in the control of tumor vasculature, due to its ability to regulate both angiogenesis and vascular maturation. We also observed that the inactivation of endothelial L1 represents a viable option for novel anti-angiogenic treatments, resulting in reduced tumor growth and progression.

Finally, L1 orchestrates several functions of endothelial cells, thus playing a major role in the aberrant pathophysiology of cancer vessels. Such a pleiotropic effect is dependent on the L1-mediated control of gene networks and biochemical pathways that, in turn, govern vascular function.

Conclusions: Besides shedding light on the molecular mechanisms that underlie the formation and function of cancer vasculature, our data may pave the way for innovative vascular targeting strategies in the context of tumor therapy**.**

V 9
ZOLEDRONIC ACID IMPAIRS STROMAL REACTIVITY BY INHIBITING M2-MACROPHAGES POLARIZATION AND PROSTATE CANCER-ASSOCIATED FIBROBLASTS

Giuseppina Comito [1] - Coral Pons Segura [1] - Luigi Ippolito [1] - Maria Letizia Taddei [1] - Elisa Giannoni [1] - Paola Chiarugi [1]

Dipartimento di Scienze Sperimentali e Cliniche, Università di Firenze, Firenze, Italy [1]

Introduction: Cancer associated fibroblasts (CAFs), the major cellular components of tumor microenvironment, promote epithelial mesenchymal transition (EMT) and acquistion of stemness traits in prostate cancer cells. Of note, prostate CAFs are active players in promoting monocyte recruitment to tumor site. CAFs secretion of stromal derived growth factor-1 (SDF-1) delivery promote macrophage trans-differentiation to the M2-macrophages phenotype. Moreover, M2 macrophages are able to induce mesenchymal- mesenchymal transition of fibroblasts, leading to their enhanced reactivity and highlighting the mutual relationship between these cell compartments. This complex interplay among cancer cells, CAFs and M2-macrophages, lead to i) an increase in tumor cell motility, that promote cancer cells escape from primary tumor and metastatic spread, and ii) an activation of both endothelial cells and their bone-marrow-derived precursors to drive *de novo* angiogenesis. Zoledronic acid (ZA), an amino-bisphosphonate compound in use for the treatment of symptomatic skeletal events, has recently been shown to have immunomodulatory properties that need to be exploited in cancer immunotherapy. There is *in vivo* evidence showing that ZA can reduce the tumorigenic phenotype of M2- polarized macrophages.

Methods: Human Monocytes were obtained from normal donor buffy coat, fibroblasts were isolated from aggressive carcinoma (CAFs) or from benign prostate hyperplasia (HPFs).

Results: Here we show the key effects of ZA on M1/M2 macrophages and CAFs in prostate carcinoma progression. First, we analyzed the phenotype of the differentiated macrophages treated with ZA evaluating their M1 and M2 phenotype, by looking at the expression of IL-12 or IL-10, respectively. Our data revealed that ZA treatment impairs M2- macrophages polarization, while it is ineffective on M1-macrophages polarization. As a consequence, ZA-treated M2-polarized macrophages lose their ability to foster the motility of prostate cancer cells. We also investigated the effect of ZA on fibroblasts activation and found that this molecule is able to reduce stromal fibroblasts reactivity, as well as their ability to elicit EMT in prostate carcinoma cells. Finally, we demonstrated that CAFs treated with ZA lack their ability to protect prostate cancer cells to docetaxel toxicity.

Conclusion: Our data suggest that the benefit of ZA in the therapy of prostate carcinoma patients, potentially goes beyond the simple skeletal/bone symptoms treatment, but is enlarged to regulation of stromal inflammatory events.

V 10
IDENTIFICATION OF STRUCTURAL FEATURES IN THREE CHEMOKINE RECEPTORS, CXCR3, CXCR4 AND CXCR7 INVOLVED IN INFLAMMATORY TUMOR MICROENVIRONMENT

Raffaele Raucci [1] - Giovanni Colonna [1] - Giuseppe Castello [2] - Susan Costantini [2]

Dipartimento di Biochimica, Biofisica e Patologia Generale, Seconda Università degli Studi di Napoli, Napoli, Italy [1] - Istituto Nazionale Per Lo Studio E La Cura Dei Tumori "Fondazione Giovanni Pascale", IRCCS, Napoli, Italy [2]

Introduction: Chemokine receptor trio composed by CXCR3, CXCR4 and CXCR7 represents a hard and interesting challenge for cancer biology because these three receptors are found to be over-expressed in different cancers as well as to bind the same chemokines. In fact, CXCR4 interacts with CXCL12, CXCR7 not only with CXCL12 but also with CXCL11, that is a natural ligand for CXCR3. For these reasons, it seems necessary to define and to identify the structural determinants of CXCR3, CXCR4 and CXCR7 and their related physic-chemical properties that permit them to bind CXCL11 and CXCL12.

Material and Methods: We performed (i) the modeling of CXCR7 and CXCR3 by a comparative modeling strategy using as template the structure of human CXCR4 published recently and that of bovine rhodopsin, and (ii) that of the complexes between CXCR7 and two ligands, CXCL11 and CXCL12, compared to CXCR3/CXCL11 and CXCR4/CXCL12 by docking methods. COCOMAPS program was used to identify the amino acids at the interface and to evaluate their solvent accessibility, the presence of putative H-bonds and contact maps.

Results: Our data showed that CXCR3, CXCR4 and CXCR7 present similar trans-membrane helices and different conformations of N-terminal and C-terminal regions as well as of three extracellular loops. Two complexes, CXCR7/CXCL11 and CXCR7/CXCL12, were modeled and compared with CXCR3/CXCL11 and CXCR4/CXCL12 to assess structural similarities and/or differences between the determinants through which these receptors bind to the same chemokines. This analysis showed that the driving force of receptor–chemokine interaction was hydrophobic but electrostatic interactions also played a substantial role as evidenced by the presence of several H-bonds and salt bridges. Moreover, the analysis of the complexes evidenced that CXCL12 binds CXCR7 with higher affinity than with CXCR4 in agreement with a recent published paper.

Conclusions: Our computational strategy is able to model correctly the interactions between these chemokines and their receptors. Therefore, our chemokine/receptor complexes show in a reliable way not only the structural determinants of these interactions but also their physico-chemical features, highlighting the interacting residues involved into the formation of complexes. Hence, in absence of better alternatives, our models can be reasonably used for designing peptides able to block at the same time CXCR3, CXCR4 and CXCR7, over-expressed in different cancers.

V 11
OVEREXPRESSION OF IL6 AND RANTES CONFERS A MORE AGGRESSIVE PHENOTYPE TO MCF7 BREAST CANCER CELLS

Marianna Gallo [1] - Daniela Frezzetti [1] - Nicola Normanno [1] - Antonella De Luca [1]

Istituto Nazionale Tumori "Fondazione G. Pascale"-IRCCS, Cell Biology and Biotherapy Unit, Naples, Italy [1]

Introduction: The interaction between mesenchymal stem cells (MSCs) and cancer cells plays an important role in promoting breast cancer progression. In this regard, MSCs greatly increase the metastatic potential of breast cancer cells through the secretion of the chemokine CCL5/RANTES. In addition, recombinant RANTES cooperate with recombinant interleukin-6 (IL6) in enhancing breast cancer cell migration. The aim of this study is to analyze whether the overexpression of IL6 and RANTES induces a more aggressive phenotype in breast cancer cells.

Methods: MCF-7 breast carcinoma cells were transfected with expression vectors coding for human IL6 and RANTES and selected with appropriate antibiotics. Levels of IL6 and RANTES secretion were measured using XMAP Bio-Plex Cytokine arrays. Growth in soft agar was assessed using anchorage-independent growth assays. Migration and invasion were evaluated using commercially available kits. To evaluate tumor growth *in vivo*, transfected cells were orthotopically injected in the mammary fat pad of nude mice.

Results: MCF7 breast cancer cells that stably overexpress both IL6 and RANTES were isolated. Stable transfectants secreted amounts of IL6 and RANTES higher as compared with control cells transfected with mock vectors. To evaluate whether IL6 and RANTES expression led to a more aggressive phenotype in breast cancer cells, we analyzed breast cancer cells proliferation, migration and invasion. Cells overexpressing IL6+RANTES showed a greater ability to form colonies in soft agar, compared with control cells. In addition, IL6+RANTES clones showed a significantly higher ability to migrate through a fibronectin-coated membrane and to invade through a matrigel-coated matrix. Clones overexpressing IL6+RANTES had an increased and persistent phosphorylation of STAT3, and an increase in the phosphorylation of ERK and AKT, compared to control clones. Finally, IL6+RANTES transduced cells showed a significant increase in *in vivo* tumor growth, compared with control cells, when clones were injected in the mammary fat pad of nude mice.

Conclusions: Taken together, our data suggest that the overexpression of IL6 and RANTES produce a more aggressive phenotype in breast cancer cells. This observation might be useful to individuate potential targets for novel therapeutic strategies aimed to block the tumor-stroma interaction.

V 12
OPPOSITE ROLES OF IL-27 AND IL-30 IN THE PROSTATE CANCER MICROENVIRONMENT

Silvia Esposito [1] - Maria Grazia Tupone [1] - Marco Vincenzo Russo [1] - Irma Airoldi [2] - Serena Di Meo [1] - Alessia Zorzoli [2] - Giulia Barbarito [2] - Carlo Sorrentino [1] - Emma Di Carlo [1]

Department of Medicine and Sciences of Aging, 'G. d'Annunzio' University, Chieti, Italy [1] - Laboratory of Oncology, Istituto Giannina Gaslini, Genova, Italy [2]

Introduction: Prostate cancer (PCa) is a leading cause of cancer-related death in men worldwide. Understanding of immune-regulatory signals skewing PCa toward dormancy or progression is of crucial importance to identify novel targets of- or tools for- therapies.

The interleukin (IL)-27 has revealed potent anti-tumor activity in a variety of tumors, but its effects on PCa is still unclear. The IL-27 cytokine subunit p28, namely IL-30, has been recognized as a novel immune-regulatory molecule, but its role in cancer biology is completely unexplored.

We investigated the roles of both cytokines in PCa microenvironment and analyzed their clinical implications.

Materials and methods: IL-30 and IL-27 regulation of hPCa cell viability and expression of selected gene clusters was tested by flow cytometry and PCR array. Expression of both cytokines and their receptors were assessed by immunohistochemistry and RT-PCR on prostate and draining lymph nodes from PCa patients and correlated with clinic-pathologic data.

Results: *In vitro,* IL-30 stimulated proliferation of hPCa cells and down-regulated their expression of CCL16, LIGHT, CKLF. IL-30 consistently down-regulated the tumor suppressor and androgen co-repressor CMTM3, and up-regulated chemerinR23. In the prostate and regional lymph node tissues from PCa patients, expression of IL-30 by CD68+, CD33+/CD11b+ and CD14+ leukocytes was associated with high-grade and metastatic stage of PCa.

In vitro, IL-27 inhibited proliferation and reduced the angiogenic potential of hPCa cells by down-regulating FLT1, COX-1, FGFR3 and up-regulating CXCL10 and TIMP3. *In vivo,* IL-27 reduced proliferation and vascularization, and promoted ischemic necrosis, of tumors developed after hPCa cell injection in nude mice. In patients' prostate tissues, IL-27 was undetectable, while IL- 27R was expressed by normal epithelia and low grade PCa and lost by high tumor grade and stages. IL-27R was also expressed by CD11c+, CD4+ and CD8+ leukocytes infiltrating PCa and draining lymph nodes.

Conclusions: IL-30 supports PCa cell growth and its expression in PCa tissue is associated with advanced disease grade and stage. IL-30 may thus constitute a valuable target for modern therapeutic approaches to PCa progression. By contrast, IL-27 exerts direct anti-proliferative and anti-angiogenic effects on PCa cells, in addition to its immune-stimulatory effects, and may be of great value to rescue from prostatectomy patients with low grade, organ confined PCa.

V 13
ARYL HYDROCARBON RECEPTOR CONTROL OF ENDOTOXIN TOLERANCE PATHWAY

Marco Gargaro [1] - Alban Bessede [2] - Maria Teresa Pallotta [1] - Davide Matino [1] - Giuseppe Servillo [1] - Cinzia Brunaccui [1] - Antonio Macchiarulo [1] - Carmine Vacca [1] - Rossana Iannitti [1] - Claudia Volpi [1] - Ciriana Orabona [1] - Roberta Bianchi [1] - Luigina Romani [1] - Ursula Grohmann [1] - Paolo Puccetti [1] - Francesca Fallarino [1]

University of Perugia, University, Perugia, Italy [1] - Immunosmol, None, Bordeaux, France [2]

Introduction: Chronic inflammation may be a causative factor in a variety of various tumors. Analysis of tolerance pathways, including metabolic pathways, could provide new approaches for treating inflammatory diseases including tumors. Typically, an initial exposure to bacterial lipopolysaccharide (LPS) induces a state of refractoriness to further LPS challenge ("endotoxin tolerance").

Materials and methods : We investigated the susceptibility toendotoxemia and the establishment of endotoxin tolerance in C57BL/6 WT, Ido1$^{-/-}$, Ido2$^{-/-}$, Tdo2$^{-/-}$, and Ahr$^{-/-}$ mice challenged with various doses of LPS.

Results: We found that a first exposure to LPS activated the ligand-operated transcription factor aryl hydrocarbon receptor (AhR) and the hepatic enzyme tryptophan 2,3-dioxygenase 2, which provided an activating ligand to the former, to downregulate early inflammatory gene expression. However, on LPS rechallenge, AhR engaged in the long-term regulation of systemic inflammation only in the presence of indoleamine 2,3-dioxygenase 1 (IDO1). AhR complex-associated Src kinase activity promoted IDO1 phosphorylation and signaling ability, resulting in the onset of a durable endotoxin-tolerant state. This fully endotoxin-tolerant state, requiring AhR, IDO1, and transforming growth factor-beta and was found to increase host fitness and protect mice against immunopathology in gram-negative and gram-positive infections.

Conclusions: Interfering with the trade-offs that the defense systems in both pathogens and their hosts impose on host fitness could ultimately help to selectively enhance pathways responsible for strong and rapid inflammatory responses that lead to effective pathogen clearance, yet do not involve immunopathology. Our current data may pave the way to new therapeutic options involving tryptophan catabolism in the control of immune responses against tumor cells.

V 14
THE RELATIONSHIP BETWEEN NLRP3 INFLAMMASOME AND P2X7 RECEPTOR IN IMMUNE AND TUMOR CELLS

Alessia Franceschini [1] - Marina Capece [1] - Paola Chiozzi [1] - Francesco Di Virgilio [1]

Department of Morphology, Surgery and Experimental Medicine. Section of Experimental pathology, Oncology and Biology, University of Ferrara, Ferrara, Italy [1]

Introduction: The NLRP3 inflammasome (composed by NLRP3 protein, the adapter protein ASC, and pro-caspase 1) is

a caspase-activating complex, responsible for the activation and release of the IL-1β and IL-18 pro-inflammatory cytokines [1]. NLRP3 inflammasome is constitutively expressed in immune cells and implicated in different cancer types [2, 3]. A wide range of stimuli associated with tumor progression activate NLRP3 inflammasome complex, in particular ATP and the decrease in K^+ intracellular concentration [4, 5]. The P2X7 receptor (P2X7R), an extracellular ATP receptor mainly expressed in immune and tumor cells, is a growth-promoting tumor factor [6] and an initiator of inflammasome cascade [2, 4]. However, the P2X7R-NLRP3 relationship and the mechanism of P2X7R-dependent inflammasome activation remain unclear. Thus, we characterized the P2X7R-NLRP3 protein expression correlation and interaction in different cells models, including a tumor cell line.

Materials and methods : NLRP3 and P2X7R mRNA and protein relative levels were evaluated microglial cells (N13) and in the B16 melanoma cell line. Direct interaction between NLRP3 and P2X7R was investigated by co-immunoprecipitation experiments and confocal microscopy after stimulation with LPS or specific P2X7 receptor agonist application (ATP and BzATP).

Results: P2X7R and NLRP3 expression is directly correlated as down-modulation of P2X7R causes NLRP3 down-modulation. Vice versa, stimulation with inflammatory stimuli causes over-expression of both the molecules. Furthermore, P2X7R and NLRP3 are co-immunoprecipitated and co-localized.

Conclusions: Our data show a direct correlation and interaction between NLRP3 and P2X7R. Possible implication for inflammasome activation in healthy and cancer cells will be discussed.

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V 15
INFLAMMATORY CELLS IN NEUROBLASTOMA AND THEIR IMPACT ON CLINICAL OUTCOME

Doriana Fruci [1] - Marco Mina [2] - Arianna Citti [3] - Anna Alisi [1] - Paolo Romania [1] - Maretta De Ioris [1] - Aurora Castellano [1] - Franco Locatelli [1] - Cesare Furlanello [2] - Renata Boldrini [3]

Paediatric Haematology/Oncology Department, Ospedale Pediatrico Bambino Gesù, Rome, Italy [1] - FBK-MPBA Research Unit, Fondazione Bruno Kessler, Trento, Italy [2] - Pathology Department, Ospedale Pediatrico Bambino Gesù, Rome, Italy [3]

Neuroblastomas (NB) grow within an intricate network of different cell types including endothelial venule cells, stromal cells and infiltrating immune cells. Despite T cell immune infiltrates is one of the most important predictive criteria for patient survival in many cancers, the complex interactions between NB and its microenvironment remain to be elucidated. Herein, we examined composition, density, architecture and functional organization of specific intratumoral immune cell populations within 84 NB lesions representing all genetic subsets and we correlated them with clinical outcome.

The density of immune cells was blinded recorded in nests and the surrounded fibrovascular septa. Intratumoral proliferating $CD3^+$ T cells were visualized by ki67 co-staining and quantitative imaging analysis was performed.

Patients with low densities of immune T cells had a poor NB prognosis similar to that of high-risk NB patients. Furthermore, stage 4S and stage 4 NB lesions displayed different T cell immune landscapes being the first characterized by proliferating T lymphocytes that come into close contact with NB cells, while the latter displaying only non-proliferating T cells far away from tumor cells. Consistent with their role in suppressing tumor progression, a high density of intratumoral proliferating T cells in stage 4S NB may explain the prognostic impact on tumor development or progression.

This study reveals that the type, density and location of T immune cells within the tumor and surrounding fibrovascular septa significantly correlates with overall survival of NB patients suggesting that the T cell immune landscape in NB is a hallmark of the tumour microenviroment associated with tumor progression and prognosis.

V 16
THE METABOLIC COOPERATION BETWEEN PROSTATE CARCINOMA CELLS AND CANCER ASSOCIATED FIBROBLASTS: PYRUVATE KINASE M2 AT THE CROSSROADS

Elisa Giannoni [1] - Maria Letizia Taddei [1] - Andrea Morandi [1] - Giuseppina Comito [1] - Paola Chiarugi [1]

Department of Experimental and Clinical Biomedical Sciences, University of Florence, Florence, Italy [1]

Introduction: The ability of cancer cells to invade and metastasize is influenced by the surrounding tumor microenvironment. It is established that cancer associated fibroblasts (CAFs) can promote tumor progression by enhancing cancer cell invasiveness and stemness. In addition the reciprocal interaction between CAFs and prostate cancer (PCa) cells induce their metabolic reprogramming. Interestingly, upon tumor-stroma interaction, CAFs undergo Warburg metabolism (i.e. increased glucose consumption and lactate extrusion), while PCa cells undergo a "reverse Warburg metabolism", endowing cancer cells to reactivate OXPHOS and exploit CAF-derived lactate to drive anabolic pathways, thereby supporting cell growth.

Material and method: Prostate carcinoma cell lines (PC3, DU145) and prostate fibroblasts isolated from aggressive prostate carcinomas (CAFs) were used. Motility and metabolism of PCa cells was evaluated by invasion assays as well as western blot analysis. Radioactive assays were used to quantify glucose and lactate uptake and to evaluate mitochondrial respiration.

Results: We demonstrate that the metabolic reprogramming of PCa cells is strictly dependent on a CAF-mediated inactivation of the M2 isoform of the pyruvate kinase (PK-M2), an enzyme largely expressed by cancer cells. We observed that CAFs induce in PCa cells (i) PK-M2 phosphorylation mediated by Src

kinase and (ii) PK-M2 oxidation mediated by the CAF-induced pro-oxidant environment. These events lead to PK-M2 inactivation, granting for its nuclear migration and association with hypoxia-inducible factor (HIF-1). In turn, the complex PK-M2/HIF-1 recruits the transcriptional repressor Differentially Expressed in Chondrocytes-1 (DEC-1), which promotes the down-regulation of miR-205, a mandatory event for the execution of epithelial-mesenchymal transition (EMT). Treatment of PCa cells with DASA-58 (a chemical activator of PK-M2) or Metformin (an inhibitor of mitochondrial respiratory chain complex I) interferes with PK-M2 nuclear translocation and association with HIF-1/DEC-1, ultimately abrogating the EMT and the pro-invasive spur in PCa cells, as well as their "reverse Warburg metabolism".

Conclusion: Our data suggest an intriguing role for PK-M2 in coupling the motile and the metabolic programs, proposing a direct connection between the EMT program and the metabolic switch. Finally, targeting PK-M2 could be a potential therapeutic strategy that allows to simultaneously impair the motile and the metabolic advantages of cancer cells.

V 17
STABLE MODULATION OF THE GLYCOLYTIC PHENOTYPE OF TUMOR CELLS BY ANTI-VEGF THERAPY

Matteo Curtarello [(1)] - Elisabetta Zulato [(1)] - Giorgia Nardo [(1)] - Silvia Valtorta [(2)] - Giulia Guzzo [(3)] - Elisabetta Rossi [(4)] - Giovanni Esposito [(1)] - Aichi Msaki [(1)] - Andrea Rasola [(3)] - Luca Persano [(5)] - Francesco Ciccarese [(1)] - Roberta Bertorelle [(1)] - Sergio Todde [(2)] - Mario Plebani [(6)] - Henrike Schroer [(7)] - Stefan Walenta [(7)] - Wolfgang Mueller-klieser [(7)] - Alberto Amadori [(4)] - Rosa Maria Moresco [(2)] - Stefano Indraccolo [(1)]

Istituto Oncologico Veneto, IRCCS, Padova, Italy [(1)] - Nuclear Medicine Department, San Raffaele Scientific Institute, Milano, - [(2)] - Department of Biomedical Sciences, University of Padova, Padova, - [(3)] - Department of Surgery, Oncology and Gastroenterology, University of Padova, Padova, - [(4)] - Department of Woman and Child Health, University of Padova, Padova, - [(5)] - Department of Laboratory Medicine, University of Padova, Padova, - [(6)] - Institute of Physiology and Pathophysiology, University Medical Center of the Johannes Gutenberg University Mainz, Mainz, Germany [(7)]

Introduction: Anti-VEGF therapy has been shown to cause metabolic perturbations in tumors, including severe impairment of glucose and ATP levels. We investigated therapeutic effects of anti-VEGF therapy in experimental tumors with different glycolytic phenotypes as well as the possible modulation of metabolic features of tumor cells by anti-VEGF therapy.

Methods: Tumor xenografts and spontaneous tumors were treated with anti-VEGF antibodies. *In situ* markers, induced metabolic bioluminescence imaging and extracellular flux analysis were used to characterize metabolic changes in tumors treated with anti-VEGF therapy or *ex vivo* cultures of tumor cells. [^{18}F]FLT and [^{18}F]FAZA PET were used to track proliferation and hypoxia in tumor xenografts, respectively.

Results: Protracted anti-VEGF therapy induced both vascular regression and necrosis in tumor xenografts; however, highly glycolytic tumors became more rapidly resistant than poorly glycolytic tumors to anti-angiogenic therapy. By PET imaging, tumors chronically treated with anti-VEGF therapy were associated with increased hypoxic and highly proliferative tumor areas. We also observed that protracted anti-VEGF therapy selects for highly glycolytic cells and that this metabolic switch is stable and associated with increased tumor aggressiveness and resistance to VEGF blockade in serially transplanted mice.

Conclusions: These results support the hypothesis that in xenograft models the highly glycolytic phenotype of tumor cells - either primary or secondary - is a cell-autonomous trait which confers resistance to VEGF blockade. Moreover, the observation that some metabolic traits of tumors can be stably modulated by anti-angiogenic therapy suggests evolutionary dynamics of tumor metabolism.

V 18
GYNECOLOGYCAL SARCOMA PROGRESSION AND METASTASIS DEPEND UPON TUMOUR ANGIOGENESIS

Stefano Caraffi [(1)] - Domenico Corradi [(1)] - Carlotta Alias [(2)] - Laura Rocchi [(1)] - Nicoletta Campanini [(1)] - Enrico Maria Silini [(1)] - Roberto Perris [(3)] - Domenica Mangieri [(1)]

Section for Histology and Surgical Pathology, University Hospital, Parma, Italy [(1)] - Department of Life Sciences, University of Parma, Parma, Italy [(2)] - Centre for Molecular and Translational Oncology, University of Parma, Parma, Italy [(3)]

Gynecologycal Soft-Tissue Sarcomas (gSTS) account for a small group of tumours of mesenchymal origin for which there are limited therapeutic interventions and often a poor clinical outcome. In recent years, novel cellular and molecular features of tumour angiogenesis, including key modulators of endothelial cells and pericytes, have been identified as useful targets for anti-angiogenic therapeutic approaches, but it remains unknown whether these can be exploited in the context of angiogenesis in gSTS. We have therefore focused our attention upon the biological relevance of angiogenesis in these tumours by adopting a dual *in vitro-in vivo* strategy combined with *in situ* immunomorphological assessments. By employing human gSTS cell lines and human microvessel-derived cells (HMC; primary endothelial cells and pericytes), and dedicated *in vitro* assays, we find that cancer cells strongly affect the behaviour of vascular cells by modulating their proliferation rates and by enhancing their capacity to organize into capillary-like structures in 3D settings. Coincidently, we observe that HMC, in turn, act on the proliferation and migration of the tested cancer cells, suggesting that a reciprocal angiocrine interaction acts on these cellular compartments. The molecular players involved in this phenomenon are being examined by using a dedicated transwell coculture system and by multiplex immunoassay of culture medium, in which we find a marked release of angiogenic factors. Consistently, gSTS cell lines inoculated onto chick embryo chorioallantoic membranes (CAM) triggered neovascularization, show enhanced ability to intra- and extravasate and give rise to intra-embryonic metastases. In parallel, using TMAs and single gSTS specimens and immune-localization of endothelial and pericyte markers, we have also addressed the main morphological and cellular features of the neovascularization occurring within STS lesions in situ, given particular attention to the correlation between microvascular density MVD and phenotypic traits of the associated pericytes. We propose that variations in

pericyte coverage and phenotype are involved in the pronounced angiogenesis in gSTS lesions and these features correlate with cancer cell growth and spreading. A better understanding of the pericyte behaviour during tumour angiogenesis in gSTS could offer new insights into the progression of these malignancies path novel avenues for the design of therapeutic strategies.

V 19

SERUM TRYPTASE, MAST CELLS POSITIVE TO TRYPTASE AND MICROVASCULAR DENSITY EVALUATION IN EARLY BREAST CANCER PATIENTS: POSSIBLE TRANSLATIONAL SIGNIFICANCE

Ilaria Marech [1] - Michele Ammendola [2] - Rosario Sacco [2] - Gennaro Capriuolo [3] - Rosa Patruno [3] - Rosangela Rubini [3] - Maria Luposella [4] - Valeria Zuccalà [5] - Eufemia Savino [6] - Nicola Zizzo [7] - Cosmo Damiano Gadaleta [1] - Domenico Ribatti [8] - Girolamo Ranieri [1]

Interventional Radiology Unit with Integrated Section of Translational Medical Oncology, National Cancer Research Centre Istituto Tumori "Giovanni Paolo II", Bari, Italy [1] - Department of Medical and Surgery Science, University of Catanzaro "Magna Graecia" Medical School, Catanzaro, Italy [2] - Department of Prevention and Health, ASL BAT, Bari, Italy [3] - Department of Cardiology, University of Catanzaro "Magna Graecia" Medical School, Catanzaro, Italy [4] - Department of Medical and Surgery Science, Pathology Unit, University of Catanzaro "Magna Graecia" Medical School, Catanzaro, Italy [5] - Department of Experimental Oncology, Clinical Pathology Laboratory, National Cancer Research Centre Istituto Tumori "Giovanni Paolo II", Bari, Italy [6] - Chair of Pathology, Veterinary Medical School, University of Bari, Bari, Italy [7] - Department of Basic Medical Sciences, Neurosciences and Sensory Organs, Section of Human Anatomy and Histology, University of Bari Medical School, Bari, Italy [8]

Tryptase is a serin protease stored and released from mast cells (MCs) that plays a role in tumor angiogenesis. In this study we aimed to evaluate serum tryptase levels in 105 female early breast cancer patients before (STLBS) and after (STLAS) radical surgical resection, mast cell density positive to tryptase (MCDPT) and microvascular density (MVD) in primary tumor tissue. Tryptase levels were assessed before and after surgery using the UniCAP Tryptase Fluoroenzyme immunoassay. Tumor sections were immunostained with a primary anti-tryptase antibody and an anti-CD-34 antibody by means of immunohistochemistry and than evaluated by image analysis system. The mean ± 1 standard deviation STLBS and STLAS was 7,18 ± 2,63 μg/L, and 5,13 ± 2,21 respectively and a significant difference between mean levels was found (p=0.0001) by student t-test. The mean ± s.d. of MCDPT and MVD was 8.13 ± 3.28 and 29.16 ± 7.39 respectively. A strong correlation between STLBS and MVD (r=0.81, p=0.0001); STLBS and MCDPT (r=0.69, p=0.003); and MCDPT and MVD (r=0.77; p=0.0001) was found. Results demonstrated higher STLBS in BC patients, indicating an involvement of MC tryptase in BC angiogenesis. Data also indicated lower STLAS, suggesting the release of tryptase from tumor-infiltrating MCs. Therefore, serum tryptase levels may play a role as a novel surrogate angiogenic marker predictive of response to radical surgery in BC patients. In this patients setting, it's intriguing to hypothesize that tryptase inhibitors, such as gabexate and nafamostat mesilate, might be evaluated in adjuvant clinical trials.

V 20

EVALUATION OF THE INTRALESIONAL NEOVESSEL DESTABILIZING EFFECT OF BEVACIZUMAB TREATMENT OF SOFT-TISSUE SARCOMAS SUGGESTS THAT THE DRUG DOES NOT ACT AS A DIRECT "ANGIOGENIC SPROUT DISRUPTER"

Pier Andrea Nicolosi [1] - Domenica Mangieri [2] - Elena Garusi [3] - Maurizio Mongiat [1] - Daniela Zanocco [1] - Stefano Caraffi [2] - Elena Ferolla [4] - Stefano Marastoni [1] - Sergio Frustaci [5] - Roberto Perris [6]

S.O.S. for Experimental Oncology 2, CRO National Cancer Institute Aviano, Pordenone, Italy;, CRO National Cancer Institute Aviano, Aviano, Italy [1] - Section for Anatomic Pathology, University Hospital Parma, Italy;, University Hospital of Parma, Parma, Italy [2] - Department of Life Sciences, University of Parma, Parma, Italy [3] - Department of Laboratory Medicine, Division of Pathology, Karolinska Institutet, Stockholm, Sweden, Karolinska Institutet, Stockholm, Sweden [4] - Division of Medical Oncology B, CRO National Cancer Institute Aviano, Aviano, Italy [5] - Centre for Molecular and Translational Oncology, University of Parma, Parma, Italy [6]

The poor efficacy of Bevacizumab as anti-neoplastic agent in mono-drug therapeutic approaches poses some doubts about its actual effectiveness as an angiogenesis inhibitor for which it was originally developed. Indeed, while there is presently no incontrovertible evidence that Bevacizumab may alone inhibit angiogenic sprouting in patients with any human cancer, its reported utility in combination therapies incites the extension of Bevacizumab to the treatment of multiple tumour types, including soft-tissue sarcomas (STS) for which there is still a scanty therapeutic success rate and no registered new generation drug compound. Concordantly, to acquire pre-clinical information on the anti-tumour activity of Bevacizumab in STS we have re-examined its effects on tumour cell growth in vitro and in vivo. The antibody inhibits VEGF-induced STS cell proliferation, but does not affect cell survival, apoptotic rates, or VEGF-promoted chemotaxis. Treatment of STS nude mice xenografts at time of detection of palpable tumour masses does not interfere with their progressive growth. Conversely, continuous administration of Bevacizumab initiated at the time of implantation of STS cells markedly reduced the size of the forming tumour masses. Assessment of neovessel density and intra-tumoral vascular network arrangements in Bevacizumab-treated and non-treated animals reveals no significant differences, after normalization to the volume of the lesion, suggesting that the Bevacizumab-induced arrest of tumour growth was not due to a blockade of neovessel formation. However, closer comparative examination of the intra-lesional vessels in Bevacizumab-treated animals showed fewer and more immature pericytes, suggesting a preferential destabilization of the neovascular structures. These findings add to the re-definition of the "anti-angiogenic" effect of Bevacizumab and provide a strong rational for the hitherto experimentally corroborated value of the antibody in agonizing cytostatic drug activity.

V 21
THROMBOSPONDIN-1 TS3R DOMAIN POTENTIATES RESPONSE TO CHEMOTHERAPY OF HUMAN OVARIAN CARCINOMA BY INCREASING DRUG DELIVERY INTO TUMORS

Denise Pinessi [1] - Andrea Resovi [1] - Lavinia Morosi [2] - Patrizia Borsotti [1] - Raffaella Giavazzi [1] - Massimo Zucchetti [2] - Giulia Taraboletti [1]

IRCCS - Istituto di Ricerche Farmacologiche Mario Negri, Tumor Angiogenesis Unit, Laboratory of Biology and Therapy of Metastasis, Bergamo, Italy [1] - IRCCS - Istituto di Ricerche Farmacologiche Mario Negri, Clinical Cancer Pharmacology Unit, Laboratory of Cancer Pharmacology, Milano, Italy [2]

Antiangiogenic therapy, aimed at preventing blood vessels formation, is most effective in combination with chemotherapy. There is emerging evidence that antiangiogenic agents can transiently 'normalize' the abnormal tumor vasculature, improving delivery and efficacy of systemically administered cytotoxic therapies.

Thrombospondin-1 (TSP-1) is a major endogenous inhibitor of angiogenesis, proposed as a model for the design of antiangiogenic therapies. Our previous studies identified a new FGF-2-binding, antiangiogenic site in the 'type III repeats' domain (TS3R) of TSP-1. Aim of this study was to investigate the effect of TS3R on tumor response to chemotherapy.

We used a human ovarian carcinoma cell line transfected with either the entire TS3R domain or a truncated form lacking the FGF-2 binding site. Expression of the TS3R domain inhibited the angiogenic activity of the tumor cells, as shown by the reduced ability of the TS3R-expressing tumor cells to induce proliferation and migration of endothelial cells, in vitro. When implanted in vivo, into nude mice, tumor cells expressing TS3R had a decreased tumorigenicity compared with the control cells and showed a greater sensitivity to paclitaxel (PTX 10 mg/kg) or cisplatin (4 mg/kg), standard-of-care drugs for ovarian carcinoma.

Preliminary histological analysis showed that tumors expressing TS3R presented a reorganization of the tumor vasculature consistent with the process of vascular normalization: increased vascular pericyte coverage (α-SMA/CD31) and decreased vessel lumen diameter, compared to controls. Pharmacokinetic analysis coupled to MALDI imaging mass spectrometry analysis of tumors collected 4h after a single administration of PTX (60 mg/Kg), showed an improved delivery of PTX in tumor expressing TS3R. The finding that the TS3R domain of TSP-1 delays tumor growth and increases tumor response to chemotherapy by improving drug delivery may lay the basis for the design of new antiantiangiogenic therapies based on TSP-1.

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V 22
LENALIDOMIDE NORMALIZES TUMOR VESSELS IN COLORECTAL CANCER IMPROVING CHEMOTHERAPY ACTIVITY

Valeria Leuci [1] - Federica Maione [1] - Ramona Rotolo [2] - Enrico Giraudo [2] - Francesco Sassi [1] - Giorgia Migliardi [1] - Maja Todorovic [1] - Loretta Gammaitoni [1] - Giulia Mesiano [1] - Lidia Giraudo [2] - Paolo Luraghi [1] - Francesco Leone [3] - Federico Bussolino [2] - Massimo Aglietta [3] - Livio Trusolino [2] - Andrea Bertotti [2] - Dario Sangiolo [2]

Fondazione del Piemonte per l'Oncologia, Istituto di Candiolo – IRCCS, Candiolo, Italy [1] - Istituto di Candiolo – IRCCS, Università di Torino, Dip. di Oncologia, Candiolo, Italy [2] - Istituto di Candiolo – Fondazione del Piemonte per l'Oncologia, IRCCS, Università di Torino, Dip. di Oncologia, Candiolo, Italy [3]

Introduction: Lenalidomide is an oral drug with immunemodulatory and anti-angiogenic activity. It is currently approved in a range of hematologic malignancies but its activity in solid tumors is mostly unexplored.

Our aim was to explore if the anti-angiogenic activity of lenalidomide might induce normalization of tumor neo-vessels eventually enhancing the benefit of conventional chemotherapy.

We tested this hypothesis within a human xenograft model of metastatic colorectal cancer (mCRC).

Materials and methods: A cohort of 32 NOD/SCID mice were subcutaneously implanted with mCRC xenografts derived from surgical biopsy. Mice were divided into 4 groups respectively treated with oral lenalidomide (n=8, 50 mg/Kg/day for 28 days), intraperitoneal 5-Fluorouracile (5FU) (n=8, 20 mg/Kg twice weekly for 3 weeks), combination of lenalidomide and 5FU (n=8, doses as previously indicated) or irrelevant vehicle. Tumor vessel functionality was evaluated by intravital perfusion with lectins HPA. Tumor vessel density was assessed by CD146 expression. Tumor hypoxic areas were evaluated by Hypoxyprobe-1Plus kit. Antitumor activity was evaluated by Ki67 proliferative index and tumor volume at the end of treatment.

Results: Oral treatment with lenalidomide reduced tumor vessel density compared to untreated controls or treated with 5FU ($p=0.005$ and $p=0.03$). Perfusion capability of tumor vessels was enhanced by lenalidomide compared to untreated controls ($p=0.006$) or treated with 5FU ($p=0.02$). Accordingly, lenalidomide reduced tumor hypoxic areas compared to untreated controls ($p=0.02$) or treated with 5FU ($p=0.008$). As consequence, lenalidomide enhanced the antitumor activity of 5FU *in vivo*. Combination of lenalidomide and 5FU delayed tumor growth ($p=0.03$) and more significantly reduced tumor Ki67 index (mean 8% ±2) compared with untreated controls (32% ±2, $p=0.0002$) or treated with 5FU alone (20% ±2, $p=0.01$); lenalidomide alone did not have antitumor activity compared to untreated controls (Ki67 22% ±5 vs 32% ±2, $p=0.2$).

Conclusions: Our data provide first evidence of tumor vessel normalization and hypoxia-reduction induced by lenalidomide in mCRC. Such effect translates into an indirect antitumor activity enhancing the therapeutic index of chemotherapy drugs.

Our findings support the investigation of synergism between lenalidomide and conventional therapies in solid tumors that might all benefit from normalization of tumor vasculature.

V 23
COOPERATION BETWEEN ADENOSINERGIC AND HYPOXIC SIGNALS IN SHAPING CHRONIC LYMPHOCYTIC LEUKEMIA MICROENVIRONMENT

Sara Serra [1] - Roberta Buonincontri [1] - Davide Brusa [1] - Valentina Audrito [1] - Tiziana Vaisitti [1] - Marta Coscia [2] - Luca Laurenti [3] - Giovanni D'Arena [4] - Davide Rossi [5] - Gianluca Gaidano [5] - Simon C. Robson [6] - Silvia Deaglio [1]

Department of Medical Sciences and HuGeF, University of Turin, Turin, Italy [1] - Division of Hematology, AO Città della Salute e della Scienza, Turin, Italy [2] - Institute of Hematology, Catholic University of the Sacred Heart, Rome, Italy [3] - Department of Onco-Hematology, IRCCS Centro di Riferimento Oncologico della Basilicata, Rionero in Vulture, Italy [4] - Division of Hematology, Department of Translational Medicine, Amedeo Avogadro University of Eastern Piedmont, Novara, Italy [5] - Division of Gastroenterology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, United States [6]

Extracellular adenosine (ADO) generated from ATP through the concerted action of the ectoenzymes CD39 and CD73 elicits potent cytoprotective and immunosuppressive effects mediated by type-1 purinergic receptors.

Chronic lymphocytic leukemia (CLL) patients expressing the ectoenzymes CD39 and CD73 can actively produce ADO. This condition activates an autocrine adenosinergic axis that supports engraftment of leukemic cells in a growth-favorable environment. These effects are mediated by the A2A ADO receptor, which inhibits chemotaxis and limits spontaneous and drug-induced apoptosis of CLL cells.

Following the reported cross-talk between hypoxia and ADO, we tested the hypothesis of a functional interplay between the adenosinergic and hypoxic axes in the CLL microenvironment.

Results confirm that the CLL cells robustly increase HIF-1α expression when cultured under hypoxia. Under these conditions a significant increase in the mRNA and protein levels of CD73, CD26 and of A2A was observed. An HPLC assay confirmed that hypoxic CLL cell cultures are characterized by higher extracellular ADO levels, further improved upon inhibition of adenosine deaminase and nucleoside transporters.

Attention was then focused on the stromal compartment, which is critical to the formation and maintenance of the leukemic niche. Here, hypoxia enhanced differentiation of circulating monocytes into nurse-like cells, macrophages of the M2 type, which play an essential role in nurturing leukemic cells. During hypoxic culture, differentiating monocytes up-regulated A2A and A3. Both receptors were overexpressed by NLC under hypoxic conditions. Furthermore, they were functional, as determined by the finding of increased AKT and ERK1/2 phosphorylation following pharmacological activation of the receptors. The enhancement of NLC differentiation under hypoxic conditions relied, at least in part, on the activation of A2A and A3: their engagement using agonists enhanced NLC differentiation in normoxia, with overexpression of IDO, CD163 and CD206. Furthermore, activation of A2A and A3 favored secretion of immunomodulatory cytokines such as IL-10 and IL-6. On the contrary, their pharmacological blockade under hypoxia prevented NLC differentiation.

Together, these results indicate that the adenosinergic and hypoxic axes synergize in shaping the CLL niche, suggesting that the pharmacological inhibition of adenosinergic signals may counteract some of the effects mediated by an hypoxic microenvironment.

V 24
NEUTROPHIL ELASTASE BUT NOT MMPS-DEPENDENT CLEAVAGE COMPROMISES THE TUMOR SUPPRESSOR ROLE OF EMILIN1.

Eliana Pivetta [1] - Teresa Maria Elisa Modica [1] - Bruna Wassermann [1] - Orlando Maiorani [1] - Alfonso Colombatti [1] - Paola Spessotto [1]

CRO-IRCCS, National Cancer Institute, Hospital, Aviano (PN), Italy [1]

Introduction: The microenvironment consists of cells, soluble molecules and extracellular matrix (ECM) constituents. Proteolysis of ECM is a key event in tumor growth and progression. The breakdown of ECM leads to the generation of bioactive fragments that can promote cell growth and spread. EMILIN1 is an ECM glycoprotein expressed in large blood vessels, lymphatic system, intestine, lung, and skin. Beside the functional significance of adhesion and migration as the consequence of the interaction between its functional gC1q domain and α4/α9 integrins, the striking aspect of this ligand/receptor pair is related to proliferation. The signals emitting from EMILIN1 engaged by α4/α9β1 integrins through its gC1q domain are anti proliferative. We have recently demonstrated that among the proteolytic enzymes released in the tumor microenvironment neutrophil elastase was able to cleave EMILIN1. Accordingly, EMILIN1 was digested in sarcomas infiltrated by neutrophils. In this study we investigated the proteolytic function of MMPs for which EMILIN1 could represent a novel potential substrate according to a recent proteomic approach on components of vasculature and compared their activity with elastase.

Materials and Methods: In vitro experiments with purified and recombinant enzymes were performed to test proteolysis on both EMILIN1 and gC1q. The resulting fragments were used in cell cultures to test their regulatory action on proliferation .

Results: We demonstrated that MMP3, MMP9 and MMP14 were able to digest EMILIN1 with an activity lower than elastase proteolytic capacity. Very interestingly, these MMPs were not able to impair gC1q integrity whereas elastase reduced gC1q into two main fragments. Consequently, elastase but not MMP proteolytic process on EMILIN1 resulted in the impairment of its suppressor role on proliferation.

Conclusions: MMP action on EMILIN1, likely contributing to weakening of the vascular wall via their capacity to digest fundamental structural ECM components of elastic fibers, could be determinant in pathological processes such as atherosclerosis. On the other hand, EMILIN1 cleavage by elastase is crucial in the tumor microenvironment since elastase is the only enzyme to display a proteolytic action on gC1q, impairing its structural integrity and its suppressor role on proliferation.

V 25
MUTANT P53 GAINS NEW FUNCTION IN PROMOTING INFLAMMATORY SIGNALS BY REPRESSION OF THE SECRETED INTERLEUKIN-1 RECEPTOR ANTAGONIST

Valentina Ubertini [1] - Silvia Baldari [1] - Daniela Agnese D'arcangelo [2] - Aymone Gurtner [3] - Eleonora Cesareo [2] - Giulia Piaggio [1] - Paola Nisticò [1] - Silvia Soddu [1] - Antonio Facchiano [2] - Gianluca Bossi [4]

Experimental Oncology Laboratories Regina Elena National Cancer Institute, Rome, Italy [1] - Istituto Dermopatico dell'Immacolata IDI-IRCSS, Rome, Italy [2] - Experimental Oncology Laboratories Regina Elena National Cancer Institute, Rome, Italy. [3] - Laboratory of Medical Physics and Expert Systems, Regina Elena National Cancer Institute, Rome, Italy. [4]

Introduction: The tumor suppressor TP53 is one of the most frequently mutated genes in human cancers. The majority of these mutations, located in the DNA binding domain, result in the production of mutated proteins that lose the wild-type functionality but can acquire new oncogenic functions (gain-of-function, GOF). We previously showed that mutant (mut) p53R273H promotes tumor growth, stromal invasion and angiogenesis in xenografted HT-29, suggesting a role of mutp53 in the modulation of the tumor microenvironment.

Materials and methods : Human lines HT-29 (Colon Adenocarcinoma), MDA-MB468 (Breast Adenocarcinoma), HepG2 (Hepatocellular Carcinoma), MCF7 (Breast Carcinoma), HUVEC (Human Umbilical Vein Endothelial Cells). Cytokines arrays, Western blot, Real-Time PCR, RT-PCR, ChIP assays, Luciferase reporter assays, Co-IP, RNAi, Bromodeoxyuridine incorporation assay, Endothelial cell injury assay, *in vivo* assay.

Results: we explored mutp53 roles in modulating cytokines/secretory factors expression. Cytokines arrays performed on conditioned medium derived from a panel of human cancer cell lines showed that endogenous mutp53 depletion increases significantly the secreted form of IL-1RA (sIL-1RA). The sIL-1RA is a naturally occurring anti-inflammatory cytokine that acts as a specific antagonist of IL-1β. Confirmatory analyses of western blot and quantitative PCR performed in cancer cell lines (HT29, MDA-MB468) suggested a transcriptional regulation. Studies of promoter activity and ChIP assays identified specific regulatory region where mutp53 is physically recruited. Moreover, we identified the transcriptional co-factor with repressor activity MafF as significantly recruited on the sIL-1RA promoter together with the mutp53. The mutp53 physically interacts with MafF and MafF endogenous depletion impairs mutp53 recruitment on the sIL-1Ra promoter restoring its activity. Functional studies revealed that mutp53 contributes to maintain a prone inflammatory response by sIL-1Ra downregulation. Indeed, either mutp53 depletion or recombinant sIL-1Ra delivery impairs IL-1b response in vitro and in vivo.

Conclusions: Taken together, these results reveal a novel oncogenic mutp53 GOF activity, exerted by the repression of sIL-1Ra that contributes to generate a pro-inflammatory tumor microenvironment promoting tumor malignancy**.**

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MAST CELL-DERIVED IL-8 INDUCES EPITHELIAL-TO-MESENCHYMAL TRANSITION AND STEM CELL FEATURES IN HUMAN THYROID CANCER CELLS

Carla Visciano [1] - Federica Liotti [1] - Nella Prevete [2] - Gaetano Calì [1] - Renato Franco [3] - Francesca Collina [3] - Amato De Paulis [2] - Gianni Marone [2] - Massimo Santoro [1] - Rosa Marina Melillo [1]

Dipartimento di Medicina Molecolare e Biotecnologie Mediche/Istituto per L'Endocrinologia e l'Oncologia Molecolare, University of Naples, Federico II, Naples, Italy [1] - Dipartimento di Scienze Mediche Traslazionali e Centro Interdipartimentale di Ricerca in Scienze Immunologiche di Base e Cliniche, University of Naples, Federico II, Naples, Italy [2] - Struttura Complessa di Anatomia Patologica, Istituto Nazionale dei Tumori Fondazione G. Pascale, Naples, Italy [3]

Introduction: We recently demonstrated that mast cell (MC) infiltrate of human papillary thyroid carcinomas (PTCs) is increased with respect to normal thyroid. MC density correlates with extrathyroidal extension of PTCs. However, the MC-derived factors and mechanisms responsible for enhanced thyroid cancer (TC) invasiveness remain unidentified. Here, we report that MC-derived IL-8 induces Epithelial-Mesenchymal Transition (EMT) and stemness of TC cells.

Materials and Methods: MC CM was obtained by HMC-1, LAD2 and human primary lung cells. By using cell cultures we assessed EMT (Immune fluorescence, Western blot, real-time PCR, Wound healing assays) and stemness phenotype (Aldefluor, Sphere-formation, Limiting dilution, Self-renewal assays) in thyroid cells (Nthy-ori 3-1, 850-5C and TPC-1). MC CM immune-depletion was performed by immunoprecipitation and verified by ELISA assay. The protumorigenic potential of IL-8 was evaluated by TC cell xenografts in immunocompromised mice. MC density and stemness in human PTCs were evaluated by immunohistochemistry (IHC).

Results: Upon incubation with MC CM, human-immortalized (Nthy-ori 3-1) and cancerous (850-5C and TPC-1) thyroid cells underwent EMT, with the acquisition of fibroblast-like morphology, expression of mesenchimal markers, down-regulation of epithelial features and increased ability to migrate. By MC CM immune-depletion, we found that, among many mediators, IL-8 was absolutely required for EMT induction in TC cells. The IL-8 receptors CXCR1 and CXCR2 are both expressed in TC cells. Recombinant IL-8 stimulation of TC cells caused increased expression of cancer stem cell (CSC) markers, expansion of $ALDH^{high}$ cell fraction, and thyrosphere formation in low-adherence. These features could be reverted by blocking the IL-8/CXCR1/CXCR2 axis. Accordingly, 850-5C engineered to overexpress IL-8 underwent EMT, displayed increased stemness and higher tumorigenic activity with respect to control cells. The analysis of TC surgical specimens by IHC demonstrated a significative association of both MC density (tryptase+ cells) and TC cell stemness features (nuclear OCT4 staining) with tumor size (T). We also found a positive correlation between MC density and stemness in TC.

Conclusions: Our data indicate that tumor-associated MCs produce proinflammatory mediators, including IL-8, that induces EMT and expands CSC population in TC, suggest that targeting IL-8/CXCR1/CXCR2 axis may be employed to directly target CSCs in TC.

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PRO-INFLAMMATORY CYTOKINES AND CHEMOKINES ANALYSIS IN HPV-POSITIVE CANCER CELLS

Maria Simona Zangrillo [1] - Giorgio Mangino [1] - Maria Vincenza Chiantore [2] - Marco Iuliano [1] - Rosita Accardi [3] - Gianna Fiorucci [4] - Massimo Tommasino [3] - Giovanna Romeo [1]

Dept. of Medico-Surgical Sciences and Biotechnologies, Sapienza University of Rome, Latina, Italy [1] - Dept. of Infectious Parasitic and Immuno-mediated Diseases, Istituto Superiore di Sanità, Rome, Italy [2] - Infections and Cancer Biology Group, International Agency for Research on Cancer-WHO, Lyon, France [3] - Institute of Molecular Biology and Pathology, Consiglio Nazionale delle Ricerche, Rome, Italy [4]

Introduction: Recent data have expanded the concept that inflammation is a critical component of tumor progression. Many cancers arise from sites of infection, chronic irritation and inflammation. It is becoming clear that the tumor microenvironment is an indispensable participant in the neoplastic process, fostering proliferation, survival and migration. Tumor cells co-opt some of the signalling molecules of the innate immune system, such as selectins, chemokines and their receptors for invasion, migration and metastasis. Virus-induced tumors, like cervical high risk Papillomavirus-induced Squamous Cell Carcinomas (SCC), represent a paradigmatic example of the tight interplay between inflammatory responses and malignant transformation as inflammation is an integral part of the innate antiviral response.

Materials and methods : To study the tumorigenic role of inflammatory mediators in cutaneous and mucosal HPV^+ cells, we analyzed by real time RT-PCR the expression of selected inflammatory cytokines, chemokines and related molecules (i.e. IL-1α, IL-1β, IL-1RA, IL-1R1, IL-6, IL-8, MCP-1, MIP-3α, RANTES, MIP-1α and MIP-1β,) in HPV^+ SiHa carcinoma cells. We also analysed human foreskin keratinocytes transduced by E6 and E7 from mucosal (HPV-16) or cutaneous (HPV-38) genotypes comparing them to primary Human Foreskin Keratinocytes (HFK).

Results: Our results indicate that IL-1α and IL-1β mRNAs are downregulated K16 and K38 cells, whereas the level of mRNA for IL-1R1 is comparable in all cell lines. Level of IL-6 mRNA is unchanged in K16 and downregulated in K38. Experiments performed by using E6/E7 siRNAs in SiHa, confirm the specificity of these effects. Chemokines mRNA levels in K16 and K38 are also deregulated compared to HFK. Deregulation of MIP-3α mRNA appears to be related to miR-21 over-expression detected in both K16 and K38. Finally, the effect of the antiviral cytokine IFN-β on the levels of these pro-inflammatory mediators will be also discussed.

Conclusions: Our results suggest that HPV is able to modify the tumor microenvironment through the synthesis and release of specific pro-inflammatory cytokines and chemokines. These effects could interfere with the leucocytes trafficking and/or allow a better tumor growth and infiltration.

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SOCIETA ITALIANA DI CANCEROLOGIA

Società Italiana di Cancerologia
Via G. Venezian, 1 - 20133 Milan, Italy
Phone +39 02 2666895 - Fax +39 02 2664342
E-mail: sic@istitutotumori.mi.it
www.cancerologia.it

centro servizi congressuali

C.S.C. S.r.l.
Via L.S. Gualtieri, 11 - 06123 Perugia
Tel+390755730617 - Fax +390755730619
www.csccongressi.it

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