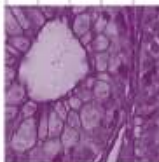# Rapporti
ISTISAN

10/2

Patologie

ISS-NIH collaborative programme
on rare diseases: reports of the projects

Edited by
D. Taruscio and M. Salvatore

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ISTITUTO SUPERIORE DI SANITÀ

ISS-NIH collaborative programme on rare diseases: reports of the projects

Edited by
Domenica Taruscio and Marco Salvatore

Centro Nazionale Malattie Rare

Istituto Superiore di Sanità
ISS-NIH collaborative programme on rare diseases: reports of the projects.
Edited by Domenica Taruscio and Marco Salvatore
2010, viii, 234 p. Rapporti ISTISAN 10/2

The bilateral Italy and USA agreement between the Istituto Superiore di Sanità (ISS) and the National Institutes of Health (NIH) was established with the purpose of developing and increasing research in different fields, rare diseases included. In this context the ISS promoted the "Call for proposals – Rare Disease, 2006" inviting the scientific community to propose projects aimed at widening scientific knowledge on rare diseases. Eighty-two projects were funded within this frame. Projects were mainly focused on three topics, namely aspects of pathogenesis, diagnosis, treatment and clinical management. Since 2007 each scientific project leader is invited to illustrate the main results during the annual International Congress on rare disease and orphan drugs, organized by the ISS. The annual Congress represents an important forum for scientific discussions and an opportunity to establish new collaborations and networks**.** Abstracts have been published in two specific issues of ISTISAN Congressi (07/C8 and 08/C10) edited by the ISS and available online (www.iss.it). The present book contains the reports of all projects, summarizing the main achievements during the 3-year period. The report is divided into three sections: pathogenesis, diagnosis, treatment and clinical management.
Key words: Rare diseases; Orphan drug production; Research

Istituto Superiore di Sanità
Accordo bilaterale ISS-NIH sulle malattie rare: relazioni sulle attività dei progetti.
A cura di Domenica Taruscio e Marco Salvatore
2010, viii, 234 p. Rapporti ISTISAN 10/2 (in inglese)

L'accordo bilaterale fra l'Italia (Istituto Superiore di Sanità, ISS) e gli Stati Uniti (National Institutes of Health, NIH) è stato sancito allo scopo di sviluppare ed incrementare le attività di ricerca scientifica in diversi settori, incluso quello delle malattie rare. In questo contesto, l'ISS ha promosso nel 2006 una specifica "Call for proposals – Rare Disease, 2006" invitando la comunità scientifica a presentare progetti di ricerca rivolti allo studio e all'approfondimento delle malattie rare. In questo contesto sono stati finanziati 82 progetti. Questi erano principalmente focalizzati su tre tematiche rivolte a patogenesi, diagnosi e trattamento e gestione clinica. Dal 2007, ciascun responsabile scientifico è stato invitato a illustrare e discutere, durante l'annuale Congresso internazionale sulle Malattie Rare e Farmaci Orfani, organizzato all'ISS, lo stato d'arte e gli avanzamenti scientifici del progetto. Il Congresso, infatti, è un'importante opportunità per la discussione scientifica e per stabilire nuove reti collaborative. Inoltre, i riassunti dei principali risultati ottenuti in ciascun progetto sono stati pubblicati nei Volumi ISTISAN Congressi (07/C8 e 08/C10) e sono disponibili on line all'indirizzo www.iss.it. Il presente volume contiene le relazioni di attività di tutti i progetti nell'ultimo triennio. Il rapporto tecnico è diviso in tre sezioni principali: patogenesi, diagnosi e trattamento e gestione clinica.
Parole chiave: Malattie rare; Produzione di farmaci orfani; Ricerca

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Il rapporto è accessibile online dal sito di questo Istituto: www.iss.it.

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INTRODUCTION

Rare Diseases (RDs) are defined on the basis of their low prevalence which has been estimated, at a European level, to be no more than 5 affected persons out of 10.000.

However, the low prevalence does not imply that a small number of patients is affected by RDs. In fact, the number of RDs ranges from 7,000 to 8,000; EURORDIS (the European umbrella patients' organization) estimates that about 36 millions of patients are affected by RDs in Europe. More than 80% of RDs is caused by genetic defects while the lasting 20% has a multifactorial origin, with the involvement of genes as well as environmental factors.

Many RDs are apparent at the birth (e.g., rare congenital anomalies, osteogenesi imperfecta) or during post-natal development (e.g., Rett syndrome); some others became evident during adult life (i.e., Huntington disease, Charcot-Marie-Tooth disease, etc.).

The RD issue implies necessarily dealing with orphan drugs. An orphan drug is a product that is potentially useful in treating an RD but which does not have a market sufficient to cover the costs of its development. Since 2000, the European Union regulation supports the development of orphan drugs (EC Reg. 141/2000).

Research and development of new technological tools are therefore fundamental in fostering health interventions towards RDs.

In this context, the bilateral Italy-USA agreement between the Istituto Superiore di Sanità (ISS) and the National Institutes of Health (NIH) was established to develop and increase research in different fields, including RDs.

In 2006 ISS promoted the "Call for proposals – Rare Disease, 2006" inviting the scientific community to propose projects aimed at widening scientific knowledge on RDs. Eighty-two projects were funded; they mainly concern topics such as: models of study for primary, secondary and tertiary prevention of RDs, characterization of conditions without a certain diagnosis, development of new diagnostic and prognostic approaches, experimental models for the development of new treatments and for the evaluation of their safety and efficacy, validation and optimisation of models to evaluate life quality of the patients as well as quality of health services.

Since 2007 during the annual International Congress on RDs and orphan drugs, organized by the ISS, each scientific project leader is invited to illustrate the main results obtained during the year. The annual Congress represents an important forum for scientific discussions and a good opportunity to establish new collaborations. Abstracts of reports presented in the 2007 and 2008 meetings have been published in two books (ISTISAN Congressi 07/C8; ISTISAN Congressi 08/C10) edited by Istituto Superiore di Sanità and available online (www.iss.it).

The present book contains the reports of all projects and summarizes the results achieved during the last 3 years (2007-2009). The report is divided into three main sections, namely aspects of pathogenesis, diagnosis, treatment and clinical management.

In particular, Section 1, dedicated to pathogenesis, is focused on molecular, cytogenetics and clinical characterization of specific syndromes (such as Cornelia de Lange, Noonan, Axenfeld Rieger syndromes, etc.) and to pathophysiological mechanisms, for instance, of neuronal development.

Section 2, dedicated to diagnosis, includes reports on new gene discovery, correlation between genotype and phenotype and development of new tools to diagnose RDs.

Finally, reports on treatment and clinical management, as well as on stem cells and new terapeutical approaches, are included in Section 3.

SECTION 1
Aspects of pathogenesis

CORNELIA DE LANGE SYNDROME: CLINICAL AND MOLECULAR DATA OF A LARGE ITALIAN COHORT

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Introduction

Cornelia de Lange syndrome (CdLS, MIM #122470, #300590, #610759) is a rare multisystem developmental disorder characterized by facial dysmorphisms, growth/mental retardation, hirsutism, and malformations of the upper limbs ranging from small hands to complete reduction defects. Mutations in NIPBL gene (5p13.2) are responsible for about half of CdLS cases while a small percentage of patients shows molecular defects in SMC1A gene (Xp11.22). SMC3 gene has been associated with CdLS, although its impact on the syndrome remains to be defined. The involved genes encode proteins of the cohesin pathway, comprising structural SMC1A and SMC3 cohesin complex subunits or regulatory elements such as NIPBL gene product both acting on multiple target genes. The essential role for the DNA-binding cohesin complex is to control sister chromatid segregation during mitosis and meiosis and facilitate the repair of damaged DNA. Recent evidence has revealed that cohesin binds to the same sites in mammalian genomes as the zing finger transcription factor CTCF suggesting that the developmental deficits of CdLS likely result from dysregulation of gene expression, due to alterations in the cohesin genes.

Methods

We report on the molecular characterization of a clinically heterogeneous sample of 137 Italian CdLS patients with a mean age of 14 years (range 1-42 years). All recruited patients with a clinical diagnosis of CdLS entered a molecular flow-chart which implies in sequence the search for mutations of i) NIPBL, ii) SMC1A (for patients negative to NIPBL mutational scan) and iii) functional candidate genes as PDS5A (for NIPBL/SMC1A-negative patients). Mutational screening of NIPBL/SMC1A/PDS5A is carried on by DHPLC and direct sequencing. In addition the NIPBL mutational scan is refined by using MLPA kit to test NIPBL exonic imbalances. Transcription analysis is performed on carriers of missense, in-frame deletion and splicing mutations.

In all analized patients we collected clinical information regarding their medical history from prenatal period, we defined the presence/absence of major malformations and of medical complications. Moreover we defined their psychomotor and mental development and their global severity using the severity score published by our research group.

Results

Molecular data

The NIPBL mutational screening allowed us to identify 42 point mutations (including 6 missense, 1 in frame deletion, 13 splice-site, 8 nonsense, 13 frameshift and 1 promoter site). The refinement of NIPBL mutational scan led to disclose five large deletions and one duplication encompassing one or several exons. One patient carrying a deletion affecting NIPBL exons 1-10 has been further characterized by FISH analysis and found to bear a 2Mb deletion which includes 14 genes apart NIPBL thus featuring a contiguous gene syndrome.

Sixty-seven out of 89 NIPBL-negative patients negative were tested for mutations in SMC1A gene. Five of them were found to have point mutations, constantly in frame deletion or missense mutations including two novel sporadic cases besides those already described.

A subgroup of 20 patients negative to NIPBL/SMC1A test has been tested for PDS5A, a gene encoding a regulatory element of cohesion complex, yielding so far negative results.

Neonatal phenotype

We analyzed prenatal and neonatal data of 101 CdLS patients. In 23% clinical diagnosis was done in the neonatal period; 75% of them shows a NIPBL mutation. In 52% patients clinical diagnosis was done within the first years of life and in this group NIPBL mutation was evident in 63%. On the contrary in the patients diagnosed after the first year of life NIPBL mutation was evident in only 29%. Intrauterine growth retardation (IUGR) was evident in 58% of them with a percentage of NIPBL mutation of 62%; NIPBL mutation's incidence in patients with no IUGR was 25%. Feeding problems were reported from parents in 71% of patients and 43% needed a nutritional support (enteral nutrition). Within patients with feeding problems the percentage of NIPBL mutation was 56% against 36% of the group without serious difficulties. This difference is confirmed looking at NIPBL mutation incidence in patients who needed support for nutrition (60%) and who did not receive any feeding support (49%).

Major malformations

We collected complete data of 103 CdLS patients. Kidney malformations were evident in 30% of our patients. Calculation of creatinine clearance value showed an abnormal function in 24% of the patients with a renal structural anomaly. NIPBL mutation were detected in 43% of the patient with structural anomalies and in 22% of the patients without malformation. NIPBL mutation was observed in 57% of patients bearing a functional renal defect versus 43% of patients with normal kidney function. Heart malformations were demonstrated in 35% of the evaluated patients, 53% of whom were carriers of NIPBL mutation, conversely patients without heart defects had a NIPBL mutation prevalence of 36%. The more common heart anomalies, isolated or as part of a more complex forms, were pulmonary stenosis (28%), atrial septal defects (17%) and ventricular septal defects (19%). The prevalence of NIPBL mutation in patients with these defects was of 70% for pulmonary stenosis, 50% for interatrial defects and 29% for interventricular defects. Limb reduction defects were evident in 17% of our cohort: 78% of these cases had a NIPBL mutation. A CNS (Central Nervous System) anomaly was observed in 32% of the patients who had an NMR: 33% of them had a NIPBL mutation. Genital anomalies were found in 31% of the patients; the incidence of NIPBL mutations in this subgroup is 52%, whereas it declined to 41% in the patients without genital anomalies. Palate

anomalies were observed in 13% of our patients being 54% the incidence of NIPBL mutations in this group. Major eyes anomalies were observed in 4% of our cohort being 50% the incidence of NIPBL mutations in this group.

Growth and medical complications

Natural history data were available on 100 CdLS patients (average age 14 years). Post natal growth showed weight retardation in 75% of the patients, 54% of them carrying a NIPBL mutation versus 25% detected in the cohort of patient with a normal weight development. Postnatal height was under 3° centile in the 84% of investigated children, 54% of them had a mutation in NIPBL; incidence of NIPBL mutations accounts for 30% in patients with normal height. Microcephaly was evident in 77% of patients; 44% showed a NIPBL mutation, while only the 22% of cases with a normal head circumpherence was associated with a NIPBL anomaly. The most common medical complication was represented by gastro-esophageal rephlux (GER, 73%) with an equal distribution between NIPBL mutated/non mutated cases (48% versus 52%); 17% of the GER positive patients underwent a surgical intervention, NIPBL mutation occurring in 58%. Some behavioural problems seem to be associated to GER: GER was evident in 93% of patients with sleep problems, in 82% of the aggressive patients. Lastly 30% of GER affected patients were described to be hyperactive. Seizures has been observed in the 26% of our cohort (15% related to hyperpyrexia and 85% not related) and among these 30% had a NIPBL mutation. Blepharitis was evident in 12%, eyelids ptosis in 17%, squint in 7%, visual refractive defects in 56%. Between the last group 70% of patients have myopia. The percentage of NIPBL mutation was 43% in the patients showing refractive diseases and 57% in those without visual problems. 70% of CdLS patients with neurosensorial deafness had a NIPBL mutation while only 30% of deaf CdLS children did not show any NIPBL anomaly

Psychomotor development and behaviour

Complete data were available on 94 CdLS patients; the distribution of the severity of psychomotor mental retardation showed that 43% had a profound/severe mental retardation (MR), 25% a moderate MR and 32% a mild MR or a borderline development. NIPBL mutations within these classes were distributed as following: 58% in the first class, 38% in the intermediate class and 30% in the last class

Fourty patients were then formally analyzed at behavioural level with DBC and CARS scale. 32,5% showed behavioural problems (pathological DBC score) while 27,5% had a CARS score in the autistic spectrum. NIPBL mutation was evident in 61% of patients with DBC pathological results and in 64% of patients with pathological CARS score.

Genetic counselling questionnaire

Thirty-eight families filled a specific questionnaire related to comprehension of the results of molecular analysis and impact of the genetic tests on their reproductive choices. The following conclusions were evident: a) there is an apparent discordance between reproductive risk understanding and anxiety about it b) it exists a confidence in genetic test application to define parents reproductive risk, offsprings' reproductive risk and proband's prognosis c) there is an apparent discordance between options of prenatal diagnosis and confidence about them.

Conclusion

Our study allows us to define the correct molecular diagnostic flow-chart to be applied in front of a patients with a clinical diagnosis of CdLS.

The available data on a large cohort of CdLS patients completely characterized at clinical level showed quite interesting correlations between the more frequent gene defect (NIPBL) and different aspects of the CdLS phenotype.

Some of these correlation confirm what is well known in medical literature but other new interesting correlations appear to be evident (positive relation between NIPBL mutation and neonatal diagnosis, more severe feeding problems, pulmonary stenosis, neurosensorial deafness, behavioural problems, autistic like features). Patients with SMC1 mutation are still few to delineate a specific medical or neuro-behavioural phenotype. The research of new candidate gene/s gave at the moment negative results.

Papers published within the project

Basile E, Villa L, Larizza L, Russo S, Gervasini C, Castronovo P, Cerutti M, Cereda A, Passarini A, Borgatti R, Selicorni A. Genotype/behavioural phenotype correlations in Cornelia de Lange Syndrome. In: 58th Annual Meeting of the American Society of Human Genetics. Philadelphia, PA, 11-15 November 2008.

Castronovo P, Gervasini C, Cereda A, Masciadri M, Milani D, Russo S, Selicorni A, Larizza L. Lack of correlation between premature chromatid separation and clinical and genetic heterogeneity in Cornelia de Lange syndrome. *Chromosome Research* 2009;17(6):763-71.

Castronovo P, Gervasini C, Russo S, Masciadri M, Milani D, Cereda A, Selicorni A, Larizza L. Separazione cromatidica prematura nella sindrome di Cornelia de Lange: criticità dell'approccio per l'individuazione di marcatori citologici nelle patologie a trasmissione dominante. In: XI Congresso Nazionale SIGU Genova 24 Novembre 2008.

Cereda A, Passarini A, Milani D, Cerutti M, Maitz S, Castronovo P, Gervasini C, Russo S, Selicorni A. Indicatori prognostici del fenotipo CdLS lieve. In: XI Congresso Nazionale SIGU Genova 24 Novembre 2008.

Gervasini C, Pfundt R, Castronovo P, Russo S, Roversi G, Masciadri M, Milani D, Zampino G, Selicorni A, Schoenmakers EF, Larizza L. Search for genomic imbalances in a cohort of 24 Cornelia de Lange patients negative for mutations in the NIPBL and SMC1L1 genes. *Clin Genet* 2008;74(6):531-8.

Gervasini C, Russo S, Masciadri M, Castronovo P, Cereda A, Selicorni A, Zampino G, Larizza L. Sindrome da geni contigui coinvolgente NIPBL associata a presentazione clinica Cornelia de Lange estremamente grave. In: XI Congresso Nazionale SIGU Genova 24 novembre 2008.

Macchini F, Selicorni A, Luzzani S, Milani D, Roggero P, Valadè A. Coeliac disease and Cornelia de Lange syndrome: lack of association. *Acta Paediatr* 2007;96(10):1518-20.

Marchisio P, Selicorni A, Pignataro L, Milani D, Baggi E, Lambertini L, Dusi E, Villa L, Capaccio P, Cerutti M, Esposito S, Principi N. Otitis media with effusion and hearing loss in children with Cornelia de Lange syndrome. *Am J Med Genet A* 2008;146A(4):426-32.

Masciadri M, Gervasini C, Castronovo P, Milani D, Cereda A, Zampino G, Di Rocco MG, Selicorni A, Larizza L, Russo S. Delezioni di interi esoni nel gene NIPBL ed un caso di sindrome di geni contigui in un campione di pazienti CdLS. In: XI Congresso Nazionale SIGU Genova 24 novembre 2008.

Olioso G, Passarini A, Atzeri F, Milani D, Cereda A, Cerutti M, Maitz S, Menni F, Selicorni A. Clinical problems and everyday abilities of a group of Italian adolescent and young adults affected by Cornelia de Lange Syndrome *Am J Med Genet A* 2009;149A(11):2532-7.

Russo S, Gervasini C, Masciadri M, Castronovo P, Milani D, Cereda A, Zampino G, Selicorni A, Larizza L. Two intragenic NIPBL deletions and one contiguous gene syndrome detected by MLPA in CdLS patients. In: 58th Annual Meeting of the American Society of Human Genetics. Philadelphia, PA, 11-15 November 2008.

Selicorni A, Colli AM, Passarini A, Milani D, Cereda A, Cerutti M, Maitz S, Alloni V, Salvini L, Galli MA, Ghiglia S, Salice P, Danzi GB. Analysis of congenital heart defects in 87 consecutive patients with Brachmann-de Lange syndrome. *Am J Med Gen A*, 2009;149A:1268-72.

Selicorni A, Russo S, Gervasini C, Masciadri M, Castronovo P, Cereda A, Passarini A, Milani D, Larizza L. Molecular characterization of a large cohort of Cornelia de Lange Syndrome Italian patients and related phenotypes. In: *58th Annual Meeting of the American Society of Human Genetics*. Philadelphia, PA, 11-15 November 2008.

DYSREGULATED RAS SIGNALLING IN NOONAN SYNDROME AND RELATED DISORDERS: DISEASE GENE DISCOVERY AND FUNCTIONAL STUDIES

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Noonan Syndrome (NS) and the clinically related LEOPARD Syndrome (LS) are genetically heterogeneous developmental disorders characterized by reduced growth, facial dysmorphisms, cardiac defects, as well as variable skin and skeletal anomalies and cognitive deficits. Increased RAS-MAPK signal traffic due to heterozygous PTPN11 and KRAS mutations occurs in approximately 50% of NS, while a bunch of amino acid changes impairing PTPN11/SHP2 catalytic activity account for 90% of LS.

Goals of this project were to identify novel NS/LS disease genes, explore the molecular mechanisms implicated in these disorders, and define genotype-phenotype correlations. Here major discoveries follow:

By using a candidacy approach focused on genes coding transducers with role relevant to RAS signaling, we discovered four novel disease genes, SOS1, RAF1, BRAF and SHOC2, implicated in NS/LS pathogenesis.

SOS1 encodes a RAS-specific GEF. SOS1 mutations account for 10% of NS, cluster at residues implicated in the maintenance of its autoinhibited conformation, and promote enhanced RAS-MAPK activation. The phenotype associated with SOS1 defects is distinctive, with a high prevalence of ectodermal abnormalities but normal cognitive development and growth. RAF1 and BRAF gene mutations were identified in a small percentage of NS and LS. These genes encode serine/threonine protein kinases functioning as RAS effectors. Most RAF1 mutations altered a motif that is critical for protein autoinhibition through 14-3-3 binding, and promoted enhanced ERK activation. RAF1 mutations in two hotspots were strongly associated with hypertrophic cardiomyopathy. BRAF mutations mapped to multiple protein domains, and largely did not overlap with cardiofaciocutaneous syndrome-causing or cancer-associated defects. Selected BRAF mutations promoted variable gain of function of the kinase, but appeared less activating compared than the oncogenic V600E protein. SHOC2 encodes for a cytoplasmic/nuclear protein involved in the activation of the MAPK pathway following growth factor stimulation.

SHOC2 functions as a regulatory subunit of the catalytic subunit of PP1C, promoting the translocation of the catalytic subunit of this phosphatase to the membrane, which is required for

RAF1activation. The 4A>G (Ser2Gly) change occues in approximately 4% of NS, and is associated with a distinctive phenotype previously reported as Noonan-like syndrome with loose anagen hair. The disease-causing mutation introduces an N-myristoylation site, resulting in aberrant targeting of SHOC2 to the plasma membrane and impaired translocation to the nucleus upon growth factor stimulation. Expression of SHOC2S2G *in vitro* enhanced MAPK activation in a cell type-specific fashion.

These results document the first example of an acquired N-terminal lipid modification of a protein causing human disease. Finally, by analyzing the biochemical behavior and ligand-binding properties of a selected panel of SHP2 mutants, we demonstrated that multiple molecular mechanisms underlie SHP2 functional dysregulation in NS.

Papers published within the project

Cordeddu V, Di Schiavi E, Pennacchio LA, Ma'ayan A, Sarkozy A, Fodale V, Cecchetti S, Cardinale A, Martin J, Schackwitz W, Lipzen A, Zampino G, Mazzanti L, Digilio MC, Martinelli S, Flex E, Lepri F, Bartholdi D, Kutsche K, Ferrero GB, Anichini C, Selicorni A, Rossi C, Tenconi R, Zenker M, Merlo D, Dallapiccola B, Iyengar R, Bazzicalupo P, Gelb BD, Tartaglia M. Mutation of SHOC2 promotes aberrant protein N-myristoylation and causes Noonan-like syndrome with loose anagen hair. Nat Genet. 2009;41(9):1022-6

Martinelli S, Torreri P, Tinti M, Stella L, Bocchinfuso G, Flex E, Grottesi A, Ceccarini M, Palleschi A, Cesareni G, Castagnoli L, Petrucci TC, Gelb BD, Tartaglia M. Diverse driving forces underlie the invariant occurrence of the T42A, E139D, I282V and T468M SHP2 amino acid substitutions causing Noonan and LEOPARD syndromes. Hum Mol Genet. 2008;17(13):2018-29

Pandit B, Sarkozy A, Pennacchio LA, Carta C, Oishi K, Martinelli S, Pogna EA, Schackwitz W, Ustaszewska A, Landstrom A, Bos JM, Ommen SR, Esposito G, Lepri F, Faul C, Mundel P, López Siguero JP, Tenconi R, Selicorni A, Rossi C, Mazzanti L, Torrente I, Marino B, Digilio MC, Zampino G, Ackerman MJ, Dallapiccola B, Tartaglia M, Gelb BD. Gain-of-function RAF1 mutations cause Noonan and LEOPARD syndromes with hypertrophic cardiomyopathy. Nat Genet. 2007;39(8):1007-12.

Sarkozy A, Carta C, Moretti S, Zampino G, Digilio MC, Pantaleoni F, Scioletti AP, Esposito G, Cordeddu V, Lepri F, Petrangeli V, Dentici ML, Mancini GM, Selicorni A, Rossi C, Mazzanti L, Marino B, Ferrero GB, Silengo MC, Memo L, Stanzial F, Faravelli F, Stuppia L, Puxeddu E, Gelb BD, Dallapiccola B, Tartaglia M. Germline BRAF mutations in Noonan, LEOPARD, and cardiofaciocutaneous syndromes: molecular diversity and associated phenotypic spectrum. Hum Mutat. 2009;30(4):695-702.

Tartaglia M, Pennacchio LA, Zhao C, Yadav KK, Fodale V, Sarkozy A, Pandit B, Oishi K, Martinelli S, Schackwitz W, Ustaszewska A, Martin J, Bristow J, Carta C, Lepri F, Neri C, Vasta I, Gibson K, Curry CJ, Siguero JP, Digilio MC, Zampino G, Dallapiccola B, Bar-Sagi D, Gelb BD. Gain-of-function SOS1 mutations cause a distinctive form of Noonan syndrome. Nat Genet. 2007;39(1):75-9.

CHARACTERIZATION OF THE MOLECULAR AND CELLULAR MECHANISMS UNDERLYING THE LIVER PATHOGENESIS IN HEMOPHAGOCYTIC SYNDROMES

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Introduction

Hemophagocytic Syndrome (HS) is a severe and often fatal syndrome resulting from potent and uncontrolled activation and proliferation of T lymphocytes, leading to excessive macrophage activation and multiple deleterious effects. This project is devoted to a better characterization of the mechanisms underlying the liver pathology of the HS. Our preliminary results indicate at least two new mechanisms potentially involved in HS progression.

In the first part of this project we have characterized the role of chemerin, the ligand of the chemotactic receptor ChemR23, in inducing the recruitment and co-localization of myeloid Dendritic Cells (M-DC), plasmacytoid DC (P-DC) and NK cells. The three cell types express high levels of functional ChemR23 and chemerin is highly expressed in the liver. Subsequent work has focussed on two different aspects of chemerin biology. First, it was investigated the source of chemerin in the liver; second, it was evaluated whether chemerin, in addition to promoting the co-localization of DC subsets and NK cells may also activate NK cell function. To address the cellular source of chemerin production, chemerin expression in M-DC, P-DC and NK cells was evaluated by quantitative PCR in resting cells as well as in stimulated cells. Chemerin was not found induced in none of the conditions investigated, suggesting that leukcocytes do not represent a relevant source for this chemotactic factor. To gain further insight in the cell type responsible for chemerin production in the liver we decided to use in situ hybridisation. To this goal a specific probe was cloned in pBSKS+ vector and sections from human liver and lymph nodes, these experiments are still ongoing. Finally, chemerin was used to stimulate NK cell *in vitro*, cytokine production, NK cell degranulation and killing of K562 cells were then evaluated. None of these activities resulted profoundly affected by the action of chemerin. In conclusion, chemerin appears to be a pure chemotactic factor for NK cells, devoid of the ability to induce cell activation. Therefore, chemerin should be viewed as the only so far described chemotactic factor that can induce the co-localization of DC subsets and NK cells. Further activation/modulation of NK cells is the result of the cross-talk among these three cell types in the absence of a direct action of chemerin on NK cell functions. We propose that chemerin, in addition to certain chemokines known to be produced by activated Kupffer cells, such as CCL20, may induce the colocalization of innate immunity effector cells in pathological conditions, such as HS.

At the Department of Pediatrics at University of Brescia, we have identified 9 patients with clinical manifestations resembling HLH. They were classified according to current diagnostic criteria of Hystiocyte Society. All patients have undergone both analysis of NK cell function and genetic studies. Patients presented the following clinical manifestations were as follows: fever was observed in 100% of patients, splenomegaly 100% of subjects, single lineage cytopenia (anemia, thrombocytopenia or neutropenia) 88%, hemophagocytosis (78%), Impaired NK cell cytotoxicity (100%). Genetic analysis of PRF1 and UNC13D was performed in all subjects. Three of them displayed mutations of PRF1, while another one carried UNC13D mutation.

We have analyzed NK and NK-T cells in two patients affected by Hermansky-Pudlak type 2. Preliminary data suggest abnormal distribution of NK cell subset and complete absence of NK-T cells. In these subjects, analysis of CD63 expression on cell surface has shown increased levels on cell surface. Moreover, CD63 expression is not regulated after activation, suggesting that CD63 transport to plasmamembrane is altered in these subjects (Figure 1).

Control   **HPS2 pt1**   **HPS2 pt2**
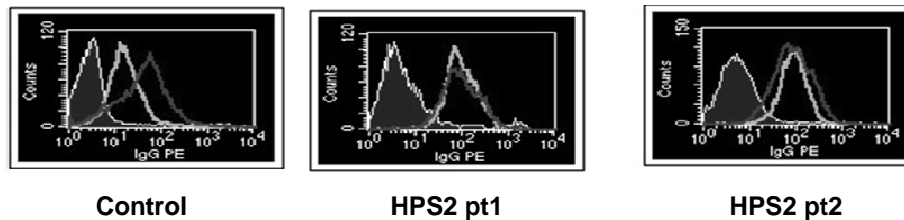
Figure 1. CD63 expression: reting cells and activated cells

PI5KIγ-dependent PIP2 pool plays a key role in Munc13-4 priming factor compartmentalization

We have previously described a critical role for phosphatidylinositol4,5bisphosphate-5 kinase type I (PI5KI)-dependent signals in the regulation of Natural Killer (NK) cell-mediated cytotoxic function by demonstrating that PI5KI product, phosphatidylinositol4,5bisphosphate (PIP2), is critically involved in the activation of cytolytic secretory pathway at a step downstream to granule polarization. More recently, our study has been focused on the analysis of the role of PIP2 in the functional regulation of Soluble N-Ethylmalemide Sensitive Factor Attachment Protein Receptor (SNARE) system responsible for secretory granule dynamics and exocytosis. In particular we focused our interest on lytic granule priming factor Munc13-4 whose mutation in Familial Hemophagocytic Lymphohistiocytosis (FHL3) patients results in a profound defect of NK cell-mediated cytotoxic function. To this purpose, primary cultured human NK cells and/or YTS NK cell line have been used and lipid raft isolation and analysis has been performed by biochemical and confocal microscopy approaches. Degranulation and internalization has been studied by cytofluorimetric and confocal microscopy analysis, respectively. Gene silencing of PI5KIα and PI5KIγ isoforms has been performed by means of GFP-bearing lentiviruses encoding shRNA specific sequences.

We observed a significant PIP2 enrichment at plasma membrane raft microdomains as demonstrated by colocalization of GM1 with PIP2 interacting domain, GFP-PH/PLCδ1; moreover, we observed that raft integrity is critically required for target-induced lytic granule exocytosis in primary human NK cells. We also found that Munc13-4 priming factor undergoes

a transient membrane raft recruitment upon FcγRIIIA activating receptor stimulation or phorbol ester plus ionomycin treatment. Interestingly, in PI5KI silenced YTS cells we demonstrate that the down-regulation of PI5KIγ-, but not of PI5KI α-dependent PIP2 pool, leads to a profound deregulation of Munc13-4 compartmentalization. Infact, we observed an increase of Munc13-4 basal levels within raft fraction associated with a lack of activation-dependent membrane raft recruitment which is possibly due to an altered PIP2-dependent clathrin-mediated recycling pathway.

Our findings suggest that PI5KIγ-dependent PIP2 pool is required to regulate Munc13-4 subcellular compartmentalization. Munc13-4 miss-localization and lipid raft retention could negatively regulate NK cells cytotoxic function.

Dendritic cells NK and NK-T cells in two patients affected by Hermansky-Pudlak type 2

Reduction of NKT number in HPS2 patients: NKT cells have been defined as lymphocytes that expresses both TCR and NK1.1, that may have important immune regulatory functions. It has been reported that AP-3-deficient mice have a significantly reduced NKT cell population, suggesting that the generation of the endogenous ligand that selects NKT cells may also be AP-3 dependent. In order to evaluate the number of Vα14 invariant NK T cells (Vα14i NK T), we stained freshly isolated PBMC from HPS2 patients and from an healthy donor with PBS57-loaded CD1d tetramer. Despite our efforts we could only identify a number of positive events that did not excide those identified using the unloaded tetramer as the negative control (Figure 2a and 2b).
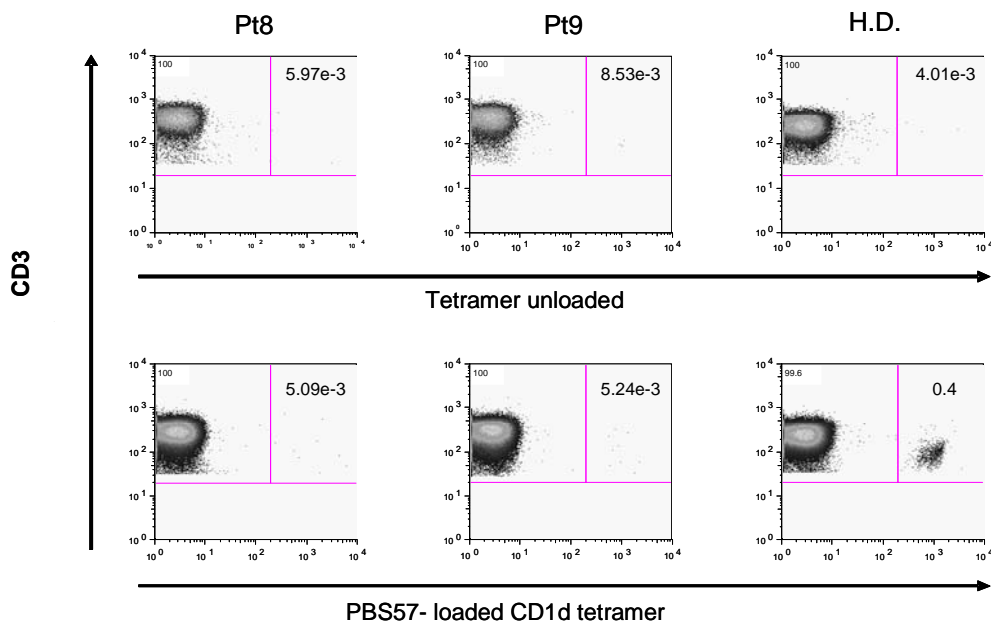
Figure 2a. NK-T cells in HPS2 patients
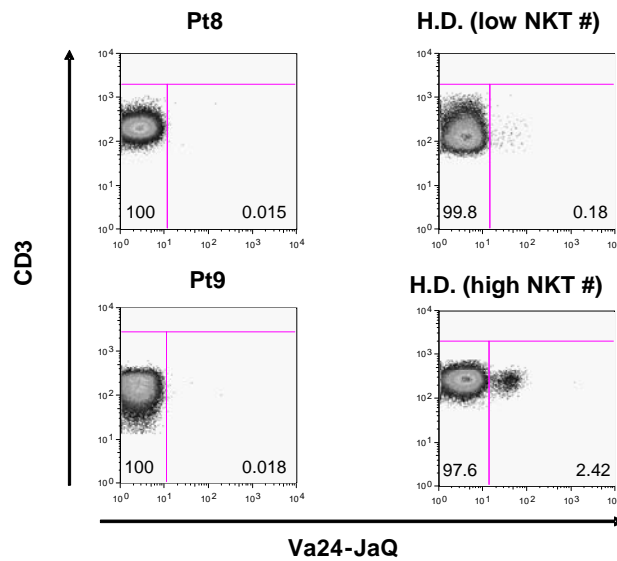
Figure2b. NK-T cells in HPS2 patients

We obtained the same results staining the cells with an antibody against the CDR3 region of the invariant chain that characterizes NKT cells. In Figure 1b, exemplificative plots of NKT cells against T cells from normal subjects, displaying particularly low and high number of NKT cells, are compared with the two HPS2 patients. These findings are consistent with an important role for the adaptor protein AP-3 in positive selection of NKT cells.

Altered expression of DC-LAMP and CD83 in monocyte derived DC stimulated with LPS and in lymph node: DC, the most specialized APC, are a subset of cells that interact with NKT and that is able to modulate their activity. Since there are indications that AP3 complex may exert an important role in the antigen presentation, we decided to evaluate the ability of patients DC to become fully competent APC. To this purpose we differentiated DCs from monocytes and we induced maturation upon LPS stimulation. The mature phenotype of monocyte derived DCs was evaluated on a cytospin preparation, through the immunostaining with anti-DC-LAMP monoclonal antibody and anti-CD83 monoclonal antibody. In these experimental conditions, it was evident the inability of patients cells to enhance a normal expression of these maturation markers, which keep still barely visible 24 hours after stimulation. Interesting results were obtained after 48 hours of LPS treatment in HPS2 patients DC, at least regarding DC-LAMP detection. In fact, after a longer stimulation, a clearly detectable cytoplasmic expression of DC-LAMP was visible by immunohistochemistry in cells derived from both HPS2 patients.

However, only few cells showing a normal capping of DC-LAMP could be noticed (Figure 3). This spread intracellular expression of DC-LAMP could be due to an intrinsic inability of DC to form a normal DC-LAMP capping in AP-3 deficient patients. *In vivo* analysis of DC infiltrating the lymphnode of pt8 confirmed the characteristic cytoplasmic expression of DC-LAMP observed in *in vitro* experiments. Also in this case the difference between the localization of DC-LAMP in normal tissues and in lymph node of this HPS2 patient is evident. Moreover the analysis of CD83 co-expression in DC cells showing altered DC-LAMP intracellular distribution display the absence of CD83 staining, supporting the idea of an intrinsic defective ability to complete DC maturation in patients carrying AP-3 mutations.

Control   **Patient 8**   **Patient 9**

magnification: 1000X
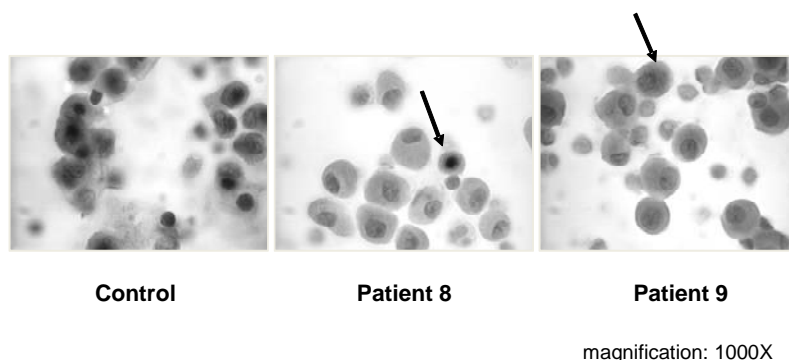
Figure 3. Cells showing a normal capping of DC-Lamp

Altered cytokine production in immature DC of HPS2 patients: Because DCs are the main producers of cytokines that regulate T-cell activation, we decided to test the production of several chemokines and cytokines by DC cultured for 24 hours with LPS or medium alone. Normal DCs derived from healthy donors and maintained in LPS for 24 hours, displayed a considerable increase in MCP-1 production (up to 17 ng/mL), IL-8 (up to 7 ng/mL), MIP1-a (up to 5 ng/mL) and MIP1-b (up to 4 ng/mL).

Surprisingly, we could no detect significative differences in chemokines production in the supernatant obtained from patients DCs treated with LPS. Interestingly, immature DCs from HPS2 patients, secreted high amounts of MCP-1 (up to 24 ng/mL) and IL-8 (up to 10 ng/mL), in contrast with immature DCs from healthy donors, that could not enhance production of these chemokines.

Papers published within the project

Badolato R, Parolini S. Novel insights from adaptor protein 3 complex deficiency. *J Allergy Clin Immunol* 2007;120(4):735-41; quiz 742-3. Review.

Fontana S, Parolini S, Vermi W, Booth S, Gallo F, Donini M, Benassi M, Gentili F, Ferrari D, Notarangelo LD, Cavadini P, Marcenaro E, Dusi S, Cassatella M, Facchetti F, Griffiths GM, Moretta A, Notarangelo LD, Badolato R. Innate immunity defects in Hermansky-Pudlak type 2 syndrome. *Blood* 2006;107:4857-64.

Micucci F, Capuano C, Marchetti E, Piccoli M, Frati L, Santoni A, Galandrini R. PI5KI-dependent signals are critical regulators of the cytolytic secretory pathway. *Blood* 2008;111(8):4165-72.

MOLECULAR TARGETS OF PITX2
IN THE PATHOGENESIS OF AXENFELD RIEGER
SYNDROME

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Introduction

Pitx2, a required factor for myoblast differentiation, is phosporylated by Akt and controls cyclin D1 mRNA decay. Paired-like homeodomain 2 (Pitx2) is a member of the bicoid-class of paired homeodomain proteins that is mutated in patients affected by Rieger syndrome, an autosomal dominant haploinsufficient disorder, that includes tooth anomalies, anterior segment eye defects, umbilical abnormalities, and facial dysmorphologies as cardinal features. The phenotype of mice with targeted deletion of Pitx2 mimics, in part, Rieger syndrome and its analysis established the critical role of Pitx2 in the development of cranio-facial structures, and multiple organs including eye, pituitary, heart, and lung. Pitx2 is expressed in myotomes and migrating myoblasts and has been implicated in growth and survival of branchiomeric muscle progenitors. At early developmental stages, Pitx2 has been involved in the establishment of left-right asymmetry.

We previously showed that Pitx2 activates the transcription of specific growth-regulating genes such as those for cyclins D1 and D2 and it is also profoundly involved in the stabilization of the same messages during wingless pathway (Wnt) activation. Moreover, Pitx2 mRNA is stabilized by Wnt activation, and this effect is mediated by AU-rich elements (AREs) in its 3' untranslated region (3'UTR)6. We showed that Pitx2 protein is required for stabilization of its own mRNA, as well as of those encoding cyclins D1 and D2. This effect is achieved, in part, by modulating the function of the stabilizing ARE-binding protein (ARE-BP) HuR as Pitx2 and HuR proteins interact.

In a screening for protein Serine/Threonine kinases able to phosphorylate *in vitro* Pitx2, we found that AKT2 is able to phosphorylate, in a concentration dependent manner, the purified recombinant N-terminal part of Pitx2 while does not phosphorylate the C-terminal part of the protein. An in silico search for potential AKT phosphorylation sites indicated Threonine 97, embedded in a nearly perfect consensus sequence for AKT (RQRTHFT), as the most probable AKT target residue. Indeed, AKT2 failed to phosphorylate the mutant Pitx2 in which Threonine 97 was substituted with Alanine. The sequence RQRTHFT is located in the homeodomain of Pitx2, is present in the three Pitx2 splicing variants, and is evolutionary conserved from Drosophila to Human.

Considering the role of Pitx2 during muscle development, and the pro-myogenic function of AKT in myoblasts7, we chose to study the functional relevance of Pitx2 phosphorylation by AKT2 in C2C12 myoblasts, able to differentiate in culture into myotubes upon shift from Growth Medium (GM) to low-serum-containing Diffentiation Medium (DM). We found that Pitx2 is a substrate for AKT at early phases of C2C12 differentiation.

To gain insight into Pitx2 function in the course of C2C12 differentiation, we transiently knocked-down Pitx2 by siRNA. The expression of some myogenic differentiation markers including myogenin, muscle creatine kinase, Myod1, and p21 was severely impaired in siPitx2-

transfected C2C12 induced to differentiate by DM. On the contrary, the expression of an early marker of myoblast progenitors and satellite cells (Pax7), as well as markers of myogenic determination such as Myf5, and Myf6 remained unchanged. Furthermore, Pitx2 knock-down caused a proliferation reduction in C2C12 maintained in GM as assessed by crystal violet staining and reduced the expression of cyclin D1, cyclin D2, and c-myc in C2C12. Interestingly, cyclin D1 knock-down in proliferating C2C12 reduced the expression of differentiation markers including myogenin, and p21 upon serum withdrawal.

Surprisingly, we found that Pitx2 is predominantly cytoplasmic in C2C12. We previously reported that Pitx2 is able to control the expression of cyclin D1 (and other transcripts) at both transcriptional and post-transcriptional levels. The subcellular localization of Pitx2 in C2C12 prompted us to investigate the consequences of Pitx2 knock-down on the decay rate of cyclin D1 mRNA in these cells. *In vitro* degradation assays revealed that cyclin D1 mRNA is stable in proliferating C2C12 and its half-life is reduced by serum withdrawal-induced differentiation. Most importantly, transient Pitx2 knock-down significantly shortened cyclin D1 transcript half-life while the decay rate of myogenin mRNA was unaffected *in vivo*.

We have previously shown that, although it is not able to directly bind to mRNA, Pitx2 participates in a ribonucleoprotein complex containing, among others, the mRNA stabilizing factor HuR6. By ribonucleoprotein complexes immunoprecipitation (RIP analysis), we found that anti-Pitx2 antibody immunoprecipitates in C2C12 extracts a protein complex bound to cyclin D1 mRNA and that this interaction is reduced by cell differentiation. Similarly, HuR binding to cyclin D1 mRNA is reduced in differentiated C2C12 when compared to proliferating C2C12.

Based on the above results, we investigated whether HuR- cyclin D1 mRNA interaction depends on Pitx2. Indeed, transient Pitx2 knock-down reduced the interaction of HuR with cyclin D1 mRNA in proliferating C2C12 without affecting HuR expression levels. Importantly, forced activation of AKT in C2C12 induced a significant reduction of cyclin D1 mRNA levels in both myoblasts and myotubes reducing the half-life of cyclin D1 mRNA. Furthermore, AKT2 activation decreased the ability of Pitx2 to participate into the cyclin D1 stabilizing complex and of HuR to interact with cyclin D1 mRNA.

It is well known that phosphorylation by AKT can change the ability of substrate proteins to interact with molecular partners. By GST-pull-down experiments, we found that phosphorylation by AKT2 increases Pitx2 ability to interact with the multifunctional protein 14-3-3 while decreases Pitx2 ability to interact with HuR. T97A mutation, that abrogates the AKT phosphorylation site, inhibited the association of Pitx2 with 14-3-3 while favored the interaction with HuR. Accordingly, co-immunoprecipitation experiments revealed that Pitx2 interaction with HuR is strongly reduced in C2C12 cultured in DM while Pitx2 interaction with 14-3-3 is increased.

Results

Identification of the RNA binding protein KSRP as a target of Pitx2

Using a genomic approach, we identified the KH-domain-containg protein KSRP as target of Pitx2 in various cell lines.

We have found that KSRP regulates gene expression in different ways: i) affecting the decay of select labile transcripts, ii) controlling the biogenesis of a class of miRNA. Interestingly, AKT, which exerts some of its cellular effects through Pitx2, phosporylates KSRP affecting its function.

Conclusions

Pitx2 is required for the proliferation of C2C12 myoblasts under high-serum growth conditions as well as for their differentiation under low-serum conditions. In C2C12, Pitx2 predominantly localizes in the cytoplasm and is part of an mRNA stabilizing complex, including the ARE-BP HuR, which controls cyclin D1 half-life. Upon phosphorylation by AKT, Pitx2 is no longer able to interact with HuR that, in turn, fails to interact with cyclin D1 transcript leading to its destabilization.

Papers published within the project

Díaz-Moreno I, Hollingworth D, Frenkiel TA, Kelly G, Martin S, Howell S, García-Mayoral M, Gherzi R, Briata P, Ramos A. Phosphorylation-mediated unfolding of a KH domain regulates KSRP localization via 14-3-3 binding. *Nat Struct Mol Biol* 2009;16:238-46.

Nechama M, Ben-Dov IZ, Briata P, Gherzi R, Naveh-Many T. The mRNA decay promoting factor K-homology splicing regulator protein post-transcriptionally determines parathyroid hormone mRNA levels. *FASEB J* 2008;22:3458-68.

Ruggiero T, Trabucchi M, De Santa F, Zupo S, Harfe BD, McManus MT, Rosenfeld MG, Briata P, Gherzi R. LPS induces KH-type splicing regulatory protein-dependent processing of microRNA-155 precursors in macrophages. *FASEB J* 2009;23(9):2898-908.

Trabucchi M, Briata P, Garcia-Mayoral M, Haase AD, Filipowicz W, Ramos A, Gherzi R, Rosenfeld MG. The RNA-binding protein KSRP promotes the biogenesis of a subset of microRNAs. *Nature* 2009; 459(7249):1010-4.

GENOTYPE/PHENOTYPE ANALYSIS OF NEURODEGENERATIVE AND AGING-PRONE SYNDROMES CAUSED BY MUTATIONS IN THE DNA DAMAGE RESPONSE/REPAIR PATHWAY

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An aim of the project concerned the production of new cell lines from patients AOA1 and AOA2. To this end, we have immortalized by EBV and stabilized lymphoblatoid cells from 10 AOA1 suspects; 3 AOA2 suspects and 2 parents; 1 AT-LD suspect; 34 A-T suspects and 5 parents. For the ATM gene mutations, 104 Italian ataxia telangiectasia patients from 91 unrelated families (detection rate 90%) were screened and found 21 recurrent mutations in 63 families. The majority (67%) of patients were compound heterozygotes, while 33% were homozygotes. To determine the existence of common haplotypes and potential founder effects, we analyzed five microsatellite markers within and flanking the ATM gene. Haplotype analysis was carried out in 48/63 families harbouring 16 of the 21 recurrent mutations. Forty different haplotypes were detected in the 48 A-T families studied. We found that the majority of patients with the same recurrent mutation originated from the same geographical area. All but one recurrent mutation analyzed displayed a common haplotype suggesting a single origin that then spread to different geographical areas. The high number of different haplotypes does not allow the screening of ATM mutations by haplotype analysis alone in the Italian population. The finding of recurrent public mutations without founder effect suggests the existence of "mild" hot spots of mutation located along the sequence of the ATM gene.

Another aim was the characterization of the senataxin (SETX) protein. For this, we have cloned the SETX cDNA and generated full length and deletion mutants GFP-SETX constructs that were transfected into U2OS and 293 cells to assess the functional effects. We found that GFP-SETX localizes in the nucleus and when overexpressed it markedly blocks the G2-M cell cycle progression, thus explaining the inability to generate clones that stably express SETX. In addition, our experiments based on the use of specific GFP reporter constructs and the use of wiled type and SETX-deficient cells, rule out its involvement in mRNA decay. We have generated 4 Hist-SETX bacterial recombinant fragments, 3 of which were used to immunize rabbits and generate polyclonal antibodies. Following affinity purification, these antibodies were validated by western blot, immunoprecipitation and immunohistochemistry (IHC). We have tested the anti-SETX antibodies on brain biopsies and found an ubiquitous expression, though much stronger in purkinje cells. Currently, we are purifying the predicted SETX helicase domain overexpressed using the vaccinia virus approach in order to test its helicase activity *in vitro*.

In relation to the devolopment of model systems of neurodegeneration, we have generated *in vitro* human neural stem cells (hNSCs) with tripotent differentiation potential in which ATM, Nbs1 and Mre11 were knocked down by shRNA lentiviral strategies. While ablation of ATM markedly impaired the DNA damage response in hNSC-shATM, it did not impair the differentiation potential into neurons, astrocytes and oligodendrocytes. However, the

differentiated oligodendrocytes were more sensitive to the treatment with oxidants. The hNSC-shNbs1 and hNSC-shMre1 presented remarkable self renewal impairements, phenotypic and morphological alterations. Stable hNSC-shSETX have been difficult to generate although the shRNA sequences used all ablate SETX in transient experiments.

Another specific aim of this collaborative project was the investigation of the molecular mechanism underlying accumulation of DNA damage, either in unstressed conditions or upon replication perturbation, in cells from Werner syndrome (WS). Specifically, we determined that accumulation of DNA damage in cells from WS occurs as a consequence of unscheduled disassembly of the replication machinery at DNA lesions or stalled replisomes and activation of a secondary pathway of replication fork recovery that depends on the MUS81 endonuclease. Channelling stalled replisomes through the MUS81 pathway leads to formation of double-strand breaks in DNA and activation of recombination even when it is detrimental to genome stability. Thus, even though the MUS81 pathway may enhance survival of WS cells upon replication stress, it functions as a double-edged sword contributing to genomic instability and possibly activating the pathway triggering premature replicative senescence.

Papers published within the project

Berni A, Meschini R, Filippi S, Palitti F, De Amicis A, Chessa L. L-Carnitine enhances resistance to oxidative stress reducing DNA damage in ataxia telangiectasia cells. *Mutat Res*, 2008;650:165-74.

Bruno T, De Nicola F, Iezzi S, Lecis D, D'Angelo C, Di Padova M, Corbi N, Dimiziani L, Zannini L, Jekimovs C, Scarsella M, Porrello A, Chersi A, Crescenzi M, Leonetti C, Khanna KK, Soddu S, Floridi A, Passananti C, Delia D, Fanciulli M. Che-1 phosphorylation by ATM/ATR and Chk2 kinases activates p53 transcription and the G2/M checkpoint. *Cancer Cell* 2006;10:473-86.

Buscemi G, Zannini L, Fontanella E, Lecis D, Lisanti S, Delia D. The shelterin protein TRF2 inhibits Chk2 activity at telomeres in the absence of DNA damage. *Curr Biol* 2009;19:874-9.

Carlessi L, De Filippis L, Lecis D, Vescovi A, Delia D. DNA-damage response, survival and differentiation *in vitro* of a human neural stem cell line in relation to ATM expression. *Cell Death Differ* 2009;16:795-806

Chessa L, Piane M, Magliozzi M, Torrente I, Savio C, Lulli P, De Luca A, Dallapiccola B. Founder effects for ATM gene mutations in Italian Ataxia Telangiectasia families. *Annals Human Genetics*, Ann Hum Genet. 2009;73(Pt 5):532-9.

Criscuolo C, Chessa L, Di Giandomenico S, Mancini P, Saccà F, Greco GS, Piane M, Barbieri F, De Michele G, Banfi S, Pierelli F, Rizzuto N, Santarelli FM, Gallosti L, Filla A, Casali C. Ataxia with oculomotor apraxia type 2: a clinical, pathology and genetic study. *Neurology* 2006;66:1207-10.

Franchitto A, Pirzio LM, Prosperi E, Sapora O, Bignami M, Pichierri P. Replication fork stalling in WRN-deficient cells is overcome by prompt activation of a MUS81-dependent pathway. *J Cell Biol* 2008;183:241-52.

Grattarola M, Borghi C, Emionite L, Lulli P, Chessa L, Vergani L. Modifications of nuclear architecture and chromatin organization in ataxia telangiectasia cells are coupled to changes of gene transcription. *J Cell Biochem* 2006;99:1148-64.

Honda M, Takagi M, Chessa L, Morio T, Mizutani S. Rapid diagnosis of ataxia-telangiectasia by flow cytometric monitoring of DNA damage-dependent ATM phosphorylation. *Leukemia* 2009;23:409-14.

Lavin MF, Delia D, Chessa L. ATM and DNA damage response *EMBO Reports* 2006;7:154-60.

Magliozzi M, Piane M, Torrente I, Sinibaldi L, Rizzo G, Savio C, Lulli P, De Luca A, Dallapiccola B, Chessa L. DHPLC screening of ATM gene in Italian patients affected by ataxia-telangiectasia: fourteen novel ATM mutations. *Disease Markers* 2006;22:257-64.

Masci A, Mastronicola D, Arese M, Piane M, De Amicis A, Blanck TJJ, Chessa L, Sarti P. Control of cell respiration by nitric oxide in Ataxia Telangiectasia lymphoblastoid cells. *Biochim Biophys Acta* 2008;1777:66-73.

Perfettini JL, Nardacci R, Bourouba M, Subra F, Gros L, Séror C, Manic G, Rosselli F, Amendola A, Masdehors P, Chessa L, Novelli G, Ojcius DM, Siwicki JK, Chechlinska M, Auclair C, Regueiro JR, de Thé H, Gougeon ML, Piacentini M, Kroemer G. Critical involvement of ATM-dependent DNA damage response in the apoptotic demise of HIV-1-elicited syncytia. *PlosOne* 2008;3:2458.

Pichierri P. Interplay between wrn and the checkpoint in s-phase. *Ital J Biochem* 2007;56:130-40. Review.

Pirzio LM, Pichierri P, Bignami M, Franchitto A. Werner syndrome helicase activity is essential in maintaining fragile site stability. *J Cell Biol* 2008;180:305-14.

Savio M, Cerri M, Cazzalini O, Perucca P, Stivala LA, Pichierri P, Franchitto A, Meijer L, Prosperi E. Replication-dependent DNA damage response triggered by roscovitine induces an uncoupling of DNA replication proteins. *Cell Cycle* 2006;65:2153-9.

Suraweera A, Becherel OJ, Chen P, Rundle N, Woods R, Nakamura J, Criscuolo C, Filla A, Chessa L, Gueven N, Lavin MF. Senataxin defective in ataxia oculomotor apraxia type 2 is involved in the defence against oxidative DNA damage. *J Cell Biol* 2007;177:969-79.

ROLE OF THE DYSTROPHIN-ASSOCIATED GLYCOPROTEIN COMPLEX IN LIMB-GIRDLE AND CONGENITAL MUSCULAR DYSTROPHIES: FROM MOLECULAR PATHOPHYSIOLOGY TO POTENTIAL THERAPY

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Muscular dystrophies are a heterogeneous group of genetic disorders characterized by the progressive loss of strength and integrity of the muscle tissue. Duchenne muscular dystrophy (DMD) is the most frequent lethal X-linked recessive disorder, due to mutation in the gene coding dystrophin. Different forms of muscular dystrophy are caused by mutations in genes coding components of the Dystrophin associated-Glycoprotein Complex (DGC), the multimeric transmembrane protein complex that links the cytoskeleton to the extracellular matrix. Limb-Girdle Muscular Dystrophies (LGMD) and Congenital Muscular Dystrophies (CMD) are two heterogeneous genetic disease groups that differ in age onset, clinical severity and muscle groups affected and, as recent studies outlined, there an overlap between them due to alterations in essential protein components of the DGC involved in the correct interaction between the sarcolemma and extracellular matrix. Although the exact function of the DGC is not completely determined, the findings that mutations in components of DGC render muscle fibers more susceptible to damage and lead to various types of muscle disorder indicate that DGC confers structural stability to the sarcolemma during contraction. However, as more genes are found and as the functions of DGC components are elucidated, it has become clear that signalling processes are crucial for normal muscle function.

First isolated from skeletal muscle membrane the DGC is widely expressed in non-muscle tissues including brain. Indeed, cognitive impairment and metal retardation are often characteristic features associated with muscle atrophy in some muscular dystrophies. The identification of high number of genes, which give rise to various forms of Congenital Muscular Dystrophy (CMD) has allowed a better understanding and has revealed the overall complexity of the pathogenesis of these diseases. CMD caused by defects in the glycosylation of α-dystroglycan (DG) are named dystroglycanopathies, which are a heterogeneous group of conditions associated with mutations in six genes so far identified encoding proven or putative glycosyltransferases. The clinical course is wide variable and in addition to muscle can compromise the brain and eyes. Our project, which involves four groups, was focused on i) the analysis of muscle tissue gene expression profile in cases of congenital muscular dystrophies (CMD) due to merosin deficiency and the analysis of mutations associated with α-DG hypoglycosylation; ii) the molecular determinants underlying the interaction between the two DG subunits (α/β interface) and the possible role that their association has for cells and tissues; iii) the emerging functions of dystrobrevin as motor adaptor and signalling scaffold protein.

During the last years the Working Unit (WU) of Dr. Ricci started to characterize muscle biopsies of patients with a known diagnosis of CMD using oligonucleotide microarrays

technology, genetic analysis and immunohistochemistry and we participated to an Italian population study aimed to establish, in patients affected by dystroglycanopathy, the prevalence of mutations in the six genes of the known or putative glycosyltrasferases and the spectrum of clinical and MRI findings. No clear-cut genotype-phenotype correlation could be observed with each gene, resulting in a wide spectrum of clinical phenotypes. As already observed with other European multicentric studies of dystroglyconopathies only a percentage, in the range of 37-53, accounted for mutations in one of the six genes indicating that other unknown genes or other mechanisms may be involved in dystroglyconopathies. In line with this hypothesis the WU of Drs. Brancaccio-Ricci extensively analyzed the dystroglycan α/β interface, which is crucial to maintain the integrity of the DGC. The importance of such interface dramatically emerges in the skeletal muscle of patients affected by muscular dystrophies, where the a-DG/b-DG interaction is weakened or sometimes completely lost, causing sarcolemmal instability and muscular necrosis.

In the characterization of the reciprocal intersubunit binding sites we have identified four amino acids that are crucial for the interaction between the two subunits. Our data collected in transfected cells indicate that the perturbation of the α/β interface has the effect to prevent the posttranslational cleavage of the DG precursor, while the uncleaved and mutated precursor seems to be still properly glycosylated and targeted to the plasma membrane. We are also analysing a group of patients affected by different types of muscular dystrophies, in order to assess the possible contribution of the DG α/β interface to the pathology of skeletal muscles.

The loss of the extracellular matrix-cytoskeleton axis DG-dependent could be also observed in CDM with lamininα2-deficience. In this form of muscular dystrophy cognitive impairment and mental retardation are often associated with muscle atrophy. The WU of Dr. Petrucci used the C57BL dy2J/dy2J mouse, an animal model of α2-deficient CMD, and littermate controls. We found that dystroglycan processing was altered and syntrophin was upregulated in skeletal muscle of dy2J/dy2J compared to control mice. Due to similarities in brain damages observed in CDM patients with lamininα2-deficiency and in patients affected by megalencephalic leukoencephalopathy with subcortical cysts, a rare congenital autosomal recessive leukodistrophy characterized the blood-brain barrier damages and enlargements of extracellular spaces, we started to analyse the organization of the DGC in relation to the protein affected in this disease.

The cytoplamic components of the DGC, syntrophin and dystrobrevin are known to be involved in signal transduction and DGC stabilization. The WU4 of Dr. Ceccarini has characterized the interaction between β-dystrobrevin and its binding partners, the molecular motor kinesin, PKA regulatory subunits and dysbindin, the product of one of the genes associated with schizophrenia and a component of the Biogenesis of Lysosome-related Organelles Complex 1 (BLOC-1) that regulates synthesis and trafficking of lysosome-related organelle. We found that dystrobrevin and dysbindin are phosphorylated by PKA suggesting that their function is subjected to potentially significant regulatory influence. We have also identified by MALDI-ToF the phosphorylation site of β-dystrobrevin that regulates the binding affinity with kinesin. Using a combination of *in vitro* and *in vivo* techniques, the WU3 of Dr. Macioce identified and characterized the association of β-dystrobrevin with a novel binding partner, the High Mobility Group (HMG)-protein iBRAF/HMG20a.

HMG proteins are ubiquitous non-histone proteins that bind to DNA and chromatin, acting as architectural elements that modulate chromatin structure and controlling the expression of a number of genes. We obtained evidence that iBRAF and β-dystrobrevin participate to the complexes that bind to regulatory elements in the promoter of neuronal genes. We also found that β-dystrobrevin directly interacts with a close homologue of iBRAF, BRAF35/HMG20b, *in vitro*. While iBRAF expression leads to the abrogation of REST-mediated transcriptional

repression and the consequent activation of the neuronal differentiation programme, BRAF35 is a component of a co-repressor complex that is required for the repression of neuronal genes. Our results corroborate the role of dystrobrevin as a multifunctional scaffold protein, and suggest that it might be involved in neuronal differentiation as a component of co-activator/co-repressor complexes required for the regulation of neuron-specific genes.

Papers published within the project

Bozzi M, Morlacchi S, Bigotti MG, Sciandra F, Brancaccio A. Functional diversity of dystroglycan. *Matrix Biol* 2009; 28(4):179-8.

Lanciotti A, Brignone MS, Camerini S, Serafini B, Macchia G, Raggi C, Molinari P, Crescenzi M, Sargiacomo M, Musumeci M, Aloisi F, Petrucci TC, Ambrosini E. MLC1 trafficking and membrane expression in astrocytes. Role of caveolin-1 and MLC1 phosphorylation. *Neurobiol Dis* 2009 Nov 25. [Epub ahead of print]

Mercuri E, Messina S, Bruno C, Mora M, Pegoraro E, Comi GP, D'Amico A, Aiello C, Biancheri R, Berardinelli A, Boffi P, Cassandrini D, Laverda A, Moggio M, Morandi L, Moroni I, Pane M, Pezzani R, Pichiecchio A, Pini A, Minetti C, Mongini T, Mottarelli E, Ricci E, Ruggieri A, Saredi S, Scuderi C, Tessa A, Toscano A, Tortorella G, Trevisan CP, Uggetti C, Vasco G, Santorelli FM, Bertini E. Congenital muscular dystrophies with defective glycosylation of dystroglycan: a population study. *Neurology* 2009;72(21):1802-9.

Sciandra F, Bozzi M, Morlacchi S, Galtieri A, Giardina B, Brancaccio A. Mutagenesis at the α/β interface impairs the cleavage of the dystroglycan precursor. *FEBS J* 2009;276(17):4933-45.

MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF THE NEWLY IDENTIFIED INTERACTION BETWEEN THE RETT SYNDROME-ASSOCIATED FACTOR MECP2 AND THE PRO-APOPTOTIC FACTOR HIPK2

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Rett Syndrome (RTT) is a devastating X-linked neurodevelopmental disorder with a prevalence of approximately 1:10,000 live female births. Affected children undergo apparently normal postnatal development until 6-18 months of age and then begin a marked neurological decline with a highly variable course. Initial manifestation of the disease may include loss of acquired speech and autistic features. Subsequently, RTT patients develop motor stereotypies, epileptiform seizures, anxiety, breathing abnormalities and autonomic dysfunction. The majority of typical RTT cases are caused by mutations in the gene encoding methyl-CpG-binding protein 2 (MeCP2). The MECP2 gene is located on the X chromosome and affected females are heterozygotes whereas most male infants carrying an MECP2 mutation die shortly after birth. Although it was originally thought that disease phenotype depends on specific mutation and X chromosome inactivation pattern, recent studies underline a role for other modifiers of the MECP2 gene function.

MeCP2 is an epigenetic transcriptional repressor, widely expressed throughout the brain, that binds methylated DNA and, mainly by recruiting repressive chromatin modifying complexes, causes the formation of a compact, transcriptionally inert chromatin structure. Recently it is becoming clear that MeCP2 activities are regulated by its phosphorylation status. In the nervous system, MeCP2 phosphorylation was shown to be influenced by extracellular stimuli and dynamically regulate gene expression. In particular, MeCP2 mediated repression of the Bdnf gene was found to be reversed by MeCP2 phosphorylation at S421 causing a change in the binding affinity to the promoter. In addition, S421 phosphorylation affects MeCP2's ability to regulate dendritic growth and spine maturation. More recently, neuronal activity was found to trigger dephosphorylation at S80 decreasing MeCP2 binding to some of its target promoters. Interestingly, Mecp2S80A and Mecp2S421A;S421A knock-in mice, carrying non-phosphorylatable MeCP2 mutants, showed altered locomotor activities, highlighting the relevance of these post-translational modifications in neurological functions. Besides S80 and S421, a number of additional residues were found phosphorylated in brain, further supporting the idea that phosphorylation might strongly influence MeCP2 activities. Whereas S421 is phosphorylated by a CaMKII/IV-dependent mechanism, the upstream events causing the phosphorylation of MeCP2 at S80 remain unknown.

There is no efficient therapy to cure RTT yet. Therefore, understanding the molecular pathogenesis of RTT is important for developing therapeutic strategies mainly, but not only, for Rett patients. Indeed, MeCP2 mutations and gene duplications result in a variety of neuropsychiatric conditions, including atypical RTT, autism, Angelman-like syndrome, schizofrenia and mental retardation. Instrumental for the studies on RTT has been the

development of mouse models in which the Mecp2 is either deleted, mutated or overexpressed. Recently, it has been proved that in Mecp2-null mice the disease is reversible by re-expressing a temporally silenced gene. This remarkable finding suggest that therapeutic strategies aimed at restoring MeCP2 dependent signaling, even in symptomatic patients, might be beneficial. However, since the slightest perturbation in MeCP2 levels is deleterious for brain functioning, gene therapy, at least with the wild-type gene, does not appear as a valid approach. Therefore RTT research is currently looking for those genes whose expression is sufficiently altered to cause the RTT symptoms and for proteins or pathways that interact with MeCP2 and might be therapeutically modulated.

Within the context described above and with the support of the Italy-USA Collaborative Program on Rare Diseases, we have performed a yeast two-hybrid screen using as bait the transcriptional repression domain and the upstream linker region of MeCP2 fused to the GAL4 DNA-binding domain. We identified, among the positive clones, the homeodomain interacting protein kinase 2 (HIPK2) that belongs to a family of Ser/Thr kinases originally identified as corepressors for homeodomain transcription factors. HIPK2 has a clear role in regulating cell growth and genotoxic stress-induced apoptosis. Furthermore, its involvement in the nervous system is indicated by the neuronal defects of null mice that partially overlap those observed in Mecp2 KO mice. Since important MeCP2 functions in the nervous system are regulated by its phosphorylation, we found it interesting to analyze the functional role of its interaction with HIPK2. We have thus confirmed that the two proteins associate *in vitro* and *in vivo* and phosphorylation assays have shown that MeCP2 is significantly phosphorylated by HIPK2 *in vitro*. Importantly, these assays have also allowed us to establish that MeCP2-Ser80 is the target of HIPK2. Functional analyses showed that MeCP2 cooperates with HIPK2 in induction of apoptosis and that S80-phosphorylation is required together with the DNA-binding of MeCP2. These data are thus the first describing a kinase associating with MeCP2 causing its specific phosphorylation *in vivo* and, moreover, they reinforce the role of MeCP2 in regulating cell growth. Importantly, HIPK2 is highly expressed in both the central and peripheral nervous system and Hipk2-null mice show an array of psychomotor abnormalities underscoring an important role of this kinase in the nervous system. Interestingly, locomotor defects were also observed in the recently developed Mecp2S80A knock-in mice in which MeCP2 cannot be phoshorylated at S80. Thus, it will be challenging to analyze whether HIPK2 is the kinase responsible for MeCP2-S80 phosphorylation in brain and whether it might be involved in the pathogenesis of the RTT.

Papers published within the project

Bracaglia G, Conca B, Bergo A, Rusconi L, Zhou Z, Greenberg ME, Landsberger N, Soddu S, Kilstrup-Nielsen C. Methyl-CpG binding protein 2 (MeCP2) is phosphorylated by HIPK2 and contributes to apoptosis. *EMBO Rep* 2009;10(12):1327-33.

X-LINKED OR AUTOSOMAL RARE MENTAL RETARDATION SYNDROMES: PHENOTYPIC ANALYSIS IN TRANSGENIC MOUSE MODELS

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Studying the function of genes that promote neuronal network architecture is a crucial step to understand the pathophysiology of inherited Mental Retardation (MR), a group of neurodevelopmental diseases with learning and behavioural deficits. Causes of MR include a number of disease loci that affect neuronal connections and remodelling. Several defects observed in MR are caused by mutations in genes encoding transcriptional regulators that orchestrate the brain differentiation and functioning.

We focussed our study on two genes associated with MR in humans, namely the X-linked methyl-CpG binding protein 2 (MeCP2) associated with Rett syndrome (RTT), and Kruppel-like factor 7 (KLF7), a gene mapping on chromosome 2q31-33, a critical chromosomal region associated with MR and autism. Two genetically modified mouse models have been used: MeCP2-308 truncated mice and KLF7 null mutant mice.

RTT is a leading cause of MR associated with autistic-like features.

This neurodevelopmental disorder primarily affects girls with a prevalence of 1 on 10.000 births. Up to five variants have been identified so far and three quarters of the cases meet the diagnostic criteria for classic RTT. One essential feature of RTT is an apparently normal prenatal and perinatal development until about 6-18 months of age, followed by a regression period, characterized by both a profound loss of acquired developmental skills in the areas of social contact, communication and hand use and a deceleration of head growth, usually leading to microcephaly. At the end of this period, which is extremely variable in duration, lasting few years in some individuals, development reaches a plateau associated with a wide variety of RTT peculiar symptoms. These include stereotyped hand movements, breathing irregularities, bloating, EEG abnormalities, sleep problems, gait dispraxia, back deformities, feeding abnormalities as well as autistic-like behaviours. During the last part of their life, RTT patients undergo a noteworthy worsening of motor performance and lifespan is extremely variable.

After a monogenic origin for RTT was proposed [mutations in the gene encoding methyl−CpG−binding protein 2 (MeCP2), more than 90% of classic RTT cases were found to have a mutation in this gene. The Mecp2 protein binds specifically to methylated CpG pairs of DNA, primarily acting as a transcriptional repressor. An X-linked dominant mode of inheritance characterizes MeCP2 gene and accounts for the skewed sex ratio observable among RTT patients: RTT mainly affects girls as hemizygous males and homozygous females hardly survive. No cure currently exists for treating this devastating disorder. Only interventional modalities aimed at improving quality of life of RTT patients are available at the moment.

Within the project "X-linked or autosomal rare mental retardation syndromes", we focussed on the following main points concerning RTT:

1. Although several RTT mouse models have been generated that recapitulate most RTT symptoms, their behavioral phenotyping is far from complete and mainly carried out at the adult stage.

A colony of a RTT mouse model which expresses a truncated form of MeCP2 [Mecp2-308], has been set up at the Istituto Superiore di Sanità. Such a mutation (C-terminal deletion) accounts for about 1% of RTT cases and has been found to be associated with milder phenotypes. In fact, Mecp2-308 male mice experience a longer developmental phase with no apparent abnormalities and live longer than Mecp2-null mice, thus allowing an accurate analysis of symptoms progression. We performed a neurobehavioral evaluation across the lifespan, starting from soon after birth till adulthood, primarily focussing on the early phases of development (spontaneous general movements and emotional communicative behavior – namely, ultrasonic vocalizations). Such analysis can be especially informative in models of human neurodevelopmental disorders with onset of neurological and cognitive symptoms already during infancy. Moreover, both RTT patients and MeCP2 mutant mice have been initially reported to show an apparently normal period before the onset of clinical symptoms, and this feature of the disease is at the moment one of the diagnostic criteria for RTT. Increasing evidence from both clinical and animal studies, however, support the presence of earlier defects (i.e. during that developmental phase previously regarded as asymptomatic). The results we obtained evidenced subtle anomalies during the first postnatal week (so-called pre-symptomatic phase) in spontaneous general movements. Specifically as early as postnatal (pnd) 3, mutant pups exhibited more intense curling and more side responses and on pnd 9 more pivoting and head rising behaviors than wild type (wt) littermates. A significant decrease in ultrasonic vocalization rate, also emerged in Mecp2-308 pups. The same mice also were characterized by increased anxiety-like behaviors during the early symptomatic phase at pnd 60, in the absence of changes in cognitive passive-avoidance task and rotarod performances. Later on, upon the clearly symptomatic stage, five-month-old Mecp2-308 mice were associated with a marked reduction in spontaneous home-cage motor activity and by a more marked profile of d-amphetamine (10mg/kg) released stereotyped behavioral syndrome than wt controls. Present results emphasize the need for an increased attention devoted to the pre-symptomatic phase which may be especially informative in mouse models of human neurodevelopmental disorders. This analysis has provided evidence of precocious behavioral markers of RTT and has identified an early developmental window of opportunities on which potential therapies could be investigated.

2. Central cholinergic hypofunction has been reported in RTT patients, but this issue has not been investigated so far in RTT mouse models. We studied the functional status of the brain cholinergic function and the long-term effects of a postnatal choline supplementation (from birth till weaning) in the truncated MeCP2-308 mouse model of RTT. When adults, in an open-field test, MeCP2-308 hemyzygous mice were characterized by reduced basal locomotor/exploratory activity. They also failed to exhibit an hyperactivity profile in response to a challenge with the cholinergic antagonist scopolamine (2 mg/kg) challenge, indicating an alteration of central cholinergic tone. Consistently, MeCP2-308 hemyzygous mice also evidenced lower striatal ChAT activity, together with increased levels of NGF protein in the hippocampus, indicative of a loss of retrograde transport of NGF by cholinergic basal forebrain nuclei. By contrast, no significant genotype differences were evident in cortical NGF protein levels. In the absence of changes in wt animals, early choline supplementation compensated the basal reduction of locomotor activity shown by MeCP2-308 hemyzygous mice compared to wt controls but did not affect scopolamine responsiveness. Choline increased ChAT activity in the striatum, as well as NGF hippocampal and cortical mRNA levels of both MeCP2-308 hemyzygous and wt mice at adulthood. By contrast, choline increased NGF protein

levels only in wt animals, whereas it did not affect the already elevated NGF protein levels of MeCP2-308 hemyzygous mice. In the absence of Mecp2-308 mutation-related changes in basal hippocampal and cortical BDNF protein and mRNA levels, choline upregulated these parameters. As a whole, postnatal choline supplementation was able to attenuate some of the behavioural as well as neurobiological abnormalities of the Mecp2-308 phenotype. In view of the cholinergic deficit evidenced in mutant mice, the choline-induced increases of ChAT activity appear particularly promising.

As for Kruppel-like factor 7 (Klf7), a brain developmental gene responsible for autosomal rare mental retardation syndromes, analyses have been carried out in collaboration with C. Perrone-Capano e M. G. Miano, Istituto di Genetica e Biofisica, CNR, Napoli, Italy. Klf7 is a transcription factor specifically expressed in the nervous system during embryogenesis; Klf7 null mice die at birth and share severe defects in olfactory bulbs development and dendritic differentiation troughout the CNS (Central Nervous System). In order to understand the KLF7 role in cellular differentiation, we analysed the effect of KLF7 depletion in various *in vitro* systems. First, we used embryonic stem (ES) cells to analyse Klf7 involvement in neuronal and cardiomyocytic differentiation and we found that Klf7 gene silencing leads to an impairment of functional cardiomyocytes and to a delay in neuronal outgrowth. Moreover, we have analysed the Klf7 potential role in ES cells self renewal pathways and we found that Oct4 or Nanog depletion results in an increase of Klf7 mRNA. Then we found that Klf7 silencing in PC12 cell line results in a strong reduction of NGF receptor TrkA and dendrite marker MAP2 transcripts. We further investigated if Klf7 has a role in mesenchymal differentiation comparing murine embryonal fibroblasts (MEF) from wt and Klf7 KO mice. Intriguingly, we found that Klf7 KO MEF show a decreased potential to differentiate to adipocytes and an increased potential to differentiate to osteocytes. In order to investigate the Klf7 function in mouse brain development, we have performed a transcriptional analysis of neuronal markers on CNS tissues dissected from Klf7 KO compared to wt mice. We found that in newborn Klf7 null mice there is a significative reduction of dopaminergic markers, such as tyrosine hydroxylase (TH) and dopamine transporter (DAT) in the ventral midbrain and in the olfactory bulbs. We have assessed the human genomic features of the homologue counterpart of Klf7. In humans, the genomic region where the Klf7 human counterpart resides (2q31-33) has been repeatedly associated with MR. Starting with in silico analysis, we carried out a genomic characterization of the human KLF7. Local alignment of the sequences demonstrated that the human KLF7 gene spans approximately 86 kbs and is composed of 4 exons. On the basis of the nucleotide sequence features coming from two predicted transcripts, we designed in the flanking intronic regions a panel of specific oligonucleotide primer pairs for each predicted exon. They will be used to set up PCR amplifications of human genomic DNA obtaining the corresponding KLF7 fragments, the sequence of which were inspected to verify the intron/exon structure.

To the aims of this project, a Ph.D position has been appointed, lasting three years.

In 2008, we entered as Partner group for neuro-biohavioural aspects in the European Network on Rett Syndrome in the frame of the E-RARE Program, funded by European Union. During the meeting organized within the EuroRETT framework, the results we obtained under this ISS-NIH project have been presented and discussed with the other European Partners.

Papers published within the project

Canese R, Adriani W, Marco EM, DePasquale F, Fabi F, Lorenzini P, deLuca N, Podo F, Laviola G. Peculiar response to methylphenidate in adolescent compared to adult rats: A phMRI study. *Psychopharmacology* 2008;203(1):143-53.

Cirulli F, Laviola G, Ricceri L (Ed.). Risk factors for mental health: translational models from behavioural neuroscience *Neuroscience & Biobehavioral Reviews* 2009;33(4):493-7.

De Filippis B, Ricceri L and Laviola G. Early postnatal behavioral changes in the Mecp2-308 truncation mouse model of Rett syndrome. *Genes, Brain and Behavior* 2009 (in press).

De Filippis B, Ricceri L, Laviola G. Investigating Rett syndrome through genetic mouse models: pre-symptomatic, clearly symptomatic phases and innovative therapeutic approaches. In: Kalueff A, Bergner CL (Ed.). *Transgenic and mutant tools to model brain disorders*. Humana Press Inc.; 2009.

Gioiosa L, Chiarotti F, Alleva E, Laviola G. A trouble shared, is a trouble halved: social context and status affect pain in mice dyads. *PLoS ONE* 2009:4(1):e4143.

Laviola G, Ognibene E, Adriani W, Romano E, Keller F. Gene-environment interaction early in development in the heterozygous reeler mouse model: Clues for modelling of major neurobehavioral syndromes. *Neuroscience & Biobehavioral Reviews* 2009;33(4):560-72.

Leo D, Adriani W, Cavaliere C, Cirillo G, Romano E, di Porzio U, Papa M, Perrone-Capano C, Laviola G. Methylphenidate administration to adolescent rats drives enduring changes of accumbal Htr7 expression, impulsive behavior and neuronal morphology. *Genes, Brain and Behavior* 2009:8(3): 356-68.

Macrì S, Granstrem O, Shumilina M, Antunes Gomes dos Santos F J, Berry A, Saso L, Laviola G. Resilience and vulnerability are dose-dependently related to neonatal stressors in mice. *Hormones and Behavior* 2009 (in press).

Macrì S, Leussis MP, Laviola G, Andersen SL. Abnormal development in the younger sibling receiving less maternal care: an environmental model. *Psychoneuroendocrinology* 2009 (in press).

Macrì S, Pasquali P, Bonsignore L, Pieretti S, Cirulli F, Chiarotti F, Laviola G. Moderate neonatal stress may decrease within-group variation and increase experimental validity in adult mice. *PloS ONE* 2007:2(10):e1015.

Ricceri L, De Filippis B and Laviola G. Altered cholinergic function and NGF levels in Mecp2-308 mice: consequences of early choline supplementation. *Neurobiology of Disease* 2009 (submitted).

Ricceri L, De Filippis B, Laviola G. Mouse models of Rett syndrome: from behavioural phenotyping to preclinical evaluation of new therapeutic approaches. *Behavioural Pharmacology* 2008;19(5-6):501-17.

Scattoni ML, Crawley J, Ricceri L. Ultrasonic vocalizations: a tool for behavioural phenotyping of mouse models of neurodevelopmental disorders. *Neurosci Biobehav Rev* 2009:33(4):508-15.

Scattoni ML, McFarlane HG, Zhodzishsky V, Caldwell HK, Young WS, Ricceri L, Crawley JN. Reduced ultrasonic vocalizations in vasopressin 1b knockout mice. *Behav Brain Res* 2008:5;187(2):371-8.

STUDY OF THE GENETIC SUSCEPTIBILITY AND ENVIRONMENTAL FACTOR INVOLVEMENT IN THE ETIOPATHOGENESIS OF AUTISM

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Introduction

Autism is a severe neurodevelopmental disorder thought to be associated with brain abnormalities and characterized by an early onset in childhood. Both genetic and non genetic causes have been involved in the pathogenesis of this condition. The wide phenotypic variability of ASD likely indicates that distinct genes and gene combinations, as well as the presence of interaction of multiple genes within an individual's genome, play a role in this disease. A great interest was risen about MHC gene involvement in ASD development. Recent data suggested the importance in autism of an extended haplotype that comprises a relatively constant sequence of DNA over a large multi-region of the MHC, inclusive of, but not limited to, the HLA-B through the HLA DR regions. This haplotype contains the complement C4B null allele in the class III region, DRβ1*04 (DR4) in the class II region, and A2 and B44 alleles in the class I region. Although the HLA−A- and HLA-B-encoded alleles have similar functions, they are in linkage disequilibrium with different genetic loci in two different polymorphic blocks (α and the ß) which are conserved in the HLA region.

On these bases we expanded our previous analyses on HLA alleles in ASD to shed further light on the possible associations between such alleles and disease development. In particular we focused on the α and the ß blocks. The α block includes HLA-A and myelin oligodendrocyte glycoprotein (MOG) genes and was investigated using D6S265, MOGc and rs2857766 markers. The ß block includes HLA-B and was analysed using MIB and TNF markers. The decision to focus our attention on MOG stems from the fact that MOG localizes in close proximity with the HLA-A region and from the observation that antiMOG antibodies have been observed in the serum of ASD children.

Finally, the SLC6A4 gene, coding for the human serotonin transporter (5-HTT) is regarded as a good candidate gene for autism susceptibility, as serotonin seems to play a key role in a range of behaviours and psychological processes, and an increase in whole blood serotonin has been observed in subjects with ASD. A previous study performed in our laboratory on a group of Sardinian families with autistic subjects, evidenced a linkage between the HLA and 5-HTTLPR genetic regions and ASD.

The principal aims of this study were: 1) The definition of the linkage between the HLA and 5-HTTLPR genetic regions and ASD in Italian population by the enrolment of Italian peninsular and Sardinian ASD subjects together with their relatives; 2) The study of the genomic regions in and around the HLA loci to find out a genetic polymorphism specifically related to ASD susceptibility 3) The verification of possible structural damages in the WM and in the GM in

ASD, using conventional and modern MRI. 4) The possible definition of distinct genetic and structural MRI pattern in the different subtypes within the spectrums of ASD.

Cases and methods

A total of 118 ASD patients with their relatives were enrolled after informed consent signature. Only 57 legal guardians gave consent to MRI analysis. We enrolled 52 families of Sardinian ancestry and 63 families from peninsular Italy, all of whom had at least one autistic child. The diagnosis was made according to DSM-IV-TR criteria. DNA was extracted both from saliva or blood depending on the available biological material.

All genetic and clinical as well as MRI data were recorded in two databases distinct regard to peninsular or Sardinian ancestry. We used an intrafamilial case control method of analysis (AFBAC Affected Family-BAsed Controls) and furthermore to assess preferential allelic transmission from heterozygous parents to affected offspring, the TDT test was performed. Moreover chi square analysis was applied to evaluate correlations between genetic polymorphisms and MRI values.

Results

HLA

A 6 Mb region spanning the HLA region ranging from the HLA-DR to the HFE gene was analysed, focusing our study on α and ß blocks. Hence we analysed α block by microsatellites (MSat): D6S265, MOGc and a single-nucleotide polymorphism (SNP) (rs2857766), ß block was evaluated using Msat (D6S2810(MIB), and two SNPs of TNF alfa gene promoter (TNF α-238 and TNF α-308). The Msat D6S2239 was analysed as delimitation at telomeric region near HFE gene. Finally, HLA–A, HLA-B and HLA-DR genes were systematically typed as well in ASD children and their relatives; both stratification and haplotype-based analysis were performed in order to take into account their potential linkage disequilibrium (LD).

The presence of positive associations with ASD for D6S265*220 ($p<0.01$); and MOGc*131 ($p<0.05$) and of negative associations for MOGc*117 and MIB*346 alelels ($p<0.01$) in ASD Sardinian children was observed. Polymorphism haplotype analysis evidenced that the D6S265 allele*220 and the MOGc allele *131 were significantly more likely to be transmitted together, as a whole haplotype to ASD children ($p<0.05$). Conversely the D6S265*224-MOGc*117-rs2857766(G) haplotype was significantly less transmitted to ASD children ($p<0.01$).

The same analysis performed in peninsular ASD revealed a negative transmission of MOGc*133 ($p<0.05$) and positive association with MIB*332 ($p<0.05$). No statistical significance were observed for haplotype transmission.

Independently by the positive or negative association with a specific allele which are different in Sardinian and in peninsular ASD subjects, we may argue that both MOGc (in block α) and MIB (in block ß) loci are associated to ASD. Since we have not evidenced possible relationships between these alleles, the modulation of the gene product, and a plausible functional explanation toward ASD development, these alleles may not be considered as directly responsible for susceptibility or protection against ASD. However they may represent gene markers in linkage disequilibrium with other, yet unidentified genetic factors that are directly involved in the pathogenesis of ASD.

5-HTTLPR

Molecular genotyping of the short (434bp) and long (528bp) variants of the 5-HTTLPR polymorphism was performed both in Sardinian and in peninsular ASD subjects. The analysis of the 5-HTTLPR allelic frequencies revealed that the short variant was present in 57% of Sardinian autistic children as well as in 56% of their healthy sibs and in 39% of peninsular ASD subjects similarly to their healthy sibs (41%). The TDT was also performed for the 5-HTTLPR polymorphism, but no differences emerged between allele transmission and non-transmission for both the long and the short variants.

MRI

The autistic group in our study was also stratified taking into account clinical aspects. Fifty six children were screened by MRI and alterations were revealed in 25%.

The HLA and 5-HTTPLR data were analyzed and compared for MRI, EEG and Childhood Autism Rating Scale (CARS) No specific correlations emerged from this analysis (data not shown).

Finally, we examined whether the "autism endophenotype" observed in some ASD sibs would extend its effects on the immune system. Multiple immune parameters were analyzed in autistic children (AC; n. 20), their siblings (HSAC; n. 15), and age-and sex-comparable healthy controls (HC; n.20) without any familiarity for autism.

The immune profiles of HSAC were significantly more similar to those of their autistic siblings than to what was observed in HC. Thus, in AC and HSAC compared to healthy controls: 1) proinflammatory and IL-10-producing immune cells were augmented ($p < 0.01$ in both comparisons); 2) CD8+ naïve (CD45RA+/CCR7+) T lymphocytes were increased ($p < 0.0001$ and $p=0.001$); and 3) CD8+ effector memory (CD45RA-/CCR7-)($p<0.0001$ and $p=0.03$) as well as CD4+ terminally differentiated (CD45RA-/CCR7+)($p<0.05$ in both comparisons) lymphocytes were diminished.

Conclusions

Results of these studies indicate a linkage disequilibrium of HLA region with ASD suggesting the involvement of immune system in ASD development. Moreover a complex immune dysfunction is present in autistic children but also and in their non-autistic siblings showing the presence of an "autism endophenotype" that expands its effects on immunologic functions. Thus, the interaction between genes, environment and other still undefined factors can result in full-blown autistic disease, or can be associated with faint, subclinical features. The data herein, although preliminary, indicate that the "autism endophenotype" extends its effects to the immune system. The observation that autism is likely associated with given alleles encoding within the HLA region, a region of pivotal importance in immunity, offers a speculative justification to our results and opens novel research avenues.

Papers published within the project

Grezzo A, Guerini FR, Bolognesi E, Matteoli M, Manca S, Sotgiu S, Bejor M, Clerici M, Chiappedi M. Neuropsycological gender differences in healthy individuals and in pediatric neurodevelopmental disorders. A role for SNAP-25. *Medical Hypotheses* 2009;73(6):978-80.

Guerini FR, Bolognesi E, Manca S, Sotgiu S, Zanzottera M, Agliardi C, Usai S, Clerici M. Family-based transmission analysis of HLA genetic markers in Sardinian children with autistic spectrum disorders. *Hum Immunol* 2009;70184-90.

Saresella M, Marventano I, Guerini FR, Mancuso R, Ceresa L, Zanzottera M, Rusconi B, Maggioni E, Tinelli C, Clerici M An Autistic Endophenotype Results in Complex Immune Dysfunction in Healthy Siblings of Autistic Children. *Biological Psychiatry* 2009;66(10):978-84.

A NOVEL PHARMACOLOGICAL APPROACH AND IDENTIFICATION OF PERIPHERAL CELLULAR BIOMARKERS IN NIEMANN-PICK TYPE C DISEASE PATIENTS

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Introduction

Niemann-Pick Type C disease (NPC) is an autosomal recessive fatal disorder (1:150,000 of newborns) caused by mutations in the NPC1 gene (~95% of the cases) or NPC2 gene. NPC shows abnormal intracellular accumulation of cholesterol and sphingolipids associated with peripheral and central organ dysfunction. Among the neurological symptoms are ataxia, dystonia, seizures and cognitive decline until severe dementia Cholesterol seems to play a major role in synapse formation and activity, and is crucial to the formation of dynamic membrane microdomains, so-called lipid rafts. Lipid rafts provide both a temporal and a spatial meeting point for crucial signalling molecules involved in myelination, growth factor signal transduction, cell adhesion, axon guidance, membrane trafficking and molecular sorting, electrical activity and synaptic function. Recent studies have provided evidence that rafts are involved in the regulation of receptors, including AMPA, Kainate and NMDA-type glutamate receptors. The loss of a correct dynamic of cholesterol-sphingolipids-enriched microdomains in the neuronal plasma membrane caused by an imbalance in the lipid trafficking due to NPDc gene mutation, could have a key role in neuronal dysfunction and the consequent neurological disturbs. Our previous results, indicate that, in cholesterol depleted neurons, AMPA-kainate- and NMDA-receptors, that are embedded in lipid rafts, play a pivotal role in the impairment of synaptic transmission and synaptic plasticity. The involvement of AMPA-and kainate-receptors was confirmed by fluorimetric analysis of intracellular calcium concentrations in hippocampal cell cultures. Moreover in *in vitro* model of ischemia, disruption of lipid rafts prevents the expression of the ischemic long term potentiation (i-LTP), which is an aberrant form of synaptic plasticity. There are indications that NPDC may be associated with alterations in membrane lipid rafts. Our published data yield a basis to investigate the mechanisms involved in neurological symptoms in NPDC. For this purpose as first step we established a colony of NPC1 -/- mice, which represent a well known experimental model of NPDC, since they display most of the clinical features of the disease, including cognitive deficits. Successively we evaluated through several experimental approaches whether the physiological properties and the neurotransmission of NPC neurons show differences from what observed in the wild type (WT). To this aim acute brain slices, primary neuronal cell cultures and synaptosomal preparation from WT and NPC1 -/- mice, were used.

Despite no specific treatment is so far available for NPC disease, a number of therapeutic options are available to improve quality of life. To achieve this, there is a pressing need for newborn screening to identify affected individuals early, before the onset of severe irreversible pathology. The possibility to mark intracellular organules rich in sphingomyelin, through specific antibody, allows to early evidentiate membrane lipid modification. In order to develop a method for a rapid and suitable diagnosis, we used some fibroblast cell lines obtained from patients.

Methods

Intracellular Calcium measurements

Intracellular calcium concentration [Ca2+]i has been measured in mouse primary hippocampal cultures (ED17) from WT or NPC1 -/- mice, prepared as previously described. Cells were used at 12-14 days *in vitro*. The fluorescent dye Fura2AM was used to evaluate the amount of [Ca2+]i. In each experiment a 3 min control recording was performed to evaluate basal [Ca2+]i. N-methyl-D-aspartate (NMDA), α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) and kainic acid (KA) have been applied by directly dropping in the cell bath. Electrophysiological recordings: Hippocampal slices were prepared by using conventional methods. Basal synaptic transmission (BST), Paired Pulse Stimulation (PPS) and Long Term Potentiation (LTP) were studied both in WT and NPC1 -/- mice slices. Orthodromic stimuli (10-500 microA, 20-90 micros, 0.1 Hz) delivered through a platinum electrode placed in the stratum radiatum in the CA3 region induced a population spike (PS) in the pyramidal layer of the CA1 subfield. After recording stable signal for 20 minutes, the modification in amplitude and in duration of the synaptic potentials after paired pulse facilitation and after tetanic stimulation by using a train at 100Hz for 1 sec was studied. In another series of experiments BST was studied during bath application of AMPA and kainic acid (KA).

Immunoblot analysis

For receptors levels determination in synaptosomal membrane fraction, subcellular fractionation of hippocampal slices from WT or NPC-/- mice was performed. Western Blot analysis and band quantitation were performed as previously described. The following antibodies were used: rabbit anti-GluR1, anti-GluR6/7 and anti KA2 antibodies (Upstate), rabbit anti-NR2A (Chemicon), rabbit anti-phospho p42/p44, anti phospho-CAMKII, anti-p42/p44, and anti-CAMKII (Cell Signaling), anti-actina antibody (Sigma). Fibroblast culture: Skin fibroblasts from two patients with Niemann-Pick disease type C and two normal subjects were established and maintained in our laboratory. The diagnosis of NPC was established by genetic analysis. Fibroblasts were cultured and maintained in RPMI medium containing 10% (v/v) fetal calf serum at 37 °C in a 5% CO2, 95% air incubator. Fibroblasts after washing with PBS, were fixed with 3.7% (w/v) formaldehyde in PBS, washed, and permeabilized with Triton X 100 (1%) for 10 min, blocked with 2% BSA-PBS for 15 min. The cells were incubated with StnII (10 ug/mL in 2% BSA-PBS) for 2 h, washed with PBS, and incubated with anti-StnII antiserum diluted 1/1000 with 2% BSA-TBS for 1 h, followed by incubation with 10 mg/mL tetramethyl rhodamine isothiocyanate-conjugated goat anti-mouse IgG (Chemicon International Inc., Temecula, CA) in 2% BSA-PBS for 1 h. Fluorescence microscopy was performed using a Olympus Fluoview 1000 Confocal Laser Scanning System Microscope. For FACScan analysis

cells were treated as above described and analyzed with a FACScan Flow Cytometer (Becton-Dickinson, CA); fluorescence was measured between 565 and 605 nm. The data was acquired and analysed by the Lysis II.

Results

In our electrophysiological results input-output curve, BST and PPF didn't show significant differences in CA1 pyramidal layer of hippocampal formation both in WT and NPC1 -/- mice slices. On the contrary, synaptic plasticity was affected: in fact induction and maintenance of NMDA-dependent LTP in the CA1 region of NPC1 -/- hippocampal slices were significantly reduced, suggesting that NMDA receptor activity is impaired. In line with these observations, we found a significantly reduction in NMDA-induced calcium influx in NPC cultured hippocampal neurons as compared with WT neurons. To evaluate if glutamate receptors display functional changes in NPC1 -/- mice slices, we studied synaptic transmission during pharmacological stimulation by adding to the bath solution KA and AMPA (2 M). While the perfusion of KA depressed synaptic transmission at CA3-CA1 synapses in both groups of animals, followed by a recovery during washout only in NPC1 -/- mice slices, AMPA application induced a disappearance of synaptic transmission only in NPC1 -/- mice slices; these results indicate that AMPA-Kainate receptors present quantitative and/or a qualitative differences probably linked to lipid rafts disruption. Moreover electrophysiological results are supported by data outcoming from cell culture experiments on excitatory aminoacid-induced intracellular calcium increase. Indeed, application of KA and AMPA in WT and NPC1 -/- cultured cells induced different calcium influxes, which were increased in homozygote cells. However, Western Blot analysis of synaptosomal membrane fractions from NPC and WT mice did not reveal any significant difference in AMPA (GluR1), KA (GluR6/7, KA2) or NMDA (R2) receptor expression in the two groups of animals. We further investigated whether this could result in compromised intracellular signal transduction pathways in NPC -/- slices following kainic acid perfusion.

To this aim, we monitored, by western blot analysis, ERK1/2 (p42/p44) and CaMKII phosphorylation status in hippocampal slices from NPC -/- and WT mice as a readout of intracellular kainic acid-mediated signalling. As expected, both p42/p44 and CaMKII were phosphorylated in a time-dependent manner following kainic acid perfusion, thus suggesting that both the kinases were indeed activated by the treatment. However, no differences between NPC-/- and WT slices were found in posphoERK and pospho CaMKII levels indicating that other pathways may underline the impairment of the excitatory neurotransmission recorded in the CA1 region of hippocampus from NPC -/- mice. Further studies are required to clarify this point.

In order to develop a method for a rapid and suitable diagnosis, we used some fibroblast cell lines obtained from patients. We investigated whether a protein (StycholysinII - StnII) purified from the Stichodactyla helianthus (Coelenterata), could be used in order to develop a precise and rapid diagnostic method for NPC disease. This assumption is based on the extremely high affinity of the toxin for the sphingomyelin, a lipid that accumulates in the endo-lysosomal compartment of NPC patients' cells.

Using a rhodamine-labelled monoclonal antibody anti-StnII, produced in our laboratory, we were able to identify with immuno-fluorescence microscopy, the sphingomyelin storages in fibroblasts obtained from NPC patients' biopsies. Using the same strategy, we also were able to quantify sphingomyelin accumulation by FACscan method. FACscan analysis revealed that the fluorescent-labelled StnII accumulation appears higher in fibroblasts from NPC patients than in

cells from control subjects, indicating that this approach could be useful to develop a novel diagnostic method to identify the disease.

Conclusions

Data obtained, might be useful not only to develop more accurate methods for NPDC diagnosis, but also as a tool to clarify the molecular basis of the disease and develop new therapeutic strategies.

Papers published within the project

Frank C, Rufini S, Tancredi V, Forcina R, Grossi D, D'Arcangelo G. Cholesterol depletion inhibits synaptic transmission and synaptic plasticity in rat hippocampus. *Exp Neurol* 2008;212:407-14.

Rufini S, Grossi D, Frank C, Luly P, Tancredi V, D'Arcangelo G. Cholesterol depletion inhibits electrophysiological changes induced by anoxia in CA1 region of rat hippocampal slices. *Br Res* 2009;1298:178-85.

RISK FACTORS INVESTIGATION
IN THE PATHOGENESIS OF THE BLADDER
EXTROPHY-EPISPADIAS COMPLEX

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The Bladder Extrophy-Epispadias Complex (BEEC) is a multifactorial disorder including congenital malformations of genitourinary tract such as epispadia, bladder extrophy and cloacal extrophy. BEEC is a defect of the early morphogenesis of the excretory and reproductive systems, originating from altered fusion of the genital tubercles and lower abdominal wall occurring during the first trimester of gestation. There is a severity gradient from epispadia (failure of upper urethral closure) through to bladder exstrophy (rupture of the cloacal membrane after separation of the genitourinary and digestive tracts) and cloacal exstrophy (rupture prior to descent of the urorectal septum).

Endocrine disrupters (http://www.iss.it/inte) are contaminants of food chains and environment that impair hormone homeostasis, and especially the estrogen/androgen balance, critical for the development of the male reproductive system. Indeed, consistent experimental data and suggestive epidemiological studies indicate an association between frequent genitourinary malformations (i.e. chryptorchidism, hypospadia) and endocrine disrupters. The endocrine disrupters involvement in the pathogenesis of the more rare and severe BEEC is plausible, but no scientific data till now can support this hypothesis. Among endocrine disrupters, polychlorinated biphenyls (PCBs) deserve particular attention both because of widespread exposure, whose type and extent are related to diet and living environment, and the ability to disrupt a number of highly relevant developmental pathways. PCBs share a general potential for bioaccumulation due to their stability and lipophilicity, but groups of congeners may have different mechanisms.

The aim of BLADE project is to investigate the possible involvement of PCBs in the BEEC pathogenesis at exposure levels and patterns detected in the general population.

The first step has been to model the internal exposure to different PCBs congener groups in the Italian population. To this purpose, PCBs congeners levels were derived from findings in human adipose tissues from the general population. Such concentrations were then transformed on the basis of the serum lipid content and grouped in three mixtures based on their structure and possible similarities in mode of action, namely: G1 (potential estrogenic action) (PCB 44, 49, 52, 101, 174, 177, 187, 201), G2 ("dioxin-like", immunotoxic, antiestrogenic) (PCB 77, 81, 105, 114, 118, 126, 169) and G3 (high potential for bioaccumulation and CYP activation) (PCB 99, 153, 180, 183, 196, 203). This result has been the starting point for the experimental hypothesis, i.e., when tested at low, realistic exposure levels, the congeners within each group might act in an additive way based on common modes of action.

Human foetal corpora cavernosa cells (hfPSMC) were used as *in vitro* model for BEEC target tissues: hfPSMC are primary foetal cells, obtained and established from four 9-10 wks fetuses. The male origin was confirmed by both chromosomal and PCR analysis for sry gene. Moreover, characterization of cells by immunocytochemistry, FACScan and RT-PCR analysis for specific muscle cells biomarkers (α-SMA, vimentin, and desmin) confirmed their smooth muscle origin. To our best knowledge, BLADE represents the first attempt to establish and

optimize this model for investigation on developmental toxicology and malformation pathogenesis.

hfPSMC were exposed to the three PCBs mixtures in order to evaluate the modulation of selected, BEEC-relevant, protein or gene expression.

The cytotoxicity of G1, G2 and G3 congeners' concentration was evaluated by the MTS assay in hFPSM; no effect on cell viability was observed, lending further support that no overt, general toxicity has to be expected from PCBs levels occurring in the general population.

Cells were then treated with the three mixtures for 72h, collected and extracted for their total protein or RNA content. The experiments were performed in triplicate.

Since several publications indicate a role of fibroblast growth factor 8 and 10 and bone morphonenetic protein 4 and 7 during genital tubercle formation and in the regulation of apoptosis in both the distal urethral epithelium and the distal mesenchyme, respectively, we assayed their possible modulation after PCBs exposure by Western Blot analysis. Sample protein levels resulted below the limit of detection with no modulation by PCBs mixtures observable.

The RNA samples were analysed for their gene expression modulation by microarray analysis using Agilent platform 4x44K featuring the whole human genome (~40.000 genes). Data were analysed and genes were considered statistically significant and differentially regulated with a fold-change greater then 1.2 and a p-value < 0.01. The choice for a 1.2 fold-change threshold has been supported by the foetal origin of the cells, where even a 20% gene expression alteration could strongly influence their development. Real-time PCR on four selected genes confirmed the microarray results as a preliminary validation.

Overall, the three PCBs mixtures displayed three different expression profiles, with only about 12% of modulated genes being altered by all three PCBs congeners groups; therefore, on the whole, the three groups appeared to behave as three different endocrine disrupters.

Moreover, the functional analysis of modulated genes showed a different enrichment in Gene Onthologies (GO) categories by PCB mixtures treatment. The genes modulated by all three PCBs mixtures are enriched in GO terms related to cell proliferation, differentiation and development, as well as terms related to cell-cell signalling. Additionally, the analysis of genes modulated by each PCBs mixture, revealed specific modes of action exerted by the different groups:

G1 - (the "potential estrogen-active") appears to affect some genes involved in lipid metabolic processes in particular those coding for enzymes with transferase activity;

G2 - (the "dioxin-like", hence more widely toxic) appears to affect the organization of actin filament processes as well as macromolecule and protein modification;

G3 - (the "CYP-modulating") appears to affect the cellular defense response (to wounding or stress) as well as the intracellular signalling cascade.

The results obtained support the previous hypothesis that the three PCBs mixture act in a different way. These findings shed new light on the toxicological assessment of the overall PCBs exposure in epidemiological and biomonitoring studies. Moreover, hfPSMC resulted a suitable model for developmental *in vitro* studies able to highlight genes modulation following realistic exposure levels to food contaminants.

All three patterns identified for the respective PCBs group may be relevant to different steps of BEEC pathogenesis since they seem to unbalance critical processes during early developmental phases, though with some genes in common indicating a total effect of PCBs exposure in the particular tissue and window of exposure observed.

Further analysis of genes network affected by PCBs mixtures are in progress in order to characterize such potential implications in the pathogenesis of this rare malformation.

Papers published within the project

La Rocca C, Alivernini S, Badiali M, Cornoldi A, Iacovella N, Silvestroni L, Spera G, Turrio-Baldassarri L. TEQs and body burden for PCDDs, PCDFs, and dioxin-like PCBs in human adipose tissue. *Chemosphere* 2008:73, 92–6.

La Rocca C, Tait S, Luconi M, Faustman EM, Maggi M, Mantovani A. Modello *in vitro* per lo studio degli effetti di contaminanti alimentari nella patogenesi dell'epispadia. XV congresso nazionale della Società Italiana di Tossicologia (Verona, 19-22/1/09), pag. 126 Abstract Book.

La Rocca C. I possibili effetti dei policlorobifenili sull'apparato riproduttivo maschile. *ARPA rivista* 2009;1:41

La Rocca C. PCBs e malformazioni del sistema riproduttivo maschile: un modello per integrare dati di esposizione e studi sul meccanismo di azione (il progetto BLADE) In: Atti del Convegno. Composti ad attività ormonale nell´ambiente e negli alimenti: quali rischi? (Bologna, 25-26/2/2009) (http://www.arpa.emr.it/pubblicazioni/alimenti/notizie_1280.asp)

Mantovani A, La Rocca C, Luconi M, Faustman EM, Lorenzetti S, Maggi S, Tait S. The bladder extrophy-epispadias complex and exogenous risk factors: the BLADE project. In: Taruscio D, Salvatore M (Ed.). *Workshop. Rare Diseases and Orphan Drugs. Rome, November 7-8, 2007. Abstract book.* Roma: Istituto Superiore di Sanità; 2007. (ISTISAN Congressi 07/C8). p. 73.

Tait S, La Rocca C, Lagatta V, Luconi M, Faustman EM, Maggi M, Mantovani A. PCBs as possible exogenous risk factors in the bladder extrophy-epispadias complex pathogenesis: the BLADE project. In: Taruscio D, Salvatore M (Ed.). *Workshop. Projects on rare diseases funded within the bilateral agreement Italy (Istituto Superiore di Sanità) and USA (NIH, Office for Rare Diseases) on joint research and development of public health actions. Istituto Superiore di Sanità. Rome, October 29-31, 2008. Abstract book.* Roma: Istituto Superiore di Sanità; 2008. (ISTISAN Congressi 08/C10). p. 134.

Tait S, La Rocca C, Luconi M, Faustman EM, Maggi M, Mantovani A. PCBs as possible exogenous risk factors in the pathogenesis of the bladder extrophy-epispadias complex: the BLADE project. In: 5th International Conference on Rare Diseases and Orphan Drugs (Rome, 23-25/2/09)

Tait S, La Rocca C, Luconi M, Faustman EM, Maggi M, Mantovani A. PCBs as possible exogenous risk factors in the pathogenesis of the bladder extrophy-epispadias complex: the BLADE project. In: 37th annual meeting of the European Teratology Society (Arles-France, 6-10/9/09)

Aknowledgements

CELLULAR AND ANIMAL MODELS
FOR THE MYH9-RELATED DISEASES

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The Milan unit has followed up on sets of evidence produced in the previous years, suggesting an interaction between MYH9, another unconventional myosin, i.e. MYO6, and the homeobox-containing transcription factor Prep1: 1) co-IP of MYH9 with the homeobox-containing transcription factor Prep1, 2) co-IP of MYH9 with MYO6, 3) nuclear colocalization, by immunofluorescence, of MYH9 with Prep1, 4) recruitment of MYO6 on the promoter of transcriptionally active genes by Prep1 (by ChIP). Therefore we decided to further investigate by immunofluorescence: 1) the presence of MYH9 in the nucleus and 2) to identify cellular regions where MYH9 interacts with MYO6 *in vivo*. We performed immunofluorescence experiments on three different human cell lines (HeLa, HepG2 and NT2/D1) susceptible of stimulation by phorbol esters. We labeled cells with anti-MYH9 and anti-MYO6 antibodies, counterstained nuclei with DAPI and analysed them by confocal microscopy. The results show that in all cell lines, in the absence of stimuli, MYH9 is abundantly present in the cytoplasm, with a distribution reminiscent of that of actin fibers. However, the protein is also present in nuclei of HeLa and HepG2 cells and is particularly abundant in those of NT2/D1 cells. MYO6 is also present in the nuclei of cells and is more abundant that MYH9. By merging the channels corresponding to the acquired fluorescence for MYH9 and MYO6 we observed protein colocalization both in the nucleus and in the cytoplasm, in particular in the perinuclear region and at the Golgi complex. These observations suggest that although the cellular distribution MYH9 and MYO6 is different, there are specific regions of interactions between the proteins.

In the presence of stimuli, the distribution of MYH9 and MYO6 changes substantially. We observed a substantial decrease of MYH9 in the nuclei of HepG2 and NT2/D1 cells (although the protein is still detectable in this organelle). However, in HeLa cells the cellular distribution of MYH9 was essentially the same as in the absence of stimulus. The signal of MYO6, on the other hand, appeared to be more considerably nuclear than in the absence of stimuli. Interstingly, while in HeLa and HepG2 cells colocalization of MYH9 and MYO6 seemed to decrease, in the latter cell line MYH9 accumulated at specific cytoplasmic sites, in close proximity to cell-cell contacts. On the other hand, in NT2/D1 cells clearly showed an increase in MYH9-MYO6 colocalization. Overlapping MYH9 and MYO6 signals were located mainly in the perinuclear cytoplasmic region, possibly corresponding to the Golgi complex.

Overall the results strongly indicate an interaction *in vivo* between MYH9, MYO6 and Prep1. The MYH9-MYO6 interaction is subject to modulation by external stimuli, mimicked by the phorbol esters treatment of cells. According to the cell line, the same stimulus can favor or impair MYH9-MYO6 interaction in different nuclear compartments. This, in turn, indicates a general "housekeeping" role for the MYH9-MYO6 interaction in the absence of stimuli and a specific, cell context-dependent role for the interaction in the presence of external stimuli, possibly linked to transcriptional activation and the role of Prep1.

The Genova unit pursued the aim of identifying new genetic events involved in the phenotypic and/or prognostic variability of MYH9-RD by investigating pathogenetic

mechanisms responsible for the pehotypic variability in different cellular models and the identification of protein partners of NMMHCIIA, coded for by the MYH9 gene.

After cloning the full-length human MYH9 cDNA and performing specific point mutations, the wild type and mutated constructs were transfected in HeLa and COS-7 cells. The results showed that the mutated protein formed rod-like aggregates in the cytoplasm, as opposed to the uniform distribution of the wild-type protein, indicating that the *in vivo*-observed rod-like structures are formed by the mutated protein. Moreover, co-transfection of the constructs showed that wild-type protein can interact with the mutated one, which, therefore, may act as a dominant-negative on the wild-type protein.

By yeast two-hybrid screening the Genova unit searched for NMMHCIIA interactors, using as bait the portion of the C-terminus of wild-type protein between aminoscids 1645-1960. The rationale for the search is that it is known that NMMHC-A, NMMHC-B and NMMHC-C, coded for by the MYH9, MYH10 and MYH14 genes, respectively, have a different cell-type distribution, although they are present in most tissues, and this may contribute to the variability of the MYH-RD phenotype. By this technique NMMHC-B (MYH10) was identified as an interactor of NMMHC-A and that this interaction occurred through the coiled-coil domain of both proteins. The interaction was confirmed by co-immunoprecipitation of the proteins both in cell systems (HeLa and COS-7) in which the proteins were exogenously co-expressed, and in a cell system (fibroblasts) where both proteins are ordinarily expressed.

The expression profiles of both NMMHC-A and NMMHC-B proteins were then analysed. The results showed that both proteins are expressed in renal glomeruli and in particular in podocytes and in tubular epithelial cells. As for the heamatopoietic lineage, it was shown that both proteins are expressed by erythrocyte precursors, whereas macrophage and granulocyte precursors only express NMMHC-A. These results raise the possibility that the expression of both NMMHC-A and -B in some tissues may have either compensatory effects or modify the pheotype (e.g. through dominant-negative interactions), thus allowing the hypothesis that these may be the molecular bases of symptoms variably associated with the MYH-RD pathogenesis.

The Pavia unit developed the *in vitro* model of human megakaryopoiesis that we subsequently used for investigating the mechanisms of platelet production in patients with MYH9-related disease (MYH9-RD). This *in vitro* model is based on the use of CD45+ cells separated from patients' peripheral blood and differentiated to megakaryocytes in the presence of IL-6, IL-11 and TPO. Large megakaryocytes at day 12 of culture are separated by a BSA gradient (3-4%) and plated in fresh Stem Span medium supplemented with TPO or cultured onto coverslips coated with different component of the extracellular matrix. After 24 hours from replating, several megakaryocytes extend proplatelets and release platelets.

By this technique we studied the *in vitro* proplatelet formation (PPF) by megakaryocytes obtained from 4 patients carrying the p.D1424N or the p.R1933X mutations of the gene MYH9. We demonstrated that MYH9-RD megakaryocytes completely lose the physiologic suppression of proplatelet extension exerted by interaction with type I collagen, thus supporting the hypothesis that a premature platelet release within bone marrow contributes to pathogenesis of MYH9-related thrombocytopenia. Moreover, proplatelets extended by MYH9-RD megakaryocytes presented a significant defect in branching in secondary processes (p=0.001) and formed a significantly lower number of proplatelet tips (p=0.005). Since platelets are assembled at the level of proplatelet tips, this defect could further contribute to pathogenesis of thrombocytopenia of MYH9-RD patients.

Results obtained by this study confirm data obtained in an *in vitro* model of mice megakaryopoiesis suggesting that myosin-IIA is an inhibitor of PPF, and indicating that it is involved in the suppression of PPF exerted by cell adhesion to type I collagen, which therefore regulates the timing of platelet release within bone marrow.

Present study not only produced new information on the pathogenetic mechanisms of thrombocytopenia of MYH9-RD, but also validated the *in vitro* model for investigating megakaryopoiesis of patients with inherited platelet defects. By this approach, we have already studied megakaryopoiesis of patients with mono-allelic Bernard-Soulier syndrome. Moreover, we programmed to use this model for investigating the *in vitro* effect of drugs that are expected to be able to increase platelet count in patients with some forms of genetic thrombocytopenia.

Papers published within the project

Balduini A, Malara A, Pecci A, Badalucco S, Bozzi V, Pallotta I, Noris P, Torti M, Balduini CL. Proplatelet formation in heterozygous bernard-soulier syndrome type bolzano. *J Thromb Haemost* 2009;7:478-84.

Chen Z, Naveiras O, Balduini A, Mammoto A, Conti MA, Adelstein RS, Ingber D, Daley GQ, Shivdasani RA. The May-Hegglin anomaly gene MYH9 is a negative regulator of platelet biogenesis modulated by the Rho-ROCK pathway. *Blood* 2007;110:171-9.

Marini M, Bruschi M, Pecci A, Romagnoli R, Musante L, Candiano G, Ghiggeri GM, Balduini C, Seri M, Ravazzolo R. Non-muscle myosin heavy chain IIA and IIB interact and co-localize in living cells: relevance for MYH9-related disease. *Int J Mol Med* 2006;17:729-36.

Panza E, Marini M, Pecci A, Giacopelli F, Bozzi V, Seri M, Balduini C, Ravazzolo R. Transfection of the mutant MYH9 cDNA reproduces the most typical cellular phenotype of MYH9-related disease in different cell lines. *PathoGenetics* 2008;1:1-5.

Pecci A, Malara A, Badalucco S, Bozzi V, Torti M, Balduini CL, Balduini A. Megakaryocytes of patients with MYH9-related thrombocytopenia present an altered proplatelet formation. *Thromb Haemost* 2009;102:90-6.

IMPROVING DIAGOSTIC SKILLS
FOR INHERITED THROMBOCYTOPENIAS

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The project was aimed at identifying tools and strategies for a correct diagnosis of inherited thrombocytopenias, a heterogeneous group of diseases at both clinical and molecular levels. In addition to diagnose known forms, it is fundamental to clone genes responsible for a series of novel nosological entities, which are relatively frequent being recognized in almost 50% of the families affected.

The results obtained are described according to the specific aims of the project as follows:

- *Identification and etiologic characterization of inherited thrombocytopenias*
  not yet described
  Two new genes, each responsible for novel forms of autosomal dominant thrombocytopenias, have been localized by linkage analysis on chromosome 10p and 17q. While the first gene called TCH2 is still being characterized as new families linked to 10p are recognized, within the candidate region 17q a novel mutation has been identified in ITGB3, a gene encoding for a subunit of the fibrinogen receptor.

- *Identification of genes responsible for inherited thrombocytopenias*
  that have been described previously but whose etiology is still unknown
  We have identified a family with a suspected diagnosis of "gray platelet syndrome". The clinical and morphological platelet features were characterized. A positional cloning strategy is being carried out to identify the gene. A few putative candidate regions are further being studied as new family members are enrolled to define the localization of the gene.

- *Characterization of the mutations causing inherited thrombocytopenia in Italy*
  We identified mutations of the c-MPL gene and clonal chromosomal anomalies in five families with congenital amegakaryocytic thrombocytopenia. Moreover, we have extended the database of Italian Registry of MYH9-Related Disease (MYH9RD) to 82 unrelated families, in 80 of which the diagnosis has been confirmed by molecular genetic testing. The screening of mutation allowed us to identify six novel mutations, extending the limited spectrum of mutations identified so far in the MYH9 gene. In order to validate an immunofluorescence test revealing the presence of MYH9 aggregates in patients' neutrophils as a suitable tool in differential diagnosis, we sequenced the entire MYH9 gene in 36 patients with the clinical features of MYH9RD but with a normal distribution of the protein. Since no mutation was detected, we concluded that neutrophil inclusions of MYH9 are a pathognomonic sign of the disorder and that at least another gene is responsible for a phenotype similar to MYH9RD. Moreover, we have excluded mutations of the cytochrome C (CYCS) gene in 70 patients with features similar to those observed in patients with a defective CYCS.

– *Identification of genotype/phenotype correlations in patients affected by diseases with known etiology and wide phenotypic variability*
In 108 MYH9RD patients from 50 unrelated families we identified a significant correlation between phenotype and genotype at least for the most four common mutations affecting 70% of patients. Thus, the risk of developing kidney failure, cataracts, deafness, and severe bleeding tendency may be predicted. Since some drugs modify the clinical course of kidney damage, patients recognized at risk of renal failure could undergo treatments to prevent or postpone the dysfunction. The genotype and phenotype correlation was also performed in CAMT patients. In this study we did not confirm previous reports and suggested that hematopoietic stem cell transplantation should not be postponed even in those patients whose c-mpl mutations may predict residual activity of c-MPL.

Papers published within the project

De Candia E, Pecci A, Ciabattoni G, Decristofaro R, Rutella S, Yao-Wu Z, Lazzareschi I, Landolfi R, Coughlin S, Balduini CL. Defective platelet responsiveness to thrombin and protease-activated receptors agonists in a novel case of gray platelet syndrome: correlation between the platelet defect and the a-granule content in the patient and four relatives. *J Thromb Haemost* 2007;5:551-9.

Gresele P, Falcinelli E, Giannini S, D'Adamo P, D'Eustacchio A, Corazzi T, Mezzasoma AM, Di Bari F, Guglielmini G, Noris P, Balduini CL, Savoia A. Dominant inheritance of a novel integrin b3 mutation associated with a hereditary macrothrombocytopenia with platelet dysfunction in two Italian families. *Hematologica* 2009;94:663-9.

Maserati E, Panarello C, Morerio C, Valli R, Pressato B, Patitucci F, Tassano E, Di Cesare-Merlone A, Cugno C, Balduini CL, Lo Curto F, Dufour C, Locatelli F, Pasquali F. Clonal chromosome anomalies and propensity to myeloid malignancies in congenital amegakaryocytic thrombocytopenia. *Haematologica* 2008;93:1271-3.

Pecci A, Panza E, Pujol-Moix N, Klersy C, Di Bari F, Bozzi V, Gresele P, Lethagen S, Fabris F, Dufour C, Granata A, Doubek M, Pecoraro C, Koivisto PA, Heller PG, Iolascon A, Alvisi P, Schwabe D, De Candia E, Rocca B, Russo U, Ramenghi U, Noris P, Seri M, Balduini CL, Savoia A. Position of NMMHC-IIA mutations predicts the natural history of MYH9-related disease. *Hum Mutat* 2008;29:409-17.

Pecci A, Granata A, Fiore CE, Balduini CL. Renin-angiotensin system blockade is effective in reducing proteinuria of patients with progressive nephropathy caused by MYH9 mutations (Fechtner-Epstein syndrome). *Nephrol Dial Transplant* 2008;23:2690-2.

Savoia A, Del Vecchio M, Totaro A, Perrotta S, Amendola G, Moretti A, Zelante L, Iolascon A. An autosomal dominant thrombocytopenia gene maps to chromosomal region 10p. *Am J Hum Genet* 1999;65:1401-5.

Savoia A, Dufour C, Locatelli F, Noris P, Ambaglio C, Rosti V, Zecca M, Ferrari S, di Bari F, Corcione A, Di Stazio M, Seri M, Balduini CL. Congenital amegakaryocytic thrombocytopenia: clinical and biological consequences of five novel mutations. *Haematologica* 2007;92:1186-93.

Savoia A, Balduini CL. MYH9-Related Disorders. 2008. In: GeneReviews at GeneTests: Medical Genetics Information Resource. Copyright, University of Washington, Seattle, 1997-2008. Available at http://www.genetests.org

Savoia A, Noris P, Perrotta S, Punzo F, De Rocco D, Oostra BA, Balduini CL. Absence of CYCS mutations in a large Italian cohort of patients with inherited thrombocytopenias of unknown origin. *Platelets* 2009;20:72-3.

HYPERTROPHIC CARDIOMYOPATHY ASSOCIATED TO FABRY'S DISEASE: CARDIOMYOCYTES AND CARDIAC STEM CELL FUNCTIONAL ANALYSIS

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Fabry's Disease (FD), is a rare X-linked recessive disorder resulting from deficient lysosomal hydrolase α-galactosidase A (α-Gal A). This enzyme plays a key role in the glycosphingolipid metabolism and the enzymatic activity defect results in the progressive intracellular glycosphingolipid globotriaosylceramide accumulation in multiple organ system including the heart. In patients with FD, cardiac involvement is characterized by progressive Left Ventricular (LV) wall thickening, mimicking hypertrophic cardiomyopathy with diastolic LV dysfunction and a preserved LV ejection fraction that may decline in the end stage of the disease. The diastolic LV dysfunction has usually been ascribed to myocardial fibrosis in addition to cardiomyocyte hypertrophy and engulfment by glycosphingolipids. Recently studies performed using Tissue Doppler Imaging (TDI) revealed reduced diastolic and systolic functions even before the pre-hypertrophic phase of the disease, suggesting that early alteration of cardiomyocyte may account for the decreased heart functions.

Objectives of this project was to investigate cellular alterations in heart biopsies from patients with Fabry's disease in order to clarify mechanisms involved in the loss of cardiac function which occurs in these patients. Specifically we planned to study cardiomyocytes, vascular cells and Cardiac Stem Cells (CSCs) in FD.

In a first study, mechanical properties of cardiomyocytes from FD endomyocardial biopsies were analyzed and correlated with LV functions. Our results demonstrated that FD derived cardiomyocytes were significantly larger and filled with glycosphingolipids. Computer-assisted histomorphometry showed a mild, although significant, increase in fibrosis in FD patients compared to controls. This data suggest that myocardial fibrosis does not appear to be the predominant mechanism causing diastolic dysfunction in FD cardiomyopathy. When isolated, cardiomyocytes were stretched a significant active and resting tension modification was observed. Active tension was four times lower in FD cardiomyocytes and correlated with extent of myofibrillolysis. Resting tension was six times higher in FD cardiomyocytes than in controls. Protein analysis revealed troponin I and desmin degradation products. Interestingly, in the presence of the Protein Kinase A-catalytic subunit (PKA), a partial decrease in resting tension was observed. Since PKA is a downstream target of the β-adreneregic stimulation, which allow to phosphorylation of myofilament proteins, this result suggest that hypophosphorylation of these target proteins may account at lest in part for the altered passive properties of FD cardiomyopathy. The *in vivo* measures of LV diastolic function, such as TDI long axis lengthening velocity, correlated with the *in vitro* measurements of resting tension indicating that in Fabry cardiomyopathy diastolic LV dysfunction is related to cardiomyocyte stiffening rather then fibrosis.

Glycosphingolipid accumulation in FD occurs not only in CM but also in vascular cells i.e endothelail cells and smooth muscle cells causing progressive vascular dysfunctions. Notably, the occurrence of angina is frequently reported in patients with FD, suggesting that vessel diseases may be present in these patients. Therefore coronary anatomy, flow and reserve as well as hystologic findings were investigated in FD patients with and without angina. At 99mTc sestamibi myocardial perfusion tomography all patients with FD with angina showed an ischemic response to stress test. This response was absent in FD patients without chest pain. Coronary angiography showed structurally normal coronary arteries in all patients with FD. However, FD patients with angina exhibited a prominent slow flow affecting each coronary artery and a slow runoff of the contrast medium. Hystology, performed on endomyocardial biopsies, showed remarkable lumen narrowing of intramural arteries because of hypertrophy and proliferation of smooth muscle and endothelial cells, both engulfed by glycosphingolipids. These results suggest that alteration of small vessels in FD may participate to the progressive myocardial dysfunction associated to the pathology.

CSCs expressing c-kit antigen have been recently identified in human hearts: these cells have been involved in the lifelong process of maintaining cardiac homeostasis. Alterations of CSC functions have been associated to the development of heart failure. Isolation of CSCs from biopsies represents a major limitation for their studies because of the low number that could be obtained. Therefore, at first we set up conditions for CSC isolation and expansion. Auricle fragments were obtained from adult patients undergoing cardiac surgery without FD. From these specimens we isolated a high proliferating plastic adherent cell population that, by FACS analysis performed after 30 days culture, expressed mesenchymal markers (CD105, CD44, CD29, CD13, CD93) and was negative for the hematopoietic markers (CD45, Cd34,CD133) and HLA-DR. Among these cells, $3.84\% \pm 2.7\%$ expressed c-kit antigen. Then, hCSC-ckit+ were selected from the *in vitro* expanded total cells and the expression of stemness and lineage differentiation markers analyzed by Real time PCR. Purified hCSC-ckit+ expressed high levels of CXCR4, KDR, and VE-cadherin compared to the total cell population. Moreover, they were negative for the early cardiac markers Nkx2.5 and Tbx5 as well as for the smooth muscle markers α-smooth muscle actin (α-SMA) and smooth muscle myosin heavy chain (smMHC). After 7 days culture, a 5 fold increase in hCSC-ckit+ cell number was observed. However, both c-kit and CXCR4 expression were strongly inhibited while endothelial markers i.e. KDR, VE-Cadherin increased. FACS analysis confirmed these data and evidenced that $96 \pm 3.7\%$ hCSC-ckit+ were KDR+. Finally, about 80% of hCSC-ckit+ cultured cells incorporated AcLDL-DiI and expressed the endothelial marker vWillembrand factor (vWF). These data demonstrate that in our culture conditions, *in vitro* expanded hCSC-ckit+ spontaneously acquired an endothelial phenotype. Therefore, hCSC-ckit+ may represent useful population for autologous cell therapy of cardiac ischemic disease in which the improvement of vascularisation is required. Ongoing experiments are focused to isolate and characterize CSC from FD patients.

Papers published within the project

Chimenti C, Hamdani N, Boontje NM, DeCobelli F, Esposito A, Bronzwaer JG, Stienen GJ, Russo MA, Paulus WJ, Frustaci A, *et al.* Myofilament degradation and dysfunction of human cardiomyocytes in Fabry disease. *Am J Pathol* 2008;172,1482-90.

TUMOR ANGIOGENESIS AND INFLAMMATION AS THERAPEUTIC TARGETS OF RETINOBLASTOMA

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Introduction

Retinoblastoma is the most common intraocular paediatric cancer, affecting 1 in 15000-20000 live births. The disease occurs in heritable form, usually bilateral, and sporadic or unilateral form. In both cases it arises from biallelic mutation of Rb gene. In bilateral form the first mutation occurs within the germline cells, while in the other form the mutational event is sporadic.

It is well established that stromal and immune cells release soluble factors that favor tumor progression by promoting the angiogenic switch through stimulation of dysregulated endothelial cell proliferation. Stromal factors further contribute to tumor growth and dissemination by inducing resistance to death and increased motility in tumor cells. Several lines of evidence indicate that inflammatory angiogenesis also promotes retinoblastoma progression. The main objectives of the project were:

1. to define the signaling pathways mediating the effects of immune and stromal-derived factors (VEGF, IGF-1) on retinoblastoma cell growth and resistance to cell death *in vitro*;
2. to define relevant signaling pathways activated in retinoblastoma cells that mediate tumor stimulated angiogenesis and inflammation *in vivo* (AKT/GSK3β, NFkB);
3. to perform in depth investigation of the biological effects of selected chemopreventive agents on retinoblastoma cells *in vitro*, in cell-free models *in vivo* (retinoblastoma conditioned medium-induced angiogenesis) and on retinoblastoma growth in cell-driven cancer models including xenotransplants and orthotopic models;
4. to study other ocular tumors (i.e., uveal melanoma) to develop intraocular xenograft murine model.

Results and methods

We studied the proapoptotic activity of the anticancer synthetic retinoid N-(4-hydroxyphenyl)retinamide (4HPR, fenretinide), a prototypical prooxidant anticancer agent, in retinoblastoma cells stimulated by the Insulin-like growth factor I (IGF-1). IGF-1 is a potent anti-apoptotic stromal-derived growth factor and sustains the autocrine growth of Y79 cells. 4HPR was used because it kills neurogenic tumor cells (neuroblastoma, retinoblastoma, glioblastoma, meningioma). 4HPR gave encouraging results in a phase I trial in children affected by neuroblastoma. We observed that 4HPR reduces retinoblastoma tumor growth *in vivo* by inducing reactive oxygen species and necrosis-like cell death in Y79 cells. As a consequence of impaired redox equilibrium, 4HPR disrupts energy balance, as indicated by ATP depletion and loss of mitochondrial membrane potential. 4HPR inhibited AKT and mTOR phosphorylation induced by IGF-1 in Y79 cells. To investigate the interference of 4HPR with IGF-1-mediated survival signaling at molecular level, we analyzed the down-stream target and

AKT effector GSK3β, a multifunctional kinase regulating cell survival, glucose metabolism and the beta-catenin/Wnt pathway. GSK3β is inactivated by phosphorylation at Ser9 by AKT and other kinases and is normally active (unphosphorylated) in unstimulated cells. 4HPR sustained GSK3β phosphorylation at Ser9 in IGF1-1-treated retinoblastoma cells. Treatment with 4HPR alone induced time-dependent GSK3β phosphorylation that was coincident with cleavage of poly (ADP-ribose) polymerase (PARP). Perturbation of IGF signaling by 4HPR correlated with the lack of effect of IGF-1 on PARP cleavage and DNA fragmentation induced by 4HPR at 24 h. Time-dependent ROS production by 4HPR preceded activation of antioxidant enzymes, GSK3 phosphorylation and PARP cleavage.

In order to characterize the pro-angiogenic potential of retinoblastoma tumors, we preliminary investigated the angiogenic molecules released by retinoblastoma cells. The Y79 cell line was used to analyze the contribution of the Insulin-like growth factor I (IGF-1)/IGF-1 receptor (IGF-1R) system. IGF-1 is a survival factor for retinal cells, a potent proangiogenic factor and promote angiogenesis by inducing hypoxia inducible factor-1alfa (HIF-1alfa) activation. We suspected that IGF-1 released by Y79 cells could substantially contribute to retinoblastoma -derived angiogenesis. In fact, IGF-1 (10 ng/mL) added to the matrigel plug effectively induced angiogenesis, that was inhibited by 4HPR. The serum-free conditioned medium (CM) from a 48h culture was concentrated and injected subcutaneously into the flanks of C57/bl6 male mice with matrigel. The treatments included the CM from Y79 cells exposed for 48h to the IGF-1R inhibitor H1356 and samples containing H1356 directly added into the matrigel plug along with the CM from untreated cells. The results obtained confirm, as previously shown 1, that Y79 cells release soluble factors capable of inducing a potent angiogenic response in the matrigel plugs, as determined by the quantification of hemoglobin content. The histologic examination of hematoxylin- and eosin-stained sections of the experimental tumors revealed the formation of large vessels. The obtained data suggest that retinoblastoma cells stimulate angiogenesis through the production of soluble factors strongly dependent on the IGF-1R, probably activated by autocrine IGF-1. We observed that retinoblastoma cells express high basal levels of HIF-1alfa. We are now investigating whether constitutive HIF-1alfa expression, which is characteristic of embrionic stem cells, could be related to the staminal properties of retinoblastoma cells. Tumor regression apparently correlates with enhanced expression of HIF-1alfa in necrotic areas, where the inflammatory infiltrate does not seem to increase. These results are in line with a proapoptotic role of HIF-1 that has been documented in several tumor model systems.

We observed that retinoblastoma cells basally produce large amounts of reactive oxygen species 1, 2, as compared with other tumor cell types including Rb-/- melanoma, suggesting that constitutive oxidative stress is of physiological importance in this tumor. Following the idea that elevated oxidative stress in tumor cells could be exploited to selectively induce cell death by prooxidant drugs, we further investigated the apoptotic effects of diverse prooxidants in retinoblastoma. This therapeutical approach received great attention recently, also in the case of retinoblastoma. We compared the activity of 4HPR with arsenic trioxide and the isothiocyanate PEITC in Y79 and Weri-Rb1 cells. We found remarkable differences between the two cell lines in the biological response to anticancer prooxidants. Retinoblastoma cells can activate an antioxidant defense response involving increased expression of the cytoprotective molecules heme oxygenase (HO-1), superoxide dismutase (SOD) and glucose 6-phosphate dehydrogenase (G6PD). The antioxidant response also activates redox cycling of glutathione (GSH). This response is regulated by survival signaling mediated by AKT, GSK3 and ERK. Flow cytometry analysis with the fluorescent dye monochlorobimane revealed the existence of subpopulations of Y79 and Weri-R1 cells containing different levels of glutathione, correlating with different basal level of oxidative stress as indicated by H2O2 content. We selected Y79 and Weri-Rb1

cells resistant to 4HPR. The resistant cells show basal elevated GSH and ROS levels and GSK3β phosphorylation. In conclusion, we found that while transient GSK3β and ERK1/2 phosphorylation is activated by mild oxidative stress, a cytoprotective response is elicited in tumor cells. This mechanism could support the development of drug resistance to anticancer agents. However, persistent GSK3 and ERK1/2 activation can lead tumor cells to death due to activation of a futile antioxidant redox cycle and energy depletion.

We adopted a gene transduction approach using a potent TH1 cytokine with strong anti-angiogenic activity, Interleukin-12 (IL-12). The 99E1, a cell line derived from a choroidal/retinal pigmented epithelial ocular tumour that arose in a transgenic FVB/N mouse bearing the SV40 oncogene, were trasfected with murine IL-12 expression construct. After transfection, the 99E1 cells were selected for transgene expression using Geneticin G-418 sulphate and the resistant clones pooled into two different pools of stably transfected cells. The enhanced levels of IL-12 mRNA expression and protein production were confirmed by RT-PCR and ELISA, respectively. About 6-7 week old female nude mice were inoculated, s.c. and intraocular transplantation, with 99E1 wild type, 99E1 null vector or IL-12 trasfected 99E1 cells.

Orthotopic intraocular injection of control cells led to invasive tumors that destroyed ocular architecture whereas the IL-12 transduced cells rarely formed tumours. When intraocular tumor invaded the eye anterior chamber the tissues were removed for histological analysis. Samples were fixed in formalin, embedded in paraffin using standard procedures, and 3 μm sections were rehydrated and stained with either hematoxylin and eosin or processed for immuno-histochemistry by standard procedures to analyze VEGF and asilo-GM1 expression. Histological analysis revealed highly invasive and angiogenic tumor growth in the controls and poorly vascularized tumors in the presence of IL-12. The tumour repression effect could be reproduced by a systemic anti-angiogenic effect, where controlateral injection of IL-12 expressing cells strongly repressed growth of tumors formed by parental 99E1 cells. This was associated with significantly lowered tumor vessel densities, a trend towards lower VEGF levels in the lesion, and significantly decreased NK cells in the parental tumors exposed to systemic IL-12. Taken together, these data suggest that IL-12 gene transfer can provide anti-angiogenic effects without toxicity and may be particularly suited for therapy of vascularized ocular tumors.

Conclusion

The study of the action of anticancer prooxidant phytochemicals or their synthetic derivatives in retinoblastoma offered us the opportunity to formulate a hypothesis on the apparently paradoxical effects of these compounds. In which way the cytoprotective and normalizing effect in neurological, cardiovascular and metabolic disorders can match the antitumor effect based on induction of apoptosis or other forms of cell death? The explanation is apparently simpler in a cancer chemopreventive setting: redox active phytochemicals that arose during evolution as toxic pesticides in plants, reinforce the stress defense by limiting genotoxicity, mutagenicity and by improving the antioxidant capacity of the cells. But what about the cytotoxic effect on established tumor cells? We hypothesize that the intolerance to drug-induced oxidative stress could be due to the particular bioenergetic requirements of tumor cells which rely on glycolysis for energy production even in the presence of normal oxygen tension (Warburg effect). Although in tumor cells an intact respiratory chain is structurally preserved, its functionality is often reduced. Interestingly, forcing a diversion on mitochondrial respiration for energy production can kill glycolytic tumor cells. The constitutive oxidative

stress found in tumor cells has been related to the overactivation and overexpression of oncogenes including ras and bcr-abl. The requirement for an increased production of reducing equivalents (NADPH) to counterbalance the constitutive oxidative stress could justify at least in part the glycolytic switch for energy production.

Papers published within the project

Albini A, Fassina G, Cammarota R, Barberis M, Noonan D. Inhibition of a vascular ocular tumor growth by IL-12 gene transfer. *Clin Exp Metastasis* 2007;24:485-93.

Albini A, Noonan DM, Ferrari N. Molecular pathways for cancer angioprevention. *Clin Cancer Res* 2007;13:4320-5.

Albini A, Sporn MB. The tumour microenvironment as a target for chemoprevention. *Nat Rev Cancer* 2007;7:139-47.

Albini A. Tumor microenvironment, a dangerous society leading to cancer metastasis. From mechanisms to therapy and prevention. *Cancer Metastasis Rev*. 2008;27:3-4.

Noonan DM, De Lerma Barbaro A, Vannini N, Mortara L, Albini A. Inflammation, inflammatory cells and angiogenesis: decisions and indecisions. *Cancer Metastasis Rev* 2008;27:31-40.

Sogno I, Vene R, Sapienza C, Ferrari N, Tosetti F, Albini A. Anti-angiogenic properties of chemopreventive drugs: fenretinide as a prototype. *Cancer Res.* 2009;181:71-6.

Tosetti F, Vene R, Arena G, Morini M, Minghelli S, Noonan DM, *et al.* N-(4-hydroxyphenyl)retinamide inhibits retinoblastoma growth through reactive oxygen species-mediated cell death. *Mol Pharmacol* 2003;63(3):565-73.

Venè R, Arena G, Poggi A, D'Arrigo C, Mormino M, Noonan DM, *et al.* Novel cell death pathways induced by N-(4-hydroxyphenyl)retinamide: therapeutic implications. *Mol Cancer Ther* 2007;6(1):286-98.

TRANSCRIPTIONAL STUDY OF P63ALPHA MUTANTS FOUND IN ECTODERMAL DYSPLASIA SYNDROMES

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The Ectodermal Dysplasia (ED) syndromes are a group of inherited autosomal dominant human diseases. The prototypic Ectrodactyly, Ectodermal dysplasia and Cleft lip/palate (EEC) syndrome is clinically characterised by ectodermal dysplasia affecting skin, hair, nails teeth and facial clefts. Heterozygous mutations in the p63 gene have been identified in EEC syndrome patient and in other ED syndromes, including Ankyloblepharon-Ectodermal Defect-Cleft Lip and/or Palate (AEC), Limb-Mammary Syndrome (LMS), Acro-Dermato-Ungual-Larimal-Tooth syndrome (ADULT), and Split hand Foot Malformation (SHFM). The majority of the p63 mutations in EEC-like syndromes involve residues present DNA binding domain (DBD), the SAM domain (SAM) and the transactivation inhibitory domain (TID domain).

Mutations in the DBD abolish p63 DNA binding ability, while mutations in the SAM domain are supposed to abolish protein-protein interaction while mutations in the TID domain are predicted to impair the suppressive effect of the TID toward the TA domain thus increasing the transactivation activity.

Here, we have performed a systematic study of the transcriptional activity of different p63 mutants using different promoters: skin-specific (K14, IKKalpha, BPAG, EVPL), apoptotic (BAX) and cell cycle (p21). In addition, we have evaluated the ability of these mutants to induce cell cycle arrest and apoptosis. From the results obtained, we conclude that: i) there is a clear difference in the transcriptional activity of SAM and TID p63 mutations on skin-specific and apoptosis-related promoters; ii) the specific activity of TAp63alpha and DeltaNp63alpha on skin-specific promoters depends on an intact SAM domain; iii) the TID mutations mostly increase the activity of p63alpha on epidermal promoters. In addition, while TAp63alpha, and its active mutants, play a role in regulating cell cycle and in inducing apoptosis, DeltaNp63alpha does not affect apoptosis and cell cycle. Gene array analysis demonstrated also that both isoforms of the transcription factor are able to regulate genes that are involved in a wide variety of biological processes.

The clusterization (using GO terms) of the regulated genes from a set of 46000 mRNA sequences showed clusters in Oxidative Metabolism, Cell cycle, Protein degradation, Skin differentiation and development, ion channels. The results have been validated using both Real Time than ChIP analysis. Furthermore a mutant involved in the pathogenesis of SHFM, showed on the regulated gene a general effect of gain of function, leading to the conclusion that the onset of this group of disease, can be correlated with alterations of different pathways, involving the development program.

Papers published within the project

Campione E, Terrinoni A, Orlandi A, Codispoti A, Melino G, Bianchi L, *et al.* Cerebral cavernomas in a family with multiple cutaneous and uterine leiomyomas associated with a new mutation in the fumarate hydratase gene. *J Invest Dermatol* 2007;127:2271-3. m

Candi E, Rufini A, Terrinoni A, Giamboi-Miraglia A, Lena AM, Mantovani R, *et al.* DeltaNp63 regulates thymic development through enhanced expression of FgfR2 and Jag2. *Proc Natl Acad Sci USA* 2007;104:11999-04.

Codispoti A, Colombo E, Zocchi L, Serra V, Pertusi G, Leigheb G, *et al.* Knuckle pads, in an epidermal palmoplantar keratoderma patient with Keratin 9 R163W transgrediens expression. *Eur J Dermatol* 2009;19:114-8.

Didona B, Codispoti A, Bertini E, Rizzo WB, Carney G, Zambruno G, *et al.* Novel and recurrent ALDH3A2 mutations in Italian patients with Sjogren-Larsson syndrome. *J Hum Genet* 2007;52:865-70.

Diluvio L, Campione E, Paterno EJ, Orlandi A, Terrinoni A, Chimenti S Peculiar clinical and dermoscopic remission pattern following imiquimod therapy of basal cell carcinoma in seborrhoeic areas of the face. *J Dermatolog Treat* 2009;20:124-9.

Porzio O, Massa O, Cunsolo V, Colombo C, Malaponti M, Bertuzzi F, *et al.* Missense mutations in the TGM2 gene encoding transglutaminase 2 are found in patients with early-onset type 2 diabetes. Mutation in brief no. 982. Online. *Hum Mutat* 2007;28:1150.

Wen Y, Liu Y, Xu Y, Zhao Y, Hua R, Wang K, *et al.* Loss-of-function mutations of an inhibitory upstream ORF in the human hairless transcript cause Marie Unna hereditary hypotrichosis. *Nat Gene*t 2009;41:228-33.

Zocchi L, Bourdon JC, Codispoti A, Knight R, Lane DP, Melino G, *et al.* Scotin: a new p63 target gene expressed during epidermal differentiation. *Biochem Biophys Res Commun* 2008;367:271-6.

Zocchi L, Terrinoni A, Candi E, Ahvazi B, Bagetta G, Corasaniti MT, *et al.* Identification of transglutaminase 3 splicing isoforms. *J Invest Dermatol* 2007;127:1791-4.

TACKLING RARE DISEASES
YET LACKING DIAGNOSIS AND/OR PROGNOSIS.
A PILOT PROJECT INTEGRATING DATA COLLECTION
AND EXPERIMENTAL STUDIES:
THE HEPATOBLASTOMA EXPERIENCE

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Liver cancers in children are rare representing only 1.1% of malignancies. Hepatoblastoma (HB) is a rare liver cancer with a prevalence of 1.5 cases per million of children with less than 15 years, and it is the most common hepatic malignancy in childhood. By contrast, Hepatocellular carcinoma (HCC) is a less common tumor in children. Both HB and HCC are complex and heterogeneous tumors with several genomic alterations. Aberrant activation of several signalling cascades (i.e. phosphoinositol-3-kinase, Wnt, apoptotic signaling) have been widely described.

Most cases of HB are sporadic, but children with Familial Adenomatous Polyposis (FAP) and Beckwith-Wiedemann syndrome (BWS) are at higher risk. Specific chromosome aberrations have not been linked with prognosis or casual factors, however, genetic unbalances have been described in chromosomes 1q, 2q, 7q, 8, 17q and 20. Activation of wingless (Wnt) signalling pathway through mutations in β-catenin (CTNNB1) may contribute to the development of HCC and HB. Beta-catenin is the central mediator of the canonical Wnt/β-catenin signalling pathway, which acts as a gene transcription (co)factor activating the T-cell factor/lymphoid enhancer factor (TCF/LEF).

In HB, mutations leading to constitutive activation of β-catenin have been identified in genes of the Wnt/β-catenin pathway: they occur with high frequency in exon 3 of CTNNB1 and are mainly characterized by deletions or point mutations.

Recent evidences show that environmental factors can contribute to liver carcinogenesis; in particular, DEHP [di-(2-ethylhexyl) phthalate], present in PVC-based plastics, is of major concern because of its ability to affect developmental pathways as well as widespread dietary and environmental exposure, particularly in vulnerable sub-groups such as newborns undergoing intensive medical care. Liver is a DEHP target in adult rodents, but the effects on liver developmental programming have not been characterized. In this respect, we explored the potential adverse effects of in utero DEHP exposure on liver development.

Inadequate knowledge on HB pathogenesis did not allow the development of early diagnostic and prognostic markers. HB diagnosis is currently performed only through histological characteristics of the tumour, whereas prognosis is variable and difficult to predict.

In this respect, new potential tools able to look for putative differences in rare diseases and tumors are strongly needed. Several reports indicate that miRNAs expression profile is a feasible method for cancer classification.

MicroRNAs (miRNAs), a large class of non-coding small RNAs ~21 nt, have been shown to play important roles in various cellular and pathogenic processes, including cellular development, immunological response, and carcinogenesis. MiRNAs can target oncogenes or tumor suppressor genes, contributing to the initiation and progression of many human cancers.

Aims of this project were: i) to investigate the molecular basis of HB development and progression using a multidisciplinary approach on human cell lines (HepG2, Hep3B, HuH6, HLE, human hepatocyte), human samples and chemical induced mice models; ii) to search for diagnostic/prognostic markers of HB. This approach could be a model for investigation on other rare conditions.

Molecular features typical of liver carcinogenesis were investigated in human cell lines and tissue samples. CTNNB1 and APC were analyzed by sequencing, and IGF-2 promoter methylation status was assessed by methylation-specific polymerase chain reaction. Although few genomic alterations were detected in our samples, an altered expression of some microRNA in HB was observed.

So far, we focused on microRNAs in order: i) to elucidate the role of specific microRNAs in the main pathways (i.e.: Wnt/beta-catenin) involved in the hepatocarcinogenesis; ii) to identify microRNAs targets; iii) to assess the role of selected microRNAs as specific early diagnostic and prognostic markers.

MicroRNA expression was assayed by microarray and quantitative reverse transcription-polymerase chain reaction in human HB samples. Unsupervised clustering shows that microRNA profile can distinguish neoplastic from non neoplastic tissues. Further analyses of microRNA contents in hepatoblastoma compared to hepatocellular carcinoma highlighted four upregulated microRNA (miR-214, miR-199a, miR-150 [$P < 0.01$], and miR-125a [$P < 0.05$]) and one downregulated microRNA (miR-148a [$P < 0.01$]). In conclusion, although our samples were poorly informative from a genetic point of view, they showed a peculiar microRNA expression pattern compared with non-tumoral tissues and hepatocellular carcinoma.

A chemically induced HB mouse established in this project was analysed: in both weanling and pubertal mice, DEHP caused a pattern of effects whose main features were i) increased hepatocyte vacuolization, ii) decreased glycogen storage, iii) increased β-catenin intracytoplasmic localization. DEHP effects were dose- and gender-related. Our results were comparable to those obtained with Benzofuran (BF, a well known hepatocarcinogen agent in mouse, inducing HB-like lesions).

Specific microRNAs have been identified as early diagnostic and prognostic tools and the identification of markers discriminating between HB and HCC. We also focused on the characterization of putative genes, regulated by microRNAs and de-regulated in HB, which are actually under investigation since they may contribute to hepatocarcinogenesis.

In utero DEHP exposure alters and delays post-natal liver development: the pattern of changes indicates that targets include programming of glycogen and lipid metabolism. Finally, mislocalization of β-catenin might hint to a relationship with liver oncogenesis.

Papers published within the project

Di Masi A, Nicolai S, Salvatore M, Viganotti M, Tosto F, Lorenzetti S, Maranghi F, Tassinari R, Azzalin G, Magrelli A, Mantovani A, Taruscio D, Macino G, Tanzarella C, Antoccia A. Characterization of Wnt/β-catenin pathway, IGF-2 methylation status, mir profiles and cell signalling in hepatocarcinoma and hepatoblastoma cell lines. In: Taruscio D, Salvatore M (Ed.). *Workshop. Projects on rare diseases funded within the bilateral agreement Italy (Istituto Superiore di Sanità) and USA (NIH, Office for Rare Diseases) on joint research and development of public health actions. Istituto Superiore di Sanità. Rome, October 29-31, 2008. Abstract book.* Roma: Istituto Superiore di Sanità; 2008. (ISTISAN Congressi 08/C10). p. 134.

Lorenzetti S, Tassinari R, Moracci G, Lagatta V, D'Ambrosio A, Marcoccia D, Salvatore M, Taruscio D, Maranghi F. Esposizione in utero a di(2-etilesil)ftalato e marcatori di effetti epatici nel topo. In: Calamandrei G, La Rocca C, Venerosi Pesciolini A, Mantovani A (Ed.). *Endocrine disrupters: evaluation and prevention of possibile risks to human health.* Roma: Istituto Superiore di Sanità. 2009; (Rapporti ISTISAN 09/18). p. 19.

Magrelli A, Azzalin G, Salvatore M, Viganotti M, Tosto F, Colombo T, Devito R, Di Masi A, Antoccia A, Lorenzetti S, Maranghi F, Mantovani A, Tanzarella C, Macino G, Taruscio D. Altered microRNA expression patterns in hepatoblastoma patients. *Transl Oncol* 2009;2(3):157-63.

Magrelli A, Salvatore M, Azzalin G, Devito R, Macino G, Mantovani A, Tanzarella C, Taruscio D. MicroRNA and rare tumours: the hepatoblastoma example. In: Moretti F, Romero M, Belardelli F (Ed.). *International Meeting. Needs and Challenges in Translational Medicine: filling the gap between basic research and clinical applications. Istituto Superiore di Sanità. Rome, Italy, October 1-3, 2008. Abstract Book.* Roma: Istituto Superiore di Sanità; 2008. (ISTISAN Congressi 08/C7). p. 126

Maranghi F, Lorenzetti S, D'Ambrosio A, Lagatta V, Marcoccia D, Moracci G, Tassinari R, Salvatore M, Viganotti M, Tosto F, Romeo A, Di Masi A, Antoccia A, Nicolai S, Magrelli A, Azzalin G, Devito R, Eusepi A, Di Virgilio A, Taruscio D, Macino G, Tanzarella C, Mantovani A. Toward the establishment of a chemically-induced mouse model of hepatoblastoma. In: Taruscio D, Salvatore M (Ed.). *Workshop. Projects on rare diseases funded within the bilateral agreement Italy (Istituto Superiore di Sanità) and USA (NIH, Office for Rare Diseases) on joint research and development of public health actions. Istituto Superiore di Sanità. Rome, October 29-31, 2008. Abstract book.* Roma: Istituto Superiore di Sanità; 2008. (ISTISAN Congressi 08/C10). p. 87.

Maranghi F, Lorenzetti S, D'Ambrosio A, Marcoccia D, Moracci G, Tassinari R, Salvatore M, Viganotti M, Tosto F, Di Masi A, Antoccia A, Nicolai S, Magrelli A, Azzalin G, Devito R, Eusepi A, Di Virgilio A, Romeo A, Taruscio D, Macino G, Tanzarella C, Mantovani A. In utero exposure to DEHP affects liver morphology, metabolism and glycogen storage in post-natal CD-1 mice. *Reproductive Toxicology* 2009;28(2):131-2.

Maranghi F, Lorenzetti S, Tassinari R, Moracci G, Tassinari V, Marcoccia D, Di Virgilio A, Eusepi A, Romeo A, Magrelli A, Salvatore M, Tosto F, Viganotti M, Antoccia A, Di Masi A, Azzalin G, Tanzarella C, Macino G, Taruscio D, Mantovani A. In utero exposure to di-(2-ethylhexyl) phthalate affects liver morphology and metabolism in post-natal CD-1 mice. *Reproductive Toxicology* 2010 (in press).

Salvatore M, Antoccia A, Azzalin G, Devito R, Di Masi A, La Rocca C, Lorenzetti S, Macino G, Magrelli A, Mantovani A, Maranghi F, Tanzarella C, Tosto F, Tait S, Viganotti M, Taruscio D. Hepatoblastoma: *in vivo* and *in vitro* models for the analysis of Wnt/b-catenin and IGFs pathways. *European Journal of Human Genetics* 2008;16(Supplement 2): P04.153

Salvatore M, Lorenzetti S, Maranghi F, Mantovani A, Taruscio D. Molecular link(s) between hepatoblastoma pathogenesis and exposure to di-(2-ethylhexyl)phthalate: a hypothesis. *Folia Med (Plovdiv)* 2008;50(4):17-23. Review.

Taruscio D, Antoccia A, Azzalin G, Devito R, Di Masi A, La Rocca C, Lorenzetti S, Macino G, Magrelli A, Mantovani A, Maranghi F, Tanzarella C, Tosto F, Salvatore M, Tait S. Tackling rare diseases yet lacking diagnosis and/or prognosis: a pilot project integrating data collection and experimental studies. In: Taruscio D, Salvatore M (Ed.). *Workshop. Rare Diseases and Orphan Drugs. Rome, November 7-8, 2007. Abstract book.* Roma: Istituto Superiore di Sanità; 2007. (ISTISAN Congressi 07/C8). p. 120.

Tassinari R, Lorenzetti S, Moracci G, D'Ambrosio A, Marcoccia D, Eusepi A, Di Virgilio A, Romeo A, Salvatore M, Taruscio D, Mantovani A, Maranghi F. Esposizione *in utero* a di-(2-etilesil) ftalato (DEHP) e marcatori di effetti epatici nel topo CD1. In: *Convegno SITOX (Convegno Annuale Società Italiana Tossicologia) 2009*;abstract book:90.

GENETIC, MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF COCKAYNE SYNDROME, A RARE TRANSCRIPTION/REPAIR DEFECTIVE HEREDITARY DISEASE

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Cockayne Syndrome (CS) is a genetically heterogeneous disease characterised by precocious ageing and progressive physical and mental impairment. To improve prevention and treatment of CS and to gain insights into its molecular and functional basis, our research has been focused on the following aspects:

- *Cellular, genetic and molecular characterisation of newly identified patients*
  In seven out of ten newly referred CS cases we have found the altered cellular response to UV light typical of CS, which reflects specific defects in transcription-coupled repair (TC-NER), the nucleotide excision repair sub-pathway that rapidly removes UV-induced damage from the transcribed strand of active genes. Two patients were assigned to the CS-A group and four to the CS-B group. The seventh case was defective in the still-unidentified gene responsible for UV-Sensitive Syndrome (UVSS), another recently recognized genetic disorder defective in TC-NER that shows only mild cutaneous symptoms and is caused by mutations in CSB or in an as-yet-unidentified gene. The mutated alleles and their inheritance have been thus far determined in two CS-A and two CS-B patients whereas four cases previously assigned to the CS-B group were characterized both at the molecular and biochemical level. Furthermore, we have collaborated to the establishment of the incidence for CS in Western Europe (2.7 per million livebirths). Finally, we have studied a French patient, designated UVSS1VI, who shows the mild clinical features suggestive for UVSS. We found that UVSS1VI was mutated in the CSA gene, thus representing a third complementation group of UVSS. The responsible mutation, which had not been described previously, is predicted to cause a trp361cys substitution. Unexpectedly, the sensitivity to oxidative stress of UVSS1VI cells appeared to be in the normal range whereas fibroblasts from CS patients mutated in the CSA or CSB gene are hypersensitive to oxidants. Accordingly, the ectopic expression of the CSA gene cloned from UVSS1VI did not restore the altered response to UV of the CS-A cells CS3BE, but it did increase their resistance to oxidative stress. These findings imply that some mutations in the CSA gene may interfere with the TC-NER dependent removal of UV-induced damage without affecting its role in the oxidative stress response. Thus, investigations on CS and UVSS patients, despite the rarity of these disorders, indicate that defects in TC-NER alone cause mild cutaneous alterations and provide further evidence that the additional features present in CS patients, namely precocious aging and deficiencies in mental and physical development, reflect additional roles of the CSA and CSB proteins in the removal of oxidative damage.

– *Clarification of the functional bases of the altered response to oxidative stress of CS cells*
Recent evidence suggests that CSA and CSB might play function(s) in transcription and in the removal of oxidative damage, and that these alterations might be of relevance in the type and severity of CS clinical symptoms. We have shown that a defect in CSA and in CSB leads to accumulation of oxidative DNA damage, i.e. 8-oxoguanine (8-OH-Gua) and cyclopurines, after exposure to oxidizing agents. Within the framework of this project we have identified another class of lesions that involve CS proteins in their repair, namely base excision repair (BER) intermediates. CS-A and CS-B human fibroblasts presented a delay in the repair of abasic sites and/or single-strand breaks (SSB) induced by alkylating agents as measured by the comet assay. This defect was fully complemented by transfection with a plasmid expressing the wild type genes. To gain mechanistic insights, oxidative DNA damage repair was analysed in a battery of CSA or CSB mouse embryo fibroblasts (MEF) with additional deficiencies in NER or BER genes (in collaboration with G.T. van der Horst). Wild-type MEF fully repaired 8-OH-Gua within 2 hr after treatment with potassium bromate.
The repair kinetics of mutant MEF was monitored within the same time frame. CS-A and CS-B MEF accumulated 8-OH-Gua in their genome at a similar extent as XPC-null MEF. A previous study from our group has shown that XPC participates to 8-OH-Gua repair by stimulating the DNA glycosylase activity of OGG1, the major repair enzyme for this oxidized guanine The lack of OGG1 led to a more significant persistence of 8-OH-Gua confirming that this is the major repair mechanism for this oxidized guanine. MEF defective in either CSA or CSB with an additional deficiency in XPC showed increased DNA oxidation levels as compared with the single mutants suggesting that the involvement of CS proteins in 8-OH-Gua repair is XPC-independent. Conversely, when OGG1 was defective the additional defect in CSB (CSB/OGG1 double knock-out MEF provided by B. Epe) did not lead to a further increase in DNA oxidation levels. A plausible interpretation of these data is that CSB requires the cleavage by OGG1 in order to affect DNA repair of 8-OH-Gua.
All together, these findings identify the involvement of CSB in the processing of AP sites/SSB that are transcription-blocking lesions, thus suggesting a possible mechanistic link between TC-NER and BER. The wide range of DNA lesions that involve in their processing CS proteins might reflect a role of CS proteins in the regulation of chromatin structure. The kinetics of loading of BER components in cell extracts defective in CS proteins was tested on DNA substrates containing a single abasic site by a cross-linking assay (in collaboration with Dr. G. Dianov). Preliminary experiments showed that in the absence of CSB the loading of PARP1 on a substrate containing a single AP site is delayed as compared with normal cell extracts. The potential interaction of PARP with CS proteins awaits to be further investigated.

– *Oxidative DNA damage repair defect in CS and its complementation by heterologous repair proteins*
Although the phenotypical consequences of defective repair of oxidatively damaged DNA in Cockayne syndrome are not determined, accumulation of oxidized lesions might contribute to delay the physical and intellectual development of these patients. To conceive new therapeutic strategies for this syndrome, we have investigated whether the oxidatively damaged DNA repair defect in Cockayne syndrome might be complemented by heterologous repair proteins, such as the Escherichia coli formamidopyrimidine-DNA glycosylase (FPG) and endonuclease III (NTH).

We have confirmed that repair of the oxidized purine 8-oxo-7,8-dihydroguanine (8-oxoGua) is inefficient in cells belonging to the B complementation group of CS (CS-B). We have further shown that cells belonging to the A complementation group (CS-A) are also defective in repair of 8-oxoGua and we have demonstrated that expression of the Escherichia coli formamidopyrimidine DNA glycosylase (FPG) completely corrected the repair deficiency in both CS-A and CS-B cells. Phenotypically, CS-A cells were normally sensitive to toxicity and micronuclei induced by the oxidizing agent potassium bromate. CS-B cells displayed sensitivity to elevated concentrations of potassium bromate but this was not compensated by FPG expression, suggesting toxicity of lesions that are not FPG substrates. In a search for such alternative lesions, we investigated DNA repair of 5-hydroxy-2'-deoxycytidine (5-OHdC), an oxidized pyrimidine with cytotoxic and mutagenic properties. Both CS-A and CS-B cells were defective in repair of 5-OHdC.

The defect in repair of oxidatively damaged DNA in CS cells thus extends to oxidized pyrimidines, indicating a general flaw in repair of oxidized lesions in this syndrome. Expression of E.coli formamidopyrimidine DNA glycosylase (FPG) or endonuclease III (NTH) complemented the 5-OHdC repair deficiency. Hence, expression of one single enzyme, FPG from E.coli, stably corrects the delayed removal of both oxidizes purines and pyrimidines in CS cells and represents a possible gene therapy tool.

Papers published within the project

D'Errico M, Parlanti E, Dogliotti E. Mechanism of oxidative DNA damage repair and relevance to human pathology. *Mutat Res*, 2008;659:4-14.

Frosina G. DNA repair and resistance of gliomas to chemo/radiotherapy. *Mol Cancer Res*. 2009;7(7):989-99.

Frosina G. DNA repair in normal and cancer stem cells with special reference to the central nervous system. *Curr Med Chem* 2009;16:854-66.

Frosina G. Oxidatively damaged DNA repair defect in Cockayne syndrome and its complementation by heterologous repair proteins. *Curr Med Chem* 2008:15:940-53.

Frosina G. The current evidence for defective repair of oxidatively damaged DNA in Cockayne's syndrome. *Free Rad Biol Med* 2007;43:165-77.

Frosina G. Tumor suppression by DNA base excision repair. *Mini Rev Med Chem* 2007;7:727-3.

Kleijer WJ, Laugel V, Berneburg M, Nardo T., Fawcett H, Gratchev A, Jaspers NGJ, Sarasin A, Stefanini M, Lehmann AR. Incidence of DNA repair deficiency disorders in Western-Europe: Xeroderma pigmentosum, Cockayne syndrome and Trichothiodystrophy. *DNA Repair* 2008;7:744-50.

Nardo T, Oneda R, Spivak G, Vaz B, Mortier L, Thomas P, Orioli D, Laugel V, Stary A, Hanawalt PC, Sarasin A, Stefanini M. A UV-sensitive syndrome patient with a specific CSA mutation reveals separable roles for CSA in response to UV and oxidative DNA damage. *Proc Natl Acad Sci USA* 2009;106:6209-14.

Ropolo M, Daga A, Griffero F, Foresta M, Casartelli G, Zunino A, Poggi A, Cappelli E, Zona G, Spaziante, R, Corte G and Frosina G. A comparative analysis of DNA repair in stem and non-stem glioma cell cultures. *Mol Cancer Res* 2009;7:383-92.

Ropolo M, Degan P, Foresta M, D'Errico MR, Lasigliè D, Dogliotti E, Casartelli G, Zupo S, Poggi A, Frosina G. Complementation of the oxidatively damaged DNA repair defect in Cockayne Syndrome A and B cells by E.coli formamidopyrimidine DNA glycosylase. *Free Rad Biol Med* 2007;42:1807-17.

Stefanini M, Ruggieri M. Cockayne syndrome. In: Ruggieri M, Pascual-Castroviejo I, Di Rocco C (Ed.). *Neurocutaneous diseases*. New York, Wien: Springer-Verlag; 2008. Chapter 52; p. 793-820.

GENETIC HETEROGENEITY OF HEREDITARY PREDISPOSITION TO BREAST CANCER: CHARACTERIZATION OF MOLECULAR MARKERS AT BOTH SOMATIC AND GERMLINE LEVEL

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It is estimated that approximately 10% to 15% of all breast carcinomas are hereditary in origin. At least 25% to 30% of them are associated with mutations in the BRCA1 and BRCA2 susceptibility genes. However, a large number of hereditary breast cancer cases cannot be attributed to mutations in these two genes (so-called BRCAX). These represent a heterogeneous class of malignancies whose genetic etiology is still poorly characterized.

The first aim of this project was the identification and validation of RNA and micro-RNA (miRNA) transcripts predictive of the different types of genetically determined breast cancers and the integration of RNA and miRNA expression profiles for the identification and functional characterization of miRNA regulated genes.

Using the microarray technology, a transcriptome analysis of coding and non coding RNAs was performed on breast cancers form carriers of germline pathogenic mutations in the BRCA1 or BRCA2 genes, BRCAX cases and sporadic cases. We identified 106 coding RNAs (P<0.005) and 100 miRNAs (P<0.05) that were differentially expressed in the above groups of cancers. Confirming previous reports, the strongest difference in gene expression profiles were observed between the BRCA-related cancers and the other groups. In addition, the largest number of expressed miRNAs was detected in sporadic cancers. Cluster analysis showed that the BRCAX group consisted of two sub-groups, one similar to the sporadic cancers and the other to the BRCA1-related cancers. These results were observed for both coding and non coding transcripts. To identify miRNA/target gene pairs deregulated in breast cancer, we integrated the two analyses and identified 24 miRNAs whose expression was inversely correlated with that 76 putative target genes. We focused on one pair consisting of miR-342, which is differentially expressed in estrogen receptor (ER)-positive and ER-negative cancers and ID4, a gene whose expression is inversely correlated with that of ER and for which a role as a negative regulator of BRCA1 has been proposed. Consistently, miR-342 was highly expressed in the BRCAX sub-group that exhibited the characteristics of the BRCA1-related cancers.

The inverse correlation between ID4 and miR-342 expression was confirmed by quantitative-real-time PCR in cancers included in the transcriptome analyses and in a panel of breast cancer cell lines, as well as in 293T and Hela cells. The interaction between ID4 and

miR-342 was functionally validated by co-transfecting the miR-342 precursor RNA oligonucleotide into 293T cells, together with a vector containing the predicted miR-342 binding site of ID4, cloned downstream to the luciferase gene. In addition, transfection of miR-342 in 293T cells resulted in 35% reduction of the endogenous ID4 expression. The activity of miR-342 was further investigated by transfection assays in different breast cancer cell lines. In all transfected cells an increase in BRCA1 expression was observed, while the levels of ESR1 (the gene coding for ER) increased only in ER-negative cell lines. No difference in ID4 expression was detected.

Finally, we observed that transfection of miR-342 induced apoptosis in ER-negative cell lines, as measured by tunnel assays, suggesting that this miRNA acts as a tumor suppressor in these cells. Apoptosis was stronger in the BRCA1-mutant HCC1937 cell line, an observation of potential relevance for the clinical treatment of BRCA1-related cancers.

The second main aim of the project was the search for new germline mutations associated with breast cancer risk and the validation of candidate breast cancer risk alleles.

The regulatory regions of BRCA1, which are usually not screened in the diagnostic setting, were sequenced in a total of 685 BRCAX cases and 803 female blood donors, as a control group. In the promoter region a 2324-bp sequence immediately upstream the translation initiation site (ATG) was examined. Five annotated single nucleotide polymorphisms (SNPs) were identified with genotypic and allelic frequencies not different in cases versus controls. Interestingly, the minor (T) allele of one of this SNP (rs1655505) was shown to increase BRCA1 promoter activity by 70%. In addition, risk association analysis performed in Chinese affected women and age matched controls showed that carriers of the T allele had a reduced risk of breast cancer. Following these observations, the analysis of the above SNP was extended to three additional series of Australian (two studies) and German (one study) breast cancer cases and controls, through the collaborations with Georgia Chenevix-Trench (University of Queensland, Brisbane, Australia) and Barbara Wappenschmidt (University of Cologne, Germany). Altogether rs11655505 was thus typed in 2,961 breast cancer cases and 2,785 controls from four different ascertainments. A logistic regression model was used to analyze the genotype and allele frequencies of the SNP rs11655505, in the four series separately and in combination. No statistically significant association was observed. These findings argue against an association of the SNP rs11655505 with breast cancer risk in Caucasian populations. Beside the 5 annotated SNPs, 30 not previously reported variants were identified in the promoter region of BRCA1. Eight of these variants were found in both cases and controls and none of them had a frequency statistically different between the two groups. Of the remaining 22 variants, 14 were found in cases only and 8 in controls only. When excluding variants identified in controls with an allelic frequency >0.15%, the overall frequency of carriers of novel rare variants was approximately 3.1% among cases and 1% among controls. These percentages, although not significantly different, suggest that a few of the rare BRCA1 promoter variants identified in cases might be associated with an increased cancer risk.

As concerned the 3'UTR, the sequencing analyses identified a total of 20 nucleotide variants. Of these, 3 were common annotated SNPs, whose frequencies were not different in cases versus controls. Of the 17 not previously reported variants, 4 were found in both cases and controls with frequencies not statistically different between the two groups. Thirteen not previously reported variants were found each in a single individual, 6 in cases and 7 in controls. The frequency of novel rare variants was similar in cases and controls (0.9%). In silico studies showed that a few of the identified variants are located within putative miRNAs binding sites of and potentially affect binding affinity. However, such variants were observed both in cases and controls. In addition, in collaboration with Melissa Brown (University of Queensland, Brisbane, Australia) the effect of these variants on gene expression was analyzed, using a luciferase based

in vitro assay. Interestingly, all constructs carrying the variants identified in cases showed a reduced expression, ranging from 40% to 70% compared to the wild-type BRCA1 3'-UTR. However, a similar reduced expression was also observed in 3 of 6 variants identified in controls. Altogether, these data indicated that variants located in the 3'UTR of BRCA1 may affect its expression, but were unable to demonstrate an involvement in breast cancer risk.

In an additional study, we sought to verify the role of the PALB2 (partner and localizer of BRCA2) gene, which ha been reported to be constitutionally mutated in approximately 1% of BRCAX cases from different populations in Italian breast cancer families, for which no data are presently available. Two distinct series are presently being analyzed. To date 154 (group 1) and 330 (group 2) BRCAX cases have been sequenced in 80% and 35% of the gene coding region, respectively. Truncating mutations were identified in 6 (3.9%) and 3 (0.9%) cases of group 1 and 2, respectively. Based on these findings, and assuming an even distribution of the mutations along the gene, the expected final mutation frequency in the two series combined is approximately 3.75%, i.e., higher than that observed in other populations. Moreover, 2 of the 6 different truncating mutations identified were recurrent. These mutations were not previously reported and might represent Italian founder mutations.

Finally, we carried out four different studies to verify, by case-control association studies, previously reported candidate alleles associated with increased or reduced breast cancer susceptibility. These included a) the Arg72Pro and Ins16bp polymorphisms in the TP53 gene; b) the -652 6Ndel polymorphism in the promoter of the CASP8 gene; c) two SNPs (rs2056116 and rs9572903),within genomic ultra-conserved regions; and d) three SNPs within miRNA-196a2, miRNA-499 and miRNA-146a. No association with breast cancer risk modification was observed for any of the investigated SNPs. However, in a case-only analysis an increasing trend of the CASP8 del/del genotype with later age at breast cancer diagnosis was detected (trend test p-value = 0.01), indicating that this polymorphism may have an effect in postponing breast cancer onset in predisposed individuals.

Papers published within the project

Catucci I, Verderio P, Pizzamiglio S, Manoukian S, Peissel B, Barile M, Tizzoni L, Bernard L, Ravagnani F, Galastri L, Pierotti MA, Radice P, Peterlongo P. Letter to the editor: SNPs in ultraconserved elements and familial breast cancer risk. *Carcinogenesis* 2009;30:544-5.

De Vecchi G, Verderio P, Pizzamiglio S, Manoukian S, Barile M, Fortuzzi S, Ravagnani F, Pierotti MA, Radice P, Peterlongo P. Evidences for association of the CASP8 –652 6N del promoter polymorphism with age at diagnosis in familial breast cancer cases. *Breast Cancer Res Treat* 2009;113:607-8.

De Vecchi G, Verderio P, Pizzamiglio S, Manoukian S, Bernard L, Pensotti V, Volorio S, Ravagnani F, Pierotti MA, Radice P, Peterlongo P. The TP53 noIns-Pro haplotype is not associated with breast and ovarian cancer in hereditary cases negative for mutations in *BRCA1* and *BRCA2*. *Cancer Detec Prev* 2008;32:140-3.

Lusa L, Peissel B, Manoukian S, Radice P, Marchesi E, Pierotti MA, Gariboldi M. Molecular basis for estrogen receptor alpha deficiency in *BRCA1*-linked breast cancer. *J Natl Cancer Inst* 2008;100:752-3.

A GENOME WIDE NON-SYNONYMOUS SNP SCAN OF AMYOTROPHIC LATERAL SCLEROSIS

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Three-stage replica study from genome wide association (GWA) screenings on different populations of European ancestry have reported a significant association of dipeptidyl-peptidase 6 (DPP6) gene with a slight increased risk to develop ALS (odds ratio, OR 1.3). To confirm this association we followed up with a replication test of the candidate polymorphism (SNP) rs10260404 on two additional European populations of Italian and British descent. The Italian cohort consisted in 904 Italian sporadic ALS cases (549 males, 360 females) and 1034 healthy controls (596 males, 453 females) and the British cohort in 406 cases (268 males, 138 females) and 303 controls (132 males, 171 females). Our results failed to replicate the association between SNP rs10260404 and sporadic ALS in the two population studied, similarly to what has been reported for many other candidate SNPs. These results highlight the genetic heterogeneity of sporadic ALS even within European populations.

In line with this view we have conducted a mutational analysis of TARDBP, another candidate gene for ALS, in our large cohort of Italian patients. Recent studies have identified rare missense mutations in this gene that encodes TDP-43, the major protein of the ubiquitinated inclusions found in affected motor neurons of both sporadic and SOD1-negative familiar cases. We screened 666 ALS samples (125 familial, FALS and 541 sporadic cases, SALS) and identified 12 different heterozygous missense mutations in 18 patients, including 6 FALS. No mutation was found in 771 matched controls. Interestingly, these variants are all located in exon 6 and 9 out of 12 represent novel mutations. We detected the c.1144G>A p.A382T change in 7 patients (5 SALS and 2 FALS), thus representing the most frequent TARDBP mutation described so far in ALS. Analysis of microsatellites surrounding the TARDBP gene indicated that p.A382T was inherited from a common ancestor in 5 out of 7 patients. Our findings strongly suggest the causative role of TARDBP gene in ALS pathogenesis and indicate that the frequency of TARDBP mutations is different in distinct ALS populations. In fact, it is particularly high in Italian patients compared to individuals of mainly Northern European origin (2.7% *vs* 1%, respectively), further confirming the genetic heterogeneity of ALS and the need to analyze large cohorts.

Recently, mutations in the FUS gene, located on chromosome 16, have been identified in ~5% of FALS patients which tested negative for SOD1 and TARDBP mutations. Most mutations are located in exon 15 and result in cytoplasmic aggregation of the protein. We screened FUS in 94 unrelated Italian FALS patients (53 males and 41 females) pre-screened for SOD1 and TARDP mutations. We identified four mutations in five cases, for a mutational frequency of 5.3%. Two mutations (R521C and R521G) have previously been described and have been shown to alter the subcellular localization of the protein. The other two mutations (G156E and R234L) are novel and presumably affect the interaction between FUS and its RNA

binding partners. Affected individuals developed early symptoms of symmetrical weakness of the scapular or pelvic girdles and of the proximal muscles of the upper or lower limbs. Also prominent was the involvement of the axial muscles of the neck and trunk. In conclusion, our results provide strong evidence that mutations in the FUS gene represent a major cause of FALS in the Italian population and raise the suggestive hypothesis that the patients carrying FUS mutations may have a common clinical phenotype.

To identify genetic variants associated with susceptibility and phenotypes in sporadic ALS, we collaboretad in performing a genome-wide SNP analysis in SALS cases and controls. A total of 288,357 SNPs were screened in a set of 1,821 SALS cases and 2,258 controls from the U.S. and Europe. Survival analysis was performed using 1,014 deceased sporadic cases. Top results for susceptibility were further screened in an independent sample set of 538 ALS cases and 556 controls. SNP rs1541160 within the KIFAP3 gene (encoding a kinesin-associated protein) yielded a genomewide significant result (P=1.84 x10_8) that withstood Bonferroni correction for association with survival. Homozygosity for the favorable allele (CC) conferred a 14.0 months survival advantage. Sequence, genotypic and functional analyses revealed that there is linkage disequilibrium between rs1541160 and SNP rs522444 within the KIFAP3 promoter and that the favorable alleles of rs1541160 and rs522444 correlate with reduced KIFAP3 expression. No SNPs were associated with risk of sporadic ALS, site of onset, or age of onset. We have identified a variant within the KIFAP3 gene that is associated with decreased KIFAP3 expression and increased survival in sporadic ALS. These findings support the view that genetic factors modify phenotypes in this disease and that cellular motor proteins are determinants of motor neuron viability. The ongoing Italian GWA will be the largest single stage GWA study of ALS in a geographically restricted population to date.

As such it promises to have a significant role in unraveling the genetics of sporadic ALS. In addition to identifying novel loci or replicating loci implicated in smaller studies that mediate risk for ALS, we will also identify loci that influence the disease phenotype, such as age and site of onset. The secondary objective is to examine the role of copy number variations (CNVs) in ALS. CNVs are a recently discovered important form of human polymorphic submicroscopic structural variations including deletions, duplications and insertions. While SNPs remain the main source of genetic and phenotypic human variation, at least 12% of the human genome contains CNVs and may account for a substantial component of phenotypic variability in complex diseases. We will use a new commercial platform developed by Illumina (Human610-Quad BeadChip), which simultaneously genotypes SNPs and CNVs in a single integrated assay and we will use sophisticated analyses to call CNV information.

The third objective is to contribute to a worldwide effort to identify ALS risk and modifier genes as part of the International GWA Consortium, led by the motor neuron disease associations ALSA and MNDA. The final objective of this project is to characterize population substructures that may be hidden in the Italian general population. A different distribution of ancestry within cases and controls may cause false positive findings leading to confounding results. In the past 1000 years, due to its central location in the Mediterranean Sea, Italy has witnessed several waves of immigration from different parts of Europe and even Middle East. Therefore, Italian history clearly points out the heterogeneity of its ancestries and the importance of further investigations with updated powerful approaches. This first large-scale GWA study on 4000 samples will facilitate to identify substructures background and admixture status of the Italian population.

Once the project will be terminated, the resulting genotypes data of the 2000 healthy controls will be made publicly available on an appropriate web-site allowing other Italian groups, who are enterprising a GWA analysis with Illumina platforms, to share the control set.In the past 14 months we have collaborated with several Neurological Centres located in North Italy to create

the first Italian ALS Consortium which aims to collect a large-scale set of Italian sporadic ALS patients for this GWA study. So far about 1700 cases and 2000 neurologically normal controls have been gathered from different Hospitals and Centres in Lombardy (IRCCS Istituto Auxologico Italiano, Institute of Neurology "Carlo Besta", the Maggiore Policlinico and the Niguarda Ca' Granda Hospitals in Milan; Mondino Institute of Neurology in Pavia), Piedmont (University of Torino and University of East Piedmont in Novara) and Veneto (University of Padua). We are still recruiting more cases from other Italian Neurological Centers all over Italy. In our GWA approach we propose to genotype all 2000 ALS samples and 2000 controls with the same marker set rather than perform a two- or multi-stage SNP genotyping strategy in which a proportion of the sample is analyzed with a full marker set and then only those markers passing a specified level of significance are genotyped in the remaining sample. This is a more powerful approach without being consistently more expensive. We will use the Illumina Human610-Quad BeadChip containing more than 550,000 SNPs derived from HapMap data and 60,000 additional markers for CNVs.

Papers published within the project

Corrado L, Ratti A, Gellera C, Buratti E, Castellotti B, Carlomagno Y, Ticozzi N, Mazzini L, Testa L, Taroni F, Baralle FE, Silani V, D' Alfonso S. High frequency of TARDBP gene mutations in Italian patiens with Amyotrophic Lateral Sclerosis. *Human Mutation* 2009;30:688-94.

Fogh I. Association of DPP6 with amyotrophic lateral sclerosis in an Italian population. *Neurobiology of Aging* 2009;12.

Landers JE, Melki J, Meininger V, Glass JD, van den Berg LH, van Es MA, Sapp PC, van Vught PW, McKenna-Yasek DM, Blauw HM, Cho TJ, Polak M, Shi L, Wills AM, Broom WJ, Ticozzi N, Silani V, Ozoguz A, Rodriguez-Leyva I, Veldink JH, Ivinson AJ, Saris CG, Hosler BA, Barnes-Nessa A, Couture N, Wokke JH, Kwiatkowski TJ Jr, Ophoff RA, Cronin S, Hardiman O, Diekstra FP, Leigh PN, Shaw CE, Simpson CL, Hansen VK, Powell JF, Corcia P, Salachas F, Heath S, Galan P, Georges F, Horvitz HR, Lathrop M, Purcell S, Al-Chalabi A, Brown RH Jr. Reduced expression of the Kinesin-Associated Protein 3 (KIFAP3) gene increases survival in sporadic amyotrophic lateral sclerosis. *Proc Natl Acad Sci USA* 2009;106(22):9004-9.

Ticozzi N, Silani V, LeClerc AL, Keagle P, Gellera C, Ratti A, Taroni F, Kwiatkowski TJ, McKenna-Yasek DM, Sapp PC, Horvitz HR, Brown RH, Landers JE. Analysis of FUS Gene mutation in familial amyotrophic lateral sclerosis within an Italian cohort. *Neurology* 2009;73(15):1180-5.

PRECLINICAL STUDIES AIMED TO DEVELOP TARGET GENES-BASED THERAPIES FOR THE TREATMENT OF AMYOTROPHIC LATERAL SCLEROSIS

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Introduction

Amyotrophic Lateral Sclerosis (ALS) is the most common form of the motor neuron disease which affects about 4000 adult people in Italy and it is still orphan of a truly effective therapy. ALS is characterised by a progressive degeneration of lower and upper motor neurons in the spinal cord, brainstem and cortex. As consequence, this neuronal loss causes weakness and muscular atrophy that evolves to paralysis and culminates in death within 2-5 years after the diagnosis, generally due to respiratory failure. Five to 10% of cases are familial, of which 20% are caused by mutations in the gene encoding an antioxidant defence protein, Cu/Zn superoxide dismutase (SOD1). However, the sporadic and familial forms of ALS are clinically indistinguishable and show the same pathological hallmarks. It is likely that they share a common death pathway. Transgenic mice overexpressing a variety of mutant human SOD1 protein found in familial ALS recapitulate several aspects of the disease and therefore they provide a powerful model system to identify the pathophysiological mechanisms associated with ALS and to screen potential therapeutics.

Studies on patients and animal models of ALS have evidenced that the pathology is the consequence of a complex interplay between several molecular pathways including excitotoxicity, mitochondrial dysfunction, accumulation of protein aggregates, proteasome dysfunction, altered axonal transport, neuroinflammation. Many of these mechanisms converge to the activation of common cell death signalling cascades in the motor neurons. In fact, recently, we and other groups have demonstrated that in the motor neurons of either SOD1 mutant mice and patients with sporadic ALS there is a remarkable activation of the p38 mitogen activated protein kinase (p38MAPK) pathway. This effect appeared at the very early stage of the disease in the motor neurons of SOD1 mutant mice suggesting a role in the genesis of the death cascade. Increased activation of p38 MAPK, in particular the alpha subunit, may induce a hyperphosphorylation of neurofilaments leading to their accumulation in the perikarya, a hallmark of neurodegeneration. The first objective of this study was to inhibit the p38MAPK to prevent motor neuronal death and block in this way the progression of the disease. Since inhibitors of p38MAPK pathway available are unable to efficiently cross the blood-brain barrier and lack of selectivity that can cause important side effects, here we proposed to use viral vector strategies to allow the transport *in vivo* of small interfering RNAs able to inhibit selectively the activation of the p38MAPK specifically in the motor neurons and/or in neighbouring glial cells. On the other hand, there are indications that motor neurons of ALS patients and transgenic SOD1 mutant mice are unable to trigger pro-survival signals such as the antiapoptotic PI3K/Akt pathway. Therefore, in addition to inhibit the detrimental mechanisms leading to motor neuron death, the second objective of this project was to use viral vectors for the delivery in motor neuron of the activated form of Akt which is known to protect cells from toxic stimuli.

Methods

We adopted a recombinant viral vector based approach to silence specifically the p38 MAPK alpha isoform and to activate the Akt pathway. Candidate shRNAs targeting the murine alpha isoform of p38MAPK were obtained from Open Biosystems and were cloned into a lentiviral vector expressing green fluorescent protein (GFP) as a reporter gene. These shRNAs were used to infect NIH3T3 murine cell line, and screened for their efficacy in reducing the levels of p38MAPK. Subsequent validation was done by infection of primary astrocytes or neuronal cultures to test the efficiency of anti-p38 sh RNAs in counteracting the activation of p38MAPK pathway or protecting cells in paradigms of neuronal death. To obtain induction of the anti-apoptotic pathway a myristilation sequence was fused at the 5' of the cDNA for Akt1 and Akt3 isoforms. This modification determines constitutive activation of the enzyme. The functionality of Akt constructs was evaluated by transfection of HEK293 cell lines and analysis of transgene protein expression in the lysates. After validation *in vitro*, constitutively activated Akt was cloned into a lentiviral vector plasmid for delivery *in vivo*, in the mice. To obtain specific and long lasting expression in the motor neurons of the spinal cord *in vivo*, EF1alpha ubiquitarious promoter was substituted by a neuro-specific promoter. The lentiviral vectors carrying potential therapeutic constructs were delivered in the spinal cord of SOD1 G93A mice (at 12 weeks of age, corresponding to presymptomatic stage) and the disease progression was monitored by assessing the grip strength, the latency to stay on rotarod and the length of survival.

Results

Three specific aims of the project have been accomplished and further experiments are underway to complete the fourth aim based on *in vivo* studies.

Aim 1: in a first set of experiments we were able to identify a single shRNA sequence (seq.nr.7) out of ten candidates, which was able to specifically downregulate p38MAPK alpha both at the mRNA and protein level. We tested this sequence in primary astrocytes cultures stimulated by TNFalpha and we found a specific and selective reduction of p38MAPK and its phosphorylated (activated) form (P-p38). Insertion of 3 point mutations in seq.7 abolished the effect of shRNA and therefore this mutated sequence (seq.7*) was used in subsequent experiments as a control of the specificity of RNAi. To highlight the therapeutic potential of RNAi of p38, we infected neuronal cultures with lentiviral vectors expressing seq.7 or its mutated seq.7* counterpart. We verified that p38 downregulation, obtained with seq.7, was able to exhibit a protective effect on neurons treated with colchicine, a paradigm of cell toxicity related to cytoskeleton alterations observed in many neurodegenerative diseases. This result reproduced the protective effect on clochicine-treated neurons obtained by pharmacologic inhibition of p38.

Aim 2: we generated a myristilated-Akt construct starting from the cDNA of Akt isoform 3. This isoform was selected because it showed a specific anti-apoptotic effect on cell death induced by mutated SOD1 *in vitro*. The activity of the construct that we generated was demonstrated *in vitro* by the increased levels of phosphorylated (activated) Akt, and by increased phosphorylation of GSK3beta at Ser9 (a specific target of Akt).

Aim 3: during this stage of the project we tested different strategies to deliver lentiviral vectors to the lumbar spinal cord of mice, by using a vector expressing GFP as reporter gene. We verified that following the intracerebroventricular injection or the lumbar puncture, the GFP signal was detected mainly in the cells of ventricle walls, the chorioid plexus and the meningeal

cells while the spinal cord parenchyma remained unlabelled. We have tried to permeabilize the blood-brain barrier by mannitol to enhance the efficiency of transfection at the lumbar level but it didn't work. We then decided to deliver the viral vectors by direct administration in the parenchyma of lumbar spinal cord. To obtain specific expression of the transgenes in the motor neurons *in vivo*, we designed and developed a construct containing the enhancer and repressor elements derived from mouse Hb9 promoter, and cloned it into a lentiviral vector. Tests *in vivo* demonstrated that specific expression in the motor neurons could be achieved by use of lentivectors carrying the Hb9-derived promoter. As an alternative approach, we used Rabies-G pseudotyped lentivectors and an Adeno-Associated Viral vector serotype 2 (AAV2) to test their ability to transduce spinal cord motor neurons after administration in peripheral muscles.

AAV2 successfully infected the motor neurons, while Rabies resulted inefficient. As subsequent step, in aim 4 we studied the effect of p38 downregulation or Akt induction in the SOD1G93A mice. Lentiviral vectors carrying shRNA seq.7 or its mutated counterpart were delivered in the parenchyma of lumbar spinal cord in SOD1 mutant mice 2 weeks before symptom onset. Surprisingly, animals treated with seq.7 showed an anticipation of the symptoms, a rapid decline of motor performances and 7 day reduction in survival as compared to animals treated with non effective shRNA or not treated animals. Histopathological examination of these animals revealed a reduced survival of motor neurons and increased gliosis in the areas of spinal cord that received the injection of seq.7 lentivirus.

In parallel, the Akt construct was cloned under Hb9 promoter in a lentiviral vector and delivered in the parenchyma of lumbar spinal cord in SOD1 mutant mice 2 weeks before symptom onset. Interestingly, we observed that the animals that received Akt vectors didn't show any increase of survival, as compared with empty vector treated mice. However, the histopathological analysis showed that, in Akt treated mice a higher number of motor neurons were found in areas of spinal cord that received the injection as compared to mice injected with empty vector.

Conclusions

We have successfully designed and developed a gene-based approach to specifically target, *in vivo*, in the mouse, intracellular molecular pathways involved in ALS. We have demonstrated for the first time that a construct derived from Hb9 promoter can be used in lentiviral vectors to restrict transgene expression to motor neuronal population *in vivo*. Moreover, we verified that AAV2-derived vectors may be regarded as alternative strategy to transduce spinal cord motor neurons through not-invasive administration roots (like muscular injection).

In particular, we have used RNAi to downregulated p38 alpha, in a mouse model of ALS, and revealed that interefering with this pathway determines an anticipation of symptom onset and reduction of survival. This might be due to the activation of complementary kinase cascades, like JNK, involved in neuronal death. At the moment, studies are underway to better characterize this phenomenon.

On the other side, we have demonstrated that induction of Akt pathway in the motor neurons, through expression of constitutively activated Akt3 isoform, prevents neuronal loss in SOD1G93A mice, though this effect does not influence their premature death. This supports the hypothesis that preservation of neuronal perikaria is not sufficient to ameliorate disease progression in ALS, and the peripheral compartment (axons, muscles) should be regarded as an additional target for potential therapeutic approaches.

DYNAMIC NAD(P)H POST SYNAPTIC AUTOFLUORESCENCE SIGNALS FOR THE ASSESSMENT OF MITOCHONDRIAL FUNCTION IN A NEURODEGENERATIVE DISEASE: MONITORING THE PRIMARY MOTOR CORTEX OF G93A MICE, AN AMYOTROPHIC LATERAL SCLEROSIS MODEL

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Amyotrophic Lateral Sclerosis (ALS) is a group of human devastating conditions which involve selective degeneration of motoneurons in spinal cord, brain stem, and cerebral cortex. The degeneration of motoneurons leads to skeletal muscle atrophy, paralysis and death. Among the early signs of damage, mitochondrial dysfunction is known to occur in patients and in models *in vivo* and *in vitro*. For instance, morphological changes in mitochondria are the earliest detected pathologic events in mouse lines overexpressing ALS- typical human SOD1 with G37R or G93A mutations.

Decreased mitochondrial respiration and abnormal enzyme activities have been reported repeatedly in G93A mice and in neuronal cultured cells. The NADH:ubiquinone oxidoreductase (Complex I), provides the input to the respiratory chain from the NAD-linked dehydrogenases of the citric acid cycle. The complex couples the oxidation of NADH and the reduction of ubiquinone, to the generation of a proton gradient which is then used for ATP synthesis.

NADH is the predominant component of tissue autofluorescence under UV excitation, but after donation of electrons to the electron transport chain, the oxidized molecule (NAD+) is nonfluorescent. Thus changes in NADH fluorescence long have been used as a measure of oxidative phosphorylation changes. Signals are attributable primarily to mitochondrial NADH dynamics but can be referred to as NAD(P)H transient signal, because the fluorescence profile of NADPH is indistinguishable from that of NADH.

Recent studies enforced the assumption that mitochondrial NADH dynamics are predominant contributors to both phases of evoked NAD(P)H transient signal; in brain slices, excitatory synaptic stimulation results typically in initial transient signal decreases in NAD(P)H fluorescence, followed by longer-lasting NAD(P)H increases, that overshoot pre-stimulus NAD(P)H levels, before returning slowly to baseline.

NAD(P)H post synaptic autofluorescence signals may therefore be used as a sensitive tool to assess mitochondrial function in neurodegenerative models which involve energy failure. In this work, we have investigated on NAD(P)H monitoring of primary motor cortex of ALS mice as a tool for a better definition of this and other murine models syndromes affecting consistently the upper motoneurons.

For the first time we evidenced, in *ex vivo* brain slices, cerebral metabolic dynamic changes in G93A SOD1 mice, a ALS murine model, through NAD(P)H autofluorescence post synaptic

signals (wild-type, wild-type human SOD1 and G93A mutant SOD1at 60, 90 and 130 day old). We found a correlation between differential amplitudes in the biphasic response and age-related metabolic alterations. The data confirm that mitochondrial complex I defective function can also induce alteration of baseline autofluorescence response level in individual groups. Indeed we found moderate (non significant) alteration between baseline of control groups versus the baseline of G93A at 130PND. We assume that in a pathological model, as G93A mouse is, an altered baseline picture compared to controls is found, without any evident effect on the post stimulus signal. To explain our findings in the overshoot amplitudes at 60 PND among the three groups, some hypotheses can be forwarded. This transient signal can be modulated either by a different glucose concentration in ACSF applied to slices during the experiment, or by a different glucose concentration in the plasma of fasting hyperglycemic (diabetic) mice from which brain slices were gathered. Furthermore, previous paper suggests that NAD(P)H overshoot signal can be modulated by an adenosine A1 receptor antagonist which can alleviate 2-deoxyglucose (chemical hypoglycemia). Our results confirm previous results observed in G93A mice at 60 PND reporting a different glucose utilization in G93A *vs* WT mice at the same age. The results of initial component are the most interesting, in our view, because they appear directly related, using spectrophotometric assay, to mitochondrial complex I activity. However the NAD(P)H post synaptic autofluorescence signals are extremely sensitive also in 90PND. We underline that these informations were obtained in living and real-time functioning brain tissue. Our data confirm and extend to upper motoneurons previous results which described an age-related progressive decrease for mitochondrial complex I activity in mitochondria-enriched fractions of G93A mice lower motoneuron (spinal cord), using a proteomic approach. These results support the hypothesis on a progressive neurodegeneration of corticospinal projections in the G93A ALS mouse, which involves not only the destruction of lower motoneurons in the spinal cord, but also additional loss of descending cortical and bulbar neurons. Present data suggest that in the G93A mouse corticospinal neuronal degeneration is apparently related to a progressive involvement of mitochondrial NADH dysfunction, whereas in previous papers only the low motoneuron progressive degeneration in the spinal cord was demonstrated.

These results demonstrate a metabolic impairment in primary motor cortex, particularly in mitochondrial electron transport chain. We speculate that in future studies, a monitoring of living tissue and cell metabolic dynamic changes in real-time, addressed also to new drugs trials in animal models, will be possible through the application of this technique.

Papers published within the project

Loizzo S, Pieri M, Ferri A, Carrì MT, Zona C, Fortuna A, Vella S. Dynamic NAD(P)H post synaptic autofluorescence signals for the assessment of mitochondrial function in a neurodegenerative disease: monitoring the primary motor cortex of G93A mice, an amyotrophic lateral sclerosis model. *Mitochondrion* 2009 Nov 10. [E-pub ahead of print].

EVALUATION OF GENETIC AND ENVIRONMENTAL FACTORS IN A COHORT OF TWINS WITH AMYOTROPHIC LATERAL SCLEROSIS (ALS)

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To evaluate genetic and environmental contribution to ALS susceptibility we performed a nation-wide study of ALS concordance in twin pairs. We linked records of 5824 ALS patients, diagnosed since 1990 and followed in 20 Centres all over Italy, with Italian Twin Registry databases and identified 52 twins (31 males and 21 females). None of the index twins belong to the same twin pair.

Gender of co-twins and living status of both twins were ascertained through registries of birth or last known residence: there were 23 same sex male, 13 same sex females and 15 unlike sex pairs. Gender of one co-twin was unknown. In 14 pairs both twins were alive, in one pair ALS twin is alive and the co-twin has moved from residence place; in 26 pairs, ALS twins were dead and the co-twins are either alive (n. 21), or dead (n. 2), or moved (n. 3); in 6 pairs ALS twins have moved and the co-twin are either alive (n. 3), or dead (n. 1), or moved (n. 2); in 4 pairs information on both twins living status were unavailable. Currently, a trained neurologist is collecting clinical records of affected twins, is seeing alive twins (both ALS and unaffected) or the closest relatives of deceased twins and is gathering information through a semi-structured questionnaire about ALS history, life exposure to putative risk factors and family recurrence of ALS or other neurodegenerative diseases.

Donation of biological material for biobanking is also asked when both twins in a pair are alive. ALS concordance, heritability and odds ratio conferred by risk factors will then be estimated.

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Papers published within the project

Nisticò L, Cotichini R, Lagalla G, Provinciali L, Logroscino G, Zoccolella S, Mandrioli J, Sola P, Monsurrò MR, Trojsi F, Mazzini L, Nasuelli N, Beghi E, Millul A, Cima V, Sorarù G, La Bella V, Spataro R, Bongioanni P, Rossi B, Giacomelli E, Inghilleri M, Antonini G, Bucci E, Pontieri E, Benincasa D, Chiò A, Vanacore N, Toccaceli V, Leone M. Evaluation of genetic and environmental factors in a cohort of twins with myotrophic Lateral Sclerosis (ALS). In: XIII National Meeting of the Italian Neuroepidemiology Association. *Neuroepidemiology* 2008;31:210 [Abstract].

GASTROESOPHAGEAL REFLUX IN PATIENTS WITH SYSTEMIC SCLEROSIS (SSc): ANY RELATIONSHIP WITH PULMONARY INVOLVEMENT?

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Background

In Systemic Sclerosis (SSc), gastrointestinal involvement is predominantly oesophageal, including impaired motility and GastroEsophageal Reflux (GER). Acid reflux has been associated with a number of respiratory diseases, such as interstitial lung disease, although a causal relationship between acid and fibrosis has not been clearly documented in humans. The aim of the study was to characterize in SSc patients the physical (gas or liquid) and the pH properties of the GER, and to evaluate whether a relationship exists between type of reflux and lung involvement.

Methods

Twenty-three consecutive patients who fulfilled the American College of Rheumatology criteria for SSc were enrolled. In all the patients an esophagogastroduodenoscopy followed by esophageal manometry and 24h-pH multichannel intraluminal impedance (pH-MII) was performed, once off from antisecretory drugs. High Resolution chest CT scan (HRCT) and pulmonary function tests (spirometry and Diffusing Capacity for Carbon Monoxide, DLCO) were used to assess lung involvement.

Results

Twenty-three patients completed the study (all female, mean age 58 yrs, 14 had limited SSc and 6 had diffuse SSc).

Fifteen patients (65%) had manometry abnormalities (oesophageal aperistalsis and/or hypotonic LES). Ten patients (43%) had endoscopic esophagitis, whereas nineteen (83%) showed GER at the 24h pH-MII recording. Analyzing the type of reflux, 3 patients (13%) showed a pure acid reflux, 7 (30%) a non-acid reflux and 9 (39%) had a mixed reflux, both acid and non acid. Imaging consistent with interstitial lung disease was detected in 6 out 23 (26%). The proximal extent of liquid refluxate into the esophagus was documented in 3 patients, all with interstitial lung disease (p=0.01 by Fisher's exact test).

Conclusions

The majority of SSc patients showed both acid and non acid GER; these findings might help for better tailoring medical treatment. Patients with proximal extent of liquid refluxate seem to be at risk of pulmonary involvement. These preliminary data need to be confirmed in a larger cohort of SSc patients.

THE ITALIAN EXTERNAL QUALITY ASSESSMENT IN GENETIC TESTING: DEVELOPMENT OF A WEB BASED SYSTEM

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Introduction

The Italian External Quality Control (EQC) started in 2001 at the Istituto Superiore di Sanità (ISS, the National Institute of Health in Italy). The major goal of this activity is the improvement of the performance in genetic tests in the clinical practice by elaboration of recommendation and guidelines, standardization of methods and diffusion of technical information. Participation is free of charge, voluntary and limited to Italian public laboratories.

The EQC programs covers seven schemes four of them in molecular genetics (Cystic Fibrosis - CFTR gene, Beta-Thalassemia - Hbb gene, Fragile-X syndrome - FMR1 gene, the Adenomatous Polyposis Coli - APC gene) and three in cytogenetic (prenatal, postnatal and oncological diagnosis).

Eightytwo public laboratories distributed on the National territory have been enrolled. Five trials have been performed and concluded. Results showed that there has been an improvement in the use and in the interpretation of molecular genetic tests. The average genotyping error rate observed over the five years was 0.6%, 0.3%, 5% and 3.7% in the Cystic Fibrosis, Beta-Thalassemia, Fragile-X syndrome and Adenomatous Polyposis Coli scheme respectively; the percentage of complete reports in cytogenetics increased over the period. However, lack of information or inadequacy in reporting are still observed. On the other hand, as has been indicated in other international surveys for quality assessment, it will be only after several years of testing experience and participation in quality assessment schemes that a significant reduction in laboratory errors will be possible.

Methods

On the basis of the experience acquired until now and in order to harmonize the activity of our schemes with existing European ones, we have developed a web-based system. The web based system is structured into three sections: i) a specific section is restricted to the ISS as scheme organizer; ii) a second section is restricted (through password) to each participating laboratory in order to load, by web, raw data and reports, consulting the manual available on the website; iii) a third section is restricted (through password) to assessors, (decided by Italian Society of Human Genetic for each scheme), that will use this area in order to perform on line the evaluation of the raw data, from they computer; the final evaluation will be performed at the ISS. The web based system allows: a) to handle about 90% less of paper; b) to manage easily materials; c) to archives performance during the years.

Participation in EQA Schemes will be open to all public laboratories enrolled during the five trials and recorded in our database. New laboratories need to be registered contacting by e-mail: testgenetici@iss.it.

Participants are informed in advance of the date of EQA opening either in cytogenetics and molecular genetics. For any problem it is the participant's responsibility to inform the scheme organiser.

Cytogenetics EQA scheme is based on a retrospective format; laboratories send to ISS images and reports of two cases analysed.

A regards molecular genetics EQA, laboratories are asked to treat the samples using current methods and to report the results (raw data and full interpretative reports), via their own account, in the website, in anonymous, and identified solely by their ID. Technical data related to samples and clinical indication must be downloaded from the website The Laboratory that does not provide returns on or before the given deadline will be deemed not evaluated.

At the end of the evaluation, participants will download their performance directly from the website. Participants who wish to present to scheme organiser claim related to their performance evaluation can do so using the address present in the website.

Results

The web based system has been used for the sixth trial. A total of 96 Public laboratories have been enrolled by website through an account, with an increase of 65.3% compared to the first trial. In particular: 35 laboratories out of 96 were registered for molecular genetics, 30 for cytogenetics, and 31 for cytogenetics and molecular genetics. The number of respondents was: 46/49 (94%) for Cystic Fibrosis; 22/23 (96%) for Beta-Thalassemia; 18/21 (86%) for Fragile-X syndrome; 6/6 (100%) for Adenomatous Polyposis Coli; 37/43 (86%) for prenatal diagnosis; 47/54 (87%) for postnatal diagnosis; 28/33 (85%) for oncological cytogenetics.

To better identify the performance of laboratories a marking system has been developed in molecular genetics and in cytogenetics in cooperation with Dr. R. Elles, Dr. S.Patton (European Molecular Genetics Quality Network) and with Dr. Ros Hastings Dr. Rod Howell (Cytogenetic European External Qualità Assessment, EUROGENTEST), on the basis of their long experience in EQA.

At the moment, assessors are evaluating raw data.

In a second time, assessors will attend a meeting at the Italian National Institute of Health to discuss and to perform the final assessment.

Conclusions

The increase of the number of participants (19 responders more than the fifth trial) suggests that a web based system is more easily approachable for laboratories. The efficiency of the proposed marking system will be evaluated in a few years time.

Papers published within the project

Falbo V, Floridia G, Salvatore M, Marra M, Tosto F, Censi F, Taruscio D. The Italian External Quality Assessment (EQA) in genetic tests: the VI EQA scheme. In: Taruscio D, Salvatore M (Ed.). *Workshop. Projects on rare diseases funded within the bilateral agreement Italy (Istituto Superiore di*

Sanità) and USA (NIH, Office for Rare Diseases) on joint research and development of public health actions. Istituto Superiore di Sanità. Rome, October 29-31, 2008. Abstract book. Roma: Istituto Superiore di Sanità; 2008. (ISTISAN Congressi 08/C10). p. 54.

Falbo V, Floridia G, Salvatore M, Tosto F, Censi F, Taruscio D. The Italian external quality assessment in genetic tests: development of a web-based system. In: Taruscio D, Salvatore M (Ed.). *Workshop. Rare Diseases and Orphan Drugs. Rome, November 7-8, 2007. Abstract book.* Roma: Istituto Superiore di Sanità; 2007. (ISTISAN Congressi 07/C8). p. 42.

Acknowledgments

RARE CANCERS IN ITALY

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Introduction

Information and health care statistics for cancer are better than for most other diseases, both because there is a long history of epidemiological studies and because population-based cancer registries have provided an invaluable source of information for decades. However, the burden of rare cancers in Italy is unknown, and no generally accepted definition exists.

In this context, RITA "Surveillance of Rare Cancers in Italy" project aims at measuring the burden of rare cancers in the Italian population and to improve the data quality of rare cancers collected by population-based cancer registries. RITA project integrates in the European Project "Surveillance of rare cancers in Europe" RARECARE (co-funded by the European Commission). This contribution reports the conclusion of the international consensus group on the definition and the list of rare cancers and provides rare cancers indicators of frequency and outcome in Italy.

Methodos

A working group including epidemiologists, pathologists and oncologists was established. This group agreed on a list of tumour entities clinically meaningful. They also agreed that incidence is the best indicator for measuring cancer frequency and set the threshold of rarity at 6/100,000/year. Accordingly to this definition a list of rare cancers was developed. The list was hierarchically structured in three layers based on various combinations of ICD-O morphology and topography to respond to different needs: layer 1) families of tumours (relevant for the health care organisation), layer 2) tumours clinically meaningful (relevant for clinical decision making and research), layer 3) WHO tumour entities. The list includes 230 rare cancers. Incidence, survival, prevalence and mortality were calculated for all the 230 rare entities identified both for Italy and for Europe. The Italian data came from 20 population-based cancer registries.

Results

Accordingly to our analysis, the annual European incidence rate of all the 230 rare cancers is about 100 per 100,000 (513,000 new diagnoses/year) and about 2,700,000 (557/100,000) European citizens live with a past diagnosis of rare tumours received during the last 15 years (1988-2002). In Italy there are 50.000 new diagnoses/year and 280.000 citizens live with a past

diagnosis of rare tumours (received in the last 15 years). Figure 1 shows the incidence of each of the 230 rare cancers in Italy and in Europe.
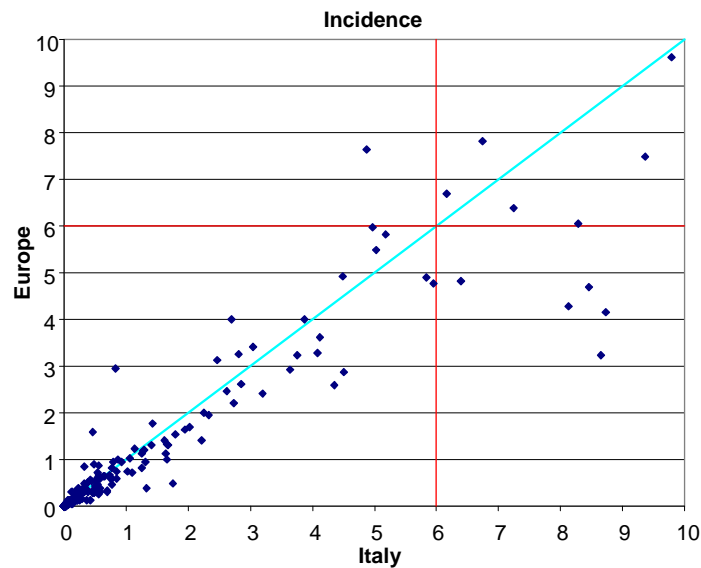
Figure 1. Rare cancers incidence in Italy and in Europe

The incidence comparison between the European and the Italian populations shows that only the epithelial tumours of the oesophagus are rare in Italy but are not rare in Europe, whereas 5 rare tumour entities (Chronic myeloid leukaemia, epithelial tumours of the larynx, epithelial tumours of the extra hepatic biliary tract, carcinoma of endocrine organs, hepatocellular carcinoma of the liver) are rare in Europe but are not rare in Italy. Figure 2 shows the prevalence of each of the 230 rare cancers in Italy and in Europe.
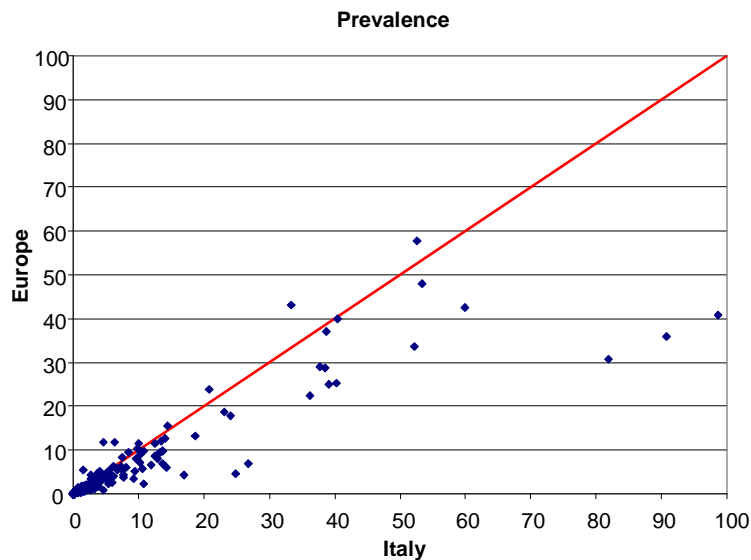
Figure 2. Rare cancers prevalence in Italy and in Europe

Accordingly to our analysis, prevalence in Italy seems higher than in EU.

Discussion

The RITA project represents a unique opportunity to study the epidemiology of rare cancers in Italy. The project provides frequency and outcome indicators by age and sex for 230 rare cancers. To our knowledge no similar large scale analysis for rare cancers have been reported in Italy. The data quality analysis for selected rare tumour is actually on going.

The RITA working group

Francesco Bellù (Registro Tumori Adige/Tumor register Südtirol); Adriano Giacomin (Biella Cancer Registry); Stefano Ferretti (Ferrara Cancer Registry); Diego Serraino (Friuli-Venezia Giulia Cancer Registry, Udine); Marina Vercelli (Liguria Cancer Registry, IST/Univ. Genova); Susanna Vitarelli (Macerata Province Cancer Registry); Massimo Federico and Claudia Cirilli (Modena Cancer Registry); Mario Fusco (Napoli Cancer Registry); Adele Traina (Palermo Breast Cancer Registry); Vincenzo De Lisi (Parma Cancer Registry); Rosario Tumino and Giuseppe Cascone (Cancer Registry Azienda Ospedaliera "Civile M.P.Arezzp", Ragusa); Lucia Mangone and Massimo Vicentini (Reggio Emilia Cancer Registry); Fabio Falcini, Flavia Foca and Rosa Vattiato (Romagna Cancer Registry – IRST); Andrea Donato (Salerno Cancer Registry); Mario Budroni (Sassari Cancer Registry); Silvano Piffer (Trento Cancer Registry); Emanuele Crocetti (Tuscan Cancer Registry, Firenze); Francesco La Rosa (Umbria Cancer Registry); Giovanna Tagliabue, Paolo Contiero and Mariarosa Ruzza (Cancer Registry Unit – Varese Cancer Registry, Fondazione IRCCS, "Istituto Nazionale dei Tumori"); Paola Zambon and Annarita Fiore (Veneto Cancer Registry, Istituto Oncologico Veneto – IRCSS, Padova); Franco Berrino, Paolo G. Casali, Lisa Licita, Gemma Gatta, Elena Palassini, Rossana Berruti (Fondazione IRCSS Istituto Nazionale Tumori, Milano); Angelo Paolo Dei Tos (Ospedale di Treviso, ASL, Treviso); Arduino Verdecchia, Riccardo Capocaccia, Roberta De Angelis (Istituto Superiore di Sanità).

SECTION 2
Diagnosis

USEFULNESS OF MLPA IN THE MOLECULAR DIAGNOSIS OF LISSENCEPHAY AND NEURONAL MIGRATION DISORDERS

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Lissencephaly (LIS), pachygyria and Subcortical Band Heterotopia (SBH) represent a malformative spectrum of abnormal neuronal migration, often resulting from mutations of either LIS1 or DCX genes. Most children have severe developmental delay and infantile spasms, but milder phenotypes are on record, including posterior SBH owing to mosaic mutations of LIS1. LIS1 mutations result in more severe LIS in the posterior brain regions (p>a gradient). LIS1 is involved in both isolated lissencephaly sequence (ILS) and Miller-Dieker syndrome (MDS). ILS is caused by intragenic mutations or by internal deletions of LIS1 whereas MDS is caused by deletions of contiguous genes in 17p13.3, including LIS1. DCX mutations usually cause anteriorly predominant LIS (a>p gradient) in males and SBH in females. Mutations of DCX have been found also in males with anterior SBH and in female relatives with normal brain magnetic resonance imaging.

We selected two distinct groups of patients. The first group of patients (n. 45) showed p>a LIS not including MDS; in all these patients fluorescence in situ hybridation (FISH) for the 17p13.3 region gave negative results. The second group of patients (n. 23) showed sporadic, diffuse, or anteriorly predominant SBH.

We initially performed DNA sequencing of LIS1 in patients with p>a LIS and DNA sequencing of DCX in patients with SBH. Subsequently, we performed Multiplex Ligation-dependent Probe Amplification (MLPA) in those patients who were mutation negative.

In our cohort of patients with p>a LIS, MLPA identified small genomic deletions/duplications of LIS1 in about 76% (19/25) of patients who had previously been tested unsuccessfully with both FISH and DNA sequencing. Overall, small genomic deletions/duplications, represented 49% (19/39) of genomic alterations and brought to 87% (39/45) the number of patients in whom any involvement of LIS1 could be demonstrated in our series. In order to characterize the breakpoint regions, we performed long range PCR in five patients with deletions. We demonstrated that, in four of them, deletions were caused by Alu elements mediated recombination, suggesting that LIS1 is particularly prone to undergo recombination between Alu elements. The high frequency of genomic deletions/duplications of LIS1 is in keeping with the over representation of Alu elements in the 17p13.3 region.

In our cohort of patients with diffuse or anteriorly predominant SBH, we found mutations of the DCX gene in 12 (11 women, 1 man) (12/23, 52%). We used MLPA to search for whole-exon deletions or duplications in the 11 remaining women. MLPA assay uncovered two deletions encompassing exons 3-5 and one involving exon 6 in 3/11 women (27%) and raised the percentage of DCX mutations from 52% (12/23) to 65% (15/23) in our series. We used semi-quantitative fluorescent multiplex PCR (SQF-PCR) and Southern blot to confirm MLPA findings.

MLPA has been demonstrated to have a high diagnostic yield and should be used as first line molecular diagnosis for p>a lissencephaly. MLPA should be also used women with diffuse or anteriorly predominant SBH to uncover DCX deletions after DNA sequencing has failed to detect mutations.

In collaboration with Prof. Orsetta Zuffardi - University of Pavia (Partner of the Project), we performed array-CGH in patients with LIS-SBH spectrum who did not harbour mutations of either the LIS1 or DCX gene. All patients included in the study had a normal karyotype and were analyzed using the 44k platform (100 kb resolution). Every possible imbalance was at first checked against the database of known polymorphisms (http://projects.tcag.ca/variation/). Array-CGH findings considered as causative were confirmed with a higher resolution platform 244k (20 kb resolution).

We identified a de novo dup(7)(q11.23) in a 2 year old boy with severe developmental delay and a simplified gyral pattern with cortical thickening and hypoplastic vermis. In addition, the patient presented axial muscular hypotonia, trigonocephaly, low-set ears, bulbous nasal tip, ogival palate, pilonidal sinus, bilateral pes cavum and cryptorchidism.

Papers published within the project

Guerrini R, Dobyns WB, Barkovich AJ. Abnormal development of the human cerebral cortex: genetics, functional consequences and treatment options. *Trends Neurosci* 2008;31(3):154-62.

Guerrini R, Parrini E. Neuronal migration disorders. *Neurobiol Dis* 2009. [Epub ahead of print].

Mei D, Lewis R, Parrini E, Lazarou LP, Marini C, Pilz DT, Guerrini R. High frequency of genomic deletions - and a duplication - in the *LIS1* gene in lissencephaly: implications for molecular diagnosis. *J Med Genet* 2008;45(6):355-61.

Mei D, Parrini E, Pasqualetti M, Tortorella G, Franzoni E, Giussani U, Marini C, Migliarini S, Guerrini R. Multiplex ligation-dependent probe amplification detects *DCX* gene deletions in band heterotopia. *Neurology* 2007;68(6):446-50.

Parrini E, Mei D, Conti V, Sgadò P, Marini C, Guerrini R. Cortical dysplasias of genetic origin. Chapter 17 in Genetics of Epilepsy and Genetic Epilepsies, G. Avanzini and J. Noebels (eds.) 2009 John Libbey Eurotext.

Torniero C, Dalla Bernardina B, Novara F, Cerini R, Bonaglia C, Pramparo T, Ciccone R, Guerrini R, Zuffardi O. Dysmorphic features, simplified gyral pattern and 7q11.23 duplication reciprocal to the Williams-Beuren deletion. *Eur J Hum Genet* 2008;16(8):880-7.

GENOMIC DIAGNOSIS AND CLASSIFICATION OF RARE DISORDERS WITH MENTAL RETARDATION USING HIGH THROUGHPUT TECHNOLOGIES

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Introduction

Mental Retardation (MR) is a quite common clinical disorder, which can result from different causes, including non-genetic and genetic mechanisms. Among genetic causes, Copy Number Variations (CNVs) represent a major mechanism, in particular in patients in which MR is associated with Multiple Congenital Anomalies (MCA). In the last few years, genomic microarrays have increased their ability to detect cryptic pathogenic CNVs. Several commercially platforms, including a large number of spotted sequences, oligonucleotides or Single-Nucleotide Polymorphisms (SNPs), are now available for high resolution whole genome analysis. This project was aiming to analyze a cohort of selected MR/MCA patients using two different microarray-based platforms, in order to detect the unbalanced cases and assess their frequency, to provide the clinical and molecular outline of new rare forms of MR and to establish which diagnostic tool is the most cost-effective.

Clinical selection of patients

Eleven centres of Medical Genetics were enrolled in this study, including 2 from Northern Italy (Mangiagalli Hospital, Milan; Modena Hospital, Modena), 6 from Central Italy (CSS-Mendel Institute, Rome; Bambino Gesù Hospital, Rome; Umberto I Polyclinic, Rome; A. Gemelli Hospital, Rome; Stella Maris Institute, Pisa; Perugia Hospital, Perugia), and 3 from Southern Italy (San Giuseppe Moscati Hospital, Avellino; G. Rummo Hospital, Benevento; Messina University Hospital, Messina). A standardized patient intake form was prepared by trained clinicians, with specific skills in dysmorphology, to include a series of mandatory items, such as family history, complete clinical assessment, and available laboratory and instrumental results. All selected patients were affected by moderate to severe developmental delay/mental retardation (DSM-IV criteria of the American Psychiatric Association) associated with at least one major malformation and/or multiple minor defects and/or facial dysmorphisms. Patients with a pre- or postnatal history suggestive for an acquired disorder were excluded from this study. All patients were previously tested negative for chromosome imbalances using standard cytogenetic and molecular analyses (e.g., FMR1 and other genes). On the whole, 204 consecutive subjects, 108 males and 96 females, were enrolled in this study. The karyotype was normal in 191 subjects, while 13 individuals were hetrerozygous for an apparently balanced chromosomal rearrangement. Na-heparinate and EDTA blood samples were collected from patients and, when possible, from their parents, together with an institutionally approved consent form, signed by the appropriate persons.

Analysis of selected patients using array-CGH

Selected patients were analyzed using two different microarray-based platforms: an oligonucleotide-array, which covers the genome with an average spacing of 75 Kb (Human Chip 44K; Agilent Technologies) and a SNP-array, including about 900,000 SNPs sequences and integrated with about 900,000 oligonucleotides, with a final genomic spacing of 0.700 Kb (Affymetrix GeneChip 6.0). These two platforms were comparable in terms of the cost and timetable of a single experiment. All detected anomalies were confirmed by additional techniques, such as FISH (Fluorescence In Situ Hybridization) and/or Q-PCR (Quantitative Polimerase Chain Reaction); whenever possible, the analysis was extended to parents in order to assess the de novo *vs* the inherited origin of CNVs.

Oligonucleotide-array analysis

At the CSS-Mendel Institute we have examined all the enrolled 204 MR/MCA individuals using the oligonucleotide-array. CNVs were considered only when at least 4 consecutive spotted oligonucleotides showed an altered signal and they did not overlap with polymorphic copy number variants. CNVs detected in regions without known genes were successively excluded. Fourty-four pathogenic CNVs were detected in 37 patients (18%). CNVs spanned 0.204-22.473 Mb and included 25 deletions and 19 duplications.

SNP-array analysis

Seventy patients selected from the original cohort of 204 subjects, were re-run on the SNP-array platform at Kimmel Cancer Center (Jefferson University, Philadelphia, PA). Nineteen turned out positive using the oligonucleotide-array analysis (SET I), while 51 were negative (SET II). SNP-array analysis detected CNVs in all patients with a highly variable number ranging from 10 to 313 per subject. The CNVs spanned 0.089-22.473 Mb. Successively, some parameters were set to consider only imbalances > 75 Kb encompassing at least 80 probe sets. Considering that the average spacing of the targets of this array is 0.7 Kb, one would expect roughly 107 SNP markers (1 SNP/0.7 kb x 75 kb = 107 SNPs). Therefore, the smallest CNVs were considered only when at least 75% of the CNV length was covered by markers (107 x 0.75 = 80). After this selection, that allowed to reduce the rate of false-positive data, an average of 21 CNVs was detected in each sample (range 8-41). Moreover, CNVs were not considered pathogenic if a rate of overlap with known non-pathogenic polymorphic CNVs was >50-70% (81% of CNVs), or if the CNV did not involve known genes (4.4% of non-polymorphic CNVs). Concerning SET I, SNP-array analysis confirmed all CNVs identified with the oligonucleotide-array platform and in 4 patients it showed additional CNVs not previously detected. In particular, one of these changes was assumed to modulate the phenotype, based on its extent and the genes involved. Concerning SET II, SNP-array analysis disclosed CNVs not detected by the oligonucleotide-array in 3 patients, including one loss and two gains, ranging in size from 0.352 to 0.583 Mb.

Genotype-phenotype correlation

Array-CGH results were compared to data recorded in a public database dedicated to genomic diseases (DECIPHER; https://decipher.sanger.ac.uk/). All but 6 CNVs mapped to genomic regions already described in MR/MCA patients. This technique allowed us to delineate the phenotype due to the X-linked OPHN1 gene duplication, whose deletion and point mutations cause a distinct form of syndromic MR, and to confirm that MSX2 duplication is causative of the Boston-type form of craniosynostosis. We have also outlined the phenotype of some new genomic disorders, including those related to duplication of 16p13 and duplication of 16p13.3, whose deletion causes Rubinstein-Taybi syndrome. In a patient with clinical gestalt of Wolf-Hirchhorn Syndrome (WHS) array-CGH analysis showed the duplication of 12q13.3q14.1 region, suggesting that this rare imbalance can result in a phenocopy of WHS. In two patients with clinical diagnosis of known mendelian disorders, previously tested negative for the relevant genes, array-CGH disclosed cryptic genomic imbalances, pointing to new pathogenic mechanisms causative of these phenotypes. In particular, in a patient affected by HDR (Hypoparathyroidism, Deafness, Renal dyslpasia) syndrome, which is generally due to GATA3 loss-of-function mutations or deletion, array-CGH disclosed the duplication of this gene, suggesting that both hypo- and hyperexpression of GATA3 result in overlapping phenotypes. In addition, in a patient with bilateral frontoparietal polymicrogyria (BFPP), an autosomal recessive disease mapping to 16q13, array-CGH detected a deletion of 16q12.1q21 region, including the BFPP gene (GPR56) and other members of the GPR family. These data suggest that hemyzygosity of 16q critical region can be a new causative mechanism for this neurologic disorder. On the other hand, the detailed characterization of two identical deletions at 3q29 region, found in two families in which this imbalance segregated from mothers, showed that all 4 affected individuals had moderate non specific mental impairment, suggesting that 3q29 deletion does not result in a clinically recognizable phenotype. However, this chromosomal region should be explored in subjects affected by moderate MR, microcephaly and aspecific dysmorphic features.

Oligonucleotide- and SNP-array platforms comparison

The increased resolution provided by SNP-array platforms showed an increased ability in detecting small CNVs. In our cohort of patients, 7 additional copy number changes were detected, including 3 possibly pathogenic imbalances, found in 3 patients tested negative with oligonucleotide-array. This means that ~6% of subjects considered "balanced" in previous analyses were found to be affected by a "genomic disorder". In one of these patients, duplication at Xp11.4 involving TSPAN7 gene was detected. TSPAN7 mutations have been associated with X-linked non-syndromic MR, and duplication of this gene has been reported in three patients, although the pathogenic function of the copy number gains remains controversial. In our case, parents were not available to assess the origin of this CNV, but a partially skewed X inactivation pattern suggested that the functionally active X chromosome was harboring the duplication. In another patient, loss of 9q33.1 including the ASTN2 and TRIM32 genes was disclosed. ASTN2 is highly expressed in the central nervous system and a relationship with schizophrenia was suggested recently. TRIM32 has been associated with Bardet-Biedl syndrome 11 (BBS11; OMIM 209900). The third patient showed 4q21.1 duplication encompassed the ANKRD56, SEPT11 and CCNI genes. Parental FISH and qPCR analyses showed the de novo origin of this CNV.

Conclusion

In conclusion, genomic microarrays are important tools for studying patients with idiopathic MR. The opportunity of identifying very small imbalances using high-resolution microarray platforms improves our ability in detecting the genetic defects in about 18-20% of subjects with rare forms of MR. However, this opportunity should be weighed against the detection of false positives and small CNVs devoid of any obvious pathogenic effect, including CNVs inherited from unaffected parents. Therefore, stringent parameters to use for data analysis and parents' DNA should be mandatory if these genomic platforms are used for diagnostic purposes.

Papers published within the project

Bedeschi MF, Novelli A, Bernardini L, Parazzini C, Bianchi V, Torres B, Natacci F, Giuffrida MG, Ficarazzi P, Dallapiccola B, Lalatta F. Association of syndromic mental retardation with an Xq12q13.1 duplication encompassing the oligophrenin 1 gene. *Am J Med Genet A*. 2008;146A:1718-24.

Bernardini L, Castori M, Capalbo A, Mokini V, Mingarelli R, Simi P, Bertuccelli A, Novelli A, Dallapiccola B. Syndromic craniosynostosis due to complex chromosome 5 rearrangement and MSX2 gene triplication. *Am J Med Genet A*. 2007;143A:2937-43.

Bernardini L, Sinibaldi L, Capalbo A, Bottillo I, Mancuso B, Torres B, Novelli A, Digilio MC, Dallapiccola B. HDR (Hypoparathyroidism, Deafness, Renal dysplasia) syndrome associated to GATA3 gene duplication. *Clin Genet* 2009;76(1):117-9.

Borgatti R, Marelli S, Bernardini L, Novelli A, Cavallini A, Tonelli A, Bassi MT, Dallapiccola B. Bilateral frontoparietal polymicrogyria (BFPP) syndrome secondary to a 16q12.1-q21 chromosome deletion involving GPR56 gene. *Clin Genet* 2009;76(6):573-6.

Dallapiccola B, Bernardini L, Novelli A, Mingarelli R Phenocopy of Wolf-Hirschhorn syndrome in a patient with duplication 12q13.3q14.1. *Am J Med Genet A*. 2009;149A(3):546-8.

Dallapiccola B, Bernardini L, Novelli A, Mingarelli R. Expanding the Phenotype of Duplication of the Rubinstein-Taybi Region on 16p13.3. *Am J Med Genet A* 2009;149A(12):2867-70.

Digilio MC, Bernardini L, Capalbo A, Capolino R, Gagliardi MG, Marino B, Novelli A, Dallapiccola B. 16p subtelomeric duplication: a clinically recognizable syndrome. *Eur J Hum Genet.* 2009;18.

Digilio MC, Bernardini L, Mingarelli R, Capolino R, Capalbo A, Giuffrida MG, Versacci P, Novelli A, Dallapiccola B. 3q29 microdeletion: a mental retardation disorder unassociated with a recognizable phenotype in two mother–daughter pairs. *Am J Med Genet* 2009;149A(8):1777-81.

USEFULNESS OF 244K ARRAY-CGH IN THE ASCERTAINMENT OF COPY NUMBER VARIATIONS IN MENTAL RETARDATION

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Introduction

We screened 100 mentally retarded patients, according DSM-IV-TR criteria, with a whole genome high density array platform (theoretical resolution of ~10Kb). The enrolled patients were divided in two clinical subgroups, made up of 50 patients each, one with a "chromosomal phenotype" and the other without major dysmorphic features. The patients were included in the appropriate group according to the score reached following the administration of the clinical checklist published by de Vries *et al.* (*J Med Genet* 2001;38:145-50). We established a total score of 3 as cut-off, separating group 1 (scoring 3 or above) from group 2 (scoring 2 or less). Array CGH analyses detected 1945 Copy Number Variations (CNVs) in 100 patients. 1918 were benign or common CNVs not related to mental retardation, 24 were rare CNVs, two were complex rearrangements and one was a mosaic chromosome 9 trisomy. Out of the 24 rare CNVs, 16 were well-defined genomics disorders associated to MR whereas 8 had an unknown clinical significance. The mean size for losses and gains was 1 Mb and 3.8 Mb, respectively. MLPA analysis performed in patients positive to Array-CGH and in their parents confirmed all deletions/duplications identified and none of these was identified in a group of at least 100 individuals in the general population. The carriers of causative CNVs between the two groups were 14/50 (28%) in dysmorphic patients and 11/50 (22%) in group 2. The high percentage in both groups could suggest that the "chromosomal phenotype" may not be the best clue for the diagnosis of pathogenic CNVs. Usefulness of 244k array platform was confirmed for 5/27 (18.5%) causative rearrangements that would have been missed by lower array resolution; three of them with a well known clinical significance and 2 unknown (mean size 49.5 Kb).

Mental Retardation (MR), associated or not to other clinical features, is a developmental disability that is marked by lower than normal intelligence (IQ<70) and limited daily living skills with onset before the age of 18 years. The 2-3% of the general population shows MR and it represents, at least in developed countries, the most frequent cause of handicap in children. A direct cause of mental retardation can actually be found in only ~50% of patients the remaining group, resulting normal to routine cytogenetic analysis, is classified having an idiopathic MR. In recent years, use of genome-wide microarrays, in patients affected by unexplained MR, detected non-recurrent submicroscopic genome imbalances with a variable percentage (10-25%) depending on techniques used and selection patients methods. Koolen *et al.* (*Hum Mutat* 2009;30:283-92), reporting 11.2% as the percentage of causative CNVs in the published genome-wide microarray

studies, highlighted that the frequency of clinically significant CNVs is currently underestimate because most of the used microarrays, discovered only genomic rearrangements >100-300kb in size. A comparative study among four high density array platforms highlighted that Agilent technologies is the most accurate and user-friendly. This background has led us to assess the usefulness of 244k Agilent array platform in a cohort of mentally retarded patients.

Methods

Patients

Two years ago we enrolled 100 patients with a diagnosis of MR according DSM-IV-TR criteria and normal routine cytogenetic analysis. Each patient was visited by a clinical geneticist who ruled out peculiar signs of well known syndromes. All patients were divided into two subgroups made up of 50 patients each, one (group 1) with a "chromosomal phenotype" and the other (group 2) without major dysmorphic features. The patients were included in the appropriate group according to the score reached following the administration of the clinical checklist. Each subject had a score between 0 and 10, depending on the presence or absence of some clinical features such as MR in family history, small birth weight, abnormal postnatal growth, some facial dysmorphisms and congenital abnormalities. We established a total score of 3 as cut-off, separating group 1 (scoring 3 or above) from group 2 (scoring 2 or less).

Array-CGH analysis

Fresh blood extracted DNA from each patient was enzymatically digested and random priming labelled with Cy3-dUTP. The labelled DNA was then mixed with an equimolar amount of Cy5 - dUTP labelled sex-matched reference DNA (NA15510 and NA10851 from Corriel Istitute), The mixture was hybridized against human Cot1 DNA to block repetitive sequences and then hybridized for 40 hours against the CGH 244K slide, from Agilent. Eventually the slide was washed and scanned by a G25651B laser (Agilent Technologies). Results were then analyzed by the CGHanalytics v 3.5 software using both the statistical algorithm Z-score and ADM-2.

For every analysed patient, we collected all the CNVs which were grouped into known CNVs, those reported in the database of genomic variants (http://projects.tcag.ca/variation/), and CNVs not reported in this database. Further criteria to assess the clinical significance of this latter subgroup were the inheritance of the CNVs, the involvement of genomic regions known to be associated with mental retardation, and gene content of the imbalances.

MLPA analysis

We tested patient positive to Array-CGH, their parents and a set of at least 100 individuals from general population. We designed new oligo probes in each region of interest. PCR reaction will be performed directly using the FAM labeled fluorescent PCR primer and protocol available from http://www.mlpa.com/pages/indexpag.html. PCR products were identified and quantified by capillary electrophoresis on an ABI 3130 genetic analyser, using the Gene Mapper software from Applied Biosystems, Foster City, CA, USA. In order to process efficiently the MLPA data, a spreadsheet was generated in Microsoft Excel. The data corresponding to each sample (patient's and control's DNAs) were normalised by dividing each probe's signal strength (i.e., the area of each peak) by the average signal strength yielded by the 10-50 control

probes to generate for each peak a Relative Peak Area (RPA). The RPA for each probe in the patient's sample was then compared to that of a control's sample by dividing, for each peak, the patient's RPA by the control's RPA. According to the latter ratio, samples could be categorised as: (i) "normal" (between 0.75 and 1.25), (ii) "deleted" (<0.6), and (iii) "duplicated" (>1.4). For samples yielding uncertain ranges (i.e., 0.6–0.75 and 1.25–1.4), the MLPA test was repeated. As a quality check for the probes, we computed the Standard deviation (SD) of the normalised signals of the ten controls' probes in patients and controls. MLPA analysis was repeated for samples yielding a SD exceeding the threshold value of 5%.

Results

We found 24 rare CNVs of clinical significance (19 deletions and 5 duplications ranging from 21Kb to 8.7 Mb):16 are well-defined genomic disorders associated to MR (OMIM182290, OMIM610443, OMIM610883, OMIM 194050, OMIM 606232, OMIM 612337, OMIM 612474) whereas eight are rare rearrangements not present in the database of CNVs variants. We also found two complex rearrangements and one mosaic chromosome 9 trisomy (Table 1).

Table 1. Patients carriers of clinical significant CNVs

Patient	Score	MR severity	Karyotype	Description	Size
1	4	severe	1q44	de novo del	1.6 Mb
2	6	profund	7q11.23	de novo del	1.5 Mb
3	4	mild	17q21.31 Xp22.32	de novo del de novo del	644K; 1Mb
4	3	moderate	22q13.33	de novo del	25kb
5	3	moderate	22qter	de novo del	0.1 Mb
6	3	moderate	10q26.3-qter	de novo del	8.7 Mb
7	4	moderate	16p11.2	de novo del	578Kb
8	3	severe	22qter	de novo del	3.4 Mb
9	5	mild	15q13.3	de novo del	1.57 Mb
10	4	severe	46,XX,+9 / 46 XX (9%)	de novo	
11	3	moderate	17p11.2	de novo dup	3.4Mb
12	4	mild	2p16.3	de novo del	21Kb
13	2	severe	17q21.31	de novo del	500kb
14	2	NOS	2p25.2	de novo del	1Mb
15	0	severe	22q13.33	de novo del	66kb
16	2	mild	Xp11. 21	mat inher dup	765Kb
17	2	mild	5q32	de novo del	200kb
18	0	severe	22qter	de novo del	1,7 Mb
19	0	moderate	6p21.32	mat inher del	128 Kb
20	4	severe	17p11.2	de novo del	3.6 Mb
21	2	severe	9p24.3 9p21	mat inher del pat inher dup	49 Kb 1.8 Mb
22	2	moderate	15	invdup	
23	3	severe	15	invdup	
24	2	severe	1q21.1	dup ND inheritance	3.2 Mb
25	2	mild	Xp22.32	mat inher dup	~500 Kb

The resulting 27 rearrangements were de novo in 22 cases and inherited in remaining five, four from unaffected parents and one by a mentally retarded mother (patient 21 in table 1). In the group 1 we detected 14/50 (28%) carriers of causative aberrations *vs* 11/50 (22%) in the group 2. Figure 1 shows a comparison of scores distribution between patients with and without causative aberrations.
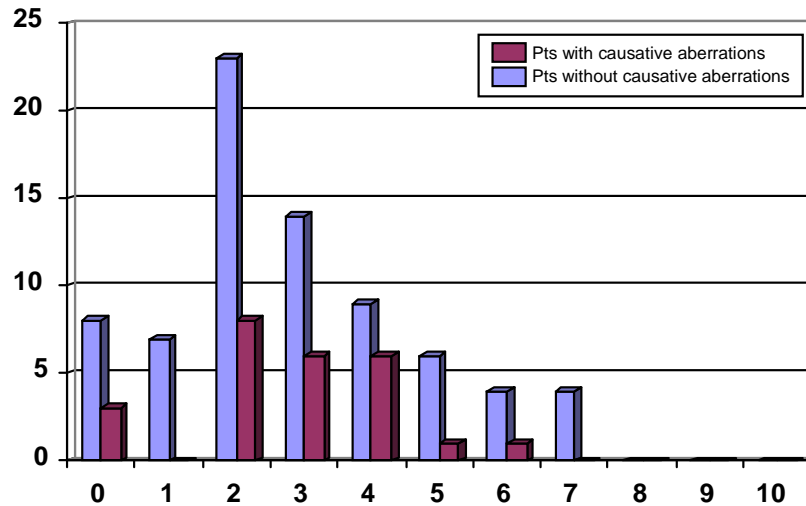
Figure 1. Clinical score distribution of patients with and without causative aberrations

MLPA analysis of RPA Ratio determined in patients and controls for 9p24.3, 9p21.2, 2p16.3 and 6p21.32 chromosomal regions was performed in all patients positive to Array-CGH. Results confirmed all deletions/duplications identified and none of these have been found in a group of at least 100 individuals in the general population.

Conclusions

Since its introduction in MR research, array-CGH has proven to be a powerful and promising method that is revolutionizing cytogenetic diagnosis, at least doubling the detection rate in MR. While highlighting many formerly unresolved cases, the array-CGH demonstrated the high degree of variability in human genome in term of structural variation. As many of these structural variations could potentially represent risk factors, and some of them have already been linked to human diseases, they will have a great impact into the study of the genetic basis of complex diseases. On the other hand, such a complexity becomes a challenge for the cytogeneticist when interpreting array-CGH results performed in clinical diagnostic settings because there is not yet a confident background about the biological significance for many of the CNVs.

The use of higher array resolution leads to the identification of a large amount of CNVs of which the potential role in developmental disorders is often unclear, raising the question of whether more resolution really increases the diagnostic yield. In this study we demonstrate that 5 out of 27 (18.5%) rearrangements identified in 100 patients would have been overlooked by using a lower resolution platform and that 3 of them are clearly clinically significant. Moreover,

the high percentage (28% *vs* 22%) in the two groups of mentally retarded patients (dysmorphic and not-dysmorphic) could suggest that the "chromosomal phenotype" may not be the best clue for the diagnosis of pathogenic CNVs. In the light of these results and giving that CNV-phenotype correlation data are rapidly growing whereas the cost of high-resolution slides is decreasing, we suggest that high-resolution array should be considered in diagnostic setting in all mentally retarded patients with and without a "chromosomal phenotype".

Papers published within the project

Greco D, Romano C, Reitano S, Barone C, Benedetto DD, Castiglia L, Fichera M, Galesi O, Zingale M, Buono S, Uliana V, Caselli R, Canitano R, Hayek G, Renieri A.Three new patients with dup(17)(p11.2p11.2) without autism. *Clin Genet* 2008;73(3):294-6.

Koolen DA, Sistermans EA, Nilessen W, Knight SJ, Regan R, Liu YT, Kooy RF, Rooms L, Romano C, Fichera M, Schinzel A, Baumer A, Anderlid BM, Schoumans J, van Kessel AG, Nordenskjold M, de Vries BB. Identification of non-recurrent submicroscopic genome imbalances: the advantage of genome-wide microarrays over targeted approaches. *Eur J Hum Genet* 2008;16(3):395-400.

Koolen DA, Vissers LE, Pfundt R, de Leeuw N, Knight SJ, Regan R, Kooy RF, Reyniers E, Romano C, Fichera M, Schinzel A, Baumer A, Anderlid BM, Schoumans J, Knoers NV, van Kessel AG, Sistermans EA, Veltman JA, Brunner HG, de Vries BB. A new chromosome 17q21.31 microdeletion syndrome associated with a common inversion polymorphism. *Nat Genet* 2006;38(9):999-1001.

Lo Castro A, Giana G, Fichera M, Castiglia L, Grillo L, Musumeci SA, Galasso C, Curatolo P Deletion 2p25.2: a cryptic chromosome abnormality in a patient with autism and mental retardation detected using aCGH. *Eur J Med Genet* 2009;52(1):67-70.

Sharp AJ, Mefford HC, Li K, Baker C, Skinner C, Stevenson RE, Schroer RJ, Novara F, De Gregori M, Ciccone R, Broomer A, Casuga I, Wang Y, Xiao C, Barbacioru C, Gimelli G, Bernardina BD, Torniero C, Giorda R, Regan R, Murday V, Mansour S, Fichera M, Castiglia L, Failla P, Ventura M, Jiang Z, Cooper GM, Knight SJ, Romano C, Zuffardi O, Chen C, Schwartz CE, Eichler EE. A recurrent 15q13.3 microdeletion syndrome associated with mental retardation and seizures. *Nat Genet* 2008;40(3):322-8.

METACHROMATIC LEUKODYSTROPHY: NEW INSIGHTS IN THE GENOTYPE-PHENOTYPE CORRELATION AND IN THE NATURAL HISTORY OF THE DISEASE

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Introduction

The project has been organized in two Work Packages (WP): WP1 based its activity on the identification of prognostic markers and tests for monitoring disease progression; WP2 was focused on the molecular characterization for genotype-phenotype correlation studies.

Identification of prognostic markers and tests for monitoring disease progression

We followed a cohort of 27 MLD (Metachromatic LeukoDystrophy) patients (16 LI, 7 EJ, 2 LJ, 2 AD) with periodical clinical and instrumental evaluations.

All patients were molecularly characterized and, based on their genotype (see WP2), classified as 0/0, 0/R, and R/R patients.

For the quantification of residual motor function, each patient was evaluated with the Gross Motor Function Measure (GMFM).

For evaluation of peripheral nervous system, sensory conduction of sural and median nerves, and motor conduction of deep peroneal and ulnar nerves were studied. We introduced the Nerve Conduction Velocity (NCV) index in order to monitor Peripheral Nervous System (PNS) involvement along time and, at the same time, to take into consideration all four tested nerves. For each tested nerve, a z-score value is calculated subtracting average NCV from control age-matched population from patient' NCV, and dividing this value by SD from control age-matched population:

$$\frac{\text{patient NCV} - \text{normal subjects' mean NCV}}{\text{normal subjects' NCV standard deviation}}$$

NCV index is then calculated as the average of z-score obtained from the four tested nerves for each patient per time.

MR studies were scored using an adapted the Loes' scoring system.

In 0/0 patients GMFM revealed profound motor deficits. The MR score, which was suggestive of minor demyelination at onset, increased rapidly with the appearance of diffuse demyelination and atrophy. The NCV index demonstrated a severe peripheral neuropathy.

In 0/R patients GMFM score was consistent with severe motor impairment within 2 years from onset. The MR score rapidly reached high values because of extensive demyelination and atrophy. The NCV Index showed the presence of peripheral neuropathy, even if less severe than in 0/0 patients.

R/R patients displayed a severe cognitive impairment, associated with mild to moderate motor deficits. The MR score did not significantly worsen throughout follow-up and remained lower than the score observed in 0/0 and 0/R patients. PNS was never involved.

From a clinical point of view, we observed, in all patients carrying at least one 0 allele, the involvement of PNS since first evaluation and a rapid disease progression. Therefore, in the absence or delay of molecular diagnosis, ENG can provide information for predicting the patient's prognosis.

Moreover, we demonstrated that Gross Motor Function Measure, ElectroNeuroGraphic recordings, and brain Magnetic Resonance represent reliable and sensitive tools to quantify and monitor the progression of neurological involvement.

Molecular characterization for genotype-phenotype correlation studies

Screening for common mutations allowed us complete genotype identification in only 5 out of the 27 patients. In the remaining patients, molecular diagnosis was possible only by sequencing of the coding region of the ARSA gene, allowing the identification of 11 rare previously described mutations and 9 new ones, which were further characterized. All the new alterations, but one, showed a null residual activity, with values below 1% of the activity measured on HeLa cells transduced with the control ARSAwt-LV. The only exception was the mutation c.412C>G, which retained a residual activity of 2.48% of the ARSAwt allele. These data were confirmed with transduction of fibroblasts from the ARSA knock-out murine model, which represent a cellular platform devoid of physiological expression of ARSA. As further confirmation that the new alleles are disease-causing, we reverted the mutations through site-specific mutagenesis of the lentiviral plasmid, and used the corresponding vector to transduce HeLa cells. All the reverted alleles showed non-pathological levels of ARSA activity, approaching the levels measured for the control wild-type enzyme.

According to residual ARSA activity, mutations with no or very low residual activity were classified as 0 alleles, while mutations with reliable residual activity (reproducibly detected above 1% of the wild-type values) were considered R alleles. Based on their genotype, we classified our patients in homogenous clinical groups (see WP1). Patients harboring two 0 alleles were the most severely affected. They had severe clinical manifestations and their disease progression was extremely rapid. Patients harboring one 0 allele in association with one R allele showed a similar presentation and disease evolution, although less rapid, to 0/0 ones. Despite some variability, all patients harboring two R mutations showed a milder disease burden and slower progression when compared with 0/0 and 0/R subjects, with stability of clinical features and instrumental findings at long-term follow-up. Overall, these data demonstrate the occurrence of a precise genotype–phenotype correlation within our cohort, which applies both to the common ARSA gene mutations as well as the rare ones.

In parallel, a second cohort of twenty-one unrelated Italian patients was characterized from a molecular and functional point of view. 17 MLD cases out of 21 were due to 20 different ARSA mutant alleles and 4 others resulted from 2 different Sap-B alleles. The eleven novel ARSA amino acid changes, accounting for the 32% of the mutants, consisted of 8 missense mutations, 1 in-frame deletion, 1 splice acceptor site mutation affecting mRNA processing. The functional relevance of this intronic splicing mutation was determined by carrying out reverse transcriptase-polymerase chain reaction analysis on mRNA, showing two anomalous transcripts: one carrying the expected skipping of an exon, whereas the other showed only a partial loss of exonic regions. The potential impact of the new missense mutants on protein function was addressed by *in vitro* expression experiments confirming that all mutants expressed extremely low residual ARSA activity ranging from 0.6 to 15.6% of the control. Moreover, to further understand the consequences of the new ARSA alleles at the protein level, the amino acid changes were also modelled into the three-dimensional structure, predicting destabilization and/or incorrect folding of the protein.

Conclusion

Molecular characterization and rare mutation analysis provide relevant prognostic indications and constitute reliable criteria for patient's classification in homogenous clinical forms. Genotype evaluation could be particularly valuable, when the prenatal and neonatal screening of lysosomal diseases becomes more widely available, and for accurate patients' selection for the new therapies under development for MLD. In addition, evaluation of nerve conduction velocities can be considered as a frontline test for all MLD patients, together with complete molecular analysis, as a further prognostic marker.

Our findings demonstrated and confirmed the necessity of a comprehensive evaluation, based on a range of diagnostic procedures including neuroradiological, neurophysiological, biochemical and molecular tests, to shed light on the underlying pathological causes of metachromatic leukodystrophy and to quantify and monitor the progression of neurological involvement.

Papers published within the project

Biffi A, Cesani M, Fumagalli F, Del Carro U, Baldoli C, Canale S, Gerevini S, Amadio S, Falautano M, Rovelli A, Comi GC, Roncarolo MG, Sessa M. Metachromatic leukodystrophy – mutation analysis provides further evidence of genotype-phenotype correlation. *Clin Genet* 2008;74:349-57.

Cesani M, Capotondo A, Plati T, Sergi Sergi L, Fumagalli F, Roncarolo MG, Naldini L, Comi GC, Sessa M, Biffi A. Characterization of new Arylsulfatase A gene mutations reinforces genotype-phenotype correlation in Metachromatic Leukodystrophy. *Human Mutations* 2009;30(10):E936-45.

Eichler F, Grodd W, Grant E, Sessa M, Biffi A, Bley A, Kohlschuetter A, Loes DJ, Kraegeloh-Mann I. Metachromatic leukodystrophy: a scoring system for brain MR observations. *Am J Neuroradiol* 2009;30:1893.

Grossi S, Regis S, Rosano C, Corsolini F, Uziel G, Sessa M, Di Rocco M, Parenti G, Deodato F, Leuzzi V, Biancheri R, Filocamo M. Molecular analysis of ARSA and PSAP genes in twenty-one Italian patients with metachromatic leukodystrophy. Identification and functional characterization of 11 novel ARSA alleles. *Hum Mutat* 2008;29:E220-E230.

GENOTYPE-PHENOTYPE CORRELATIONS IN THE CMT NEUROPATHIES: DEFINITION OF A CLINICAL AND GENETIC DIAGNOSTIC FLOW-CHART

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Introduction

Charcot-Marie-Tooth neuropathies (CMTs) are diseases characterized by progressive impairment of motricity and sensibility affecting quality and duration of life. On the basis of electrophysiology and histopathology, CMTs has been divided into demyelinating and axonal neuropathies. Due to the rare occurrence of each clinical form, a proper genotype-phenotype correlation has not been yet established and pathogenetic mechanisms remain still poorly understood. Therapeutical devices are at present simply symptomatic. CMTs have been associated to > 40 loci, inherited with dominant or recessive fashion, and > 25 genes. Nerve biopsy may sometimes facilitate the identification of the genetic defect, however it is often impossible to predict the gene associated with a specific CMT on the basis of the phenotype. Molecular screening process is therefore expensive and time-consuming, mostly following as a unique criterion the frequency of the various forms of CMTs. Moreover, only few centers can analyze a comprehensive panel of CMT genes, therefore in many cases patients need to visit several Institutes to expand genetic screening.

Methods

In order to facilitate the achievement of a definite diagnosis for CMT patients, we therefore collect clinical and histopathological data from subjects referring to the neurological department. A systematic molecular screening of 15 most frequently mutated CMT genes is employed to achieve a genetic diagnosis in most patients, allowing better definition of clinical prognosis, efficient familial counseling, better psychological coping with the disease and improving the definition of the disease for possible future treatments. Moreover, a better clinical and genetic characterization of different forms of CMTs will allow to establish genotype-phenotype correlations and to run hypothesis for pathogenetic mechanisms.

Results

In the last year, thanks to the support of ISS, we were able to increase the number of CMT patients studied by our center. Overall, our cohort now includes 180 patients, including index cases and their relatives. These patients were all carefully evaluated from the clinical, electrophysiological and, whenever possible, histopathological point of view, in order to better define clinical diagnosis and orient molecular testing. All the data were inserted in an on-purpose electronic database shared by all the participants to the project. Genetic analysis was also implemented, both increasing the sensitivity of detection of 17p11.2 rearrangements and the number of CMT genes analysed. We set up MLPA (Multiplex Ligation-dependent Probe Amplification) method to detect 17p11.2 duplications/deletions by quantifying the number of copies of PMP22 gene in the sample. This method was validated on a cohort of wild-type, duplicated and deleted patients previously genotyped with standard Southern Blotting analysis. With MLPA we were able to correctly identify all the mutated samples, while we detected a duplication and two deletions in patients previously considered as wild-type. This discrepancy is due to the well known low sensitivity (around 70%) of Southern blot method relying on junction fragment detection. We are therefore now introducing MLPA in routine diagnostics to ensure highly sensitive PMP22 gene dosage, allowing to correctly diagnose most patients affected by demyelinating CMT. We also set up molecular analysis of four additional CMT genes by PCR amplification and direct sequencing: YARS, HSP22, HSP27 and GARS and we are now developing the screening of DNM2 gene. This will allow us to widen the panel of genes associated with axonal and intermediate CMTs and therefore to increase the efficacy of our screening flow chart. Results of molecular analyses are communicated during a post-test counseling session, involving a geneticist and a neurologist, aimed at explaining the implications of the results for the individual and his family. In case of positive results, testing is then available for all the interested relatives. To date, 126 probands have now concluded the molecular screening process. Among them, 54% are affected by demyelinating, 27% by axonal and 19% by intermediate forms of CMT. In this cohort, both sporadic (60%) and familial (40%) cases are represented. Fifteen CMT genes (PMP22, MPZ, GJB1, NFL, MFN2, GDAP1, MTMR2, MTMR13, EGR2, LMNA, GARS, YARS, HSP22, HSP27, DNM2) were analysed by conventional approaches, such as direct sequencing with Sanger method preceded or not by DHPLC (Denaturing High Pressure Liquid Chromatography) analysis, following our clinical-genetic flow-chart in which genes are screened on the basis of the type of neuropathy and on the frequency of mutations occurring in each gene. In our cohort, molecular analysis led to the identification of mutations in 52/126 cases (41%), which rises to 66% for patients with positive family history. We could detect a genetic alteration in 47% of demyelinating cases (90% if we consider patients with positive family history), 44% of axonal (65% in familial cases) and 21% in intermediate CMTs (25% in familial cases). Genetic variants identified include PMP22 duplications/deletions, MPZ, GJB1, PMP22, EGR2 and NFL point mutations in demyelinating cases, and MPZ, GJB1, MFN2, GDAP1 and HSP27 mutations in axonal and intermediate cases.

Conclusion

Overall, we identified seven new mutations in the following genes: MPZ (H81Y), EGR2 (D383H), MFN2 (I714V, A738V), HSP27 (S135C), NFL (P440L). In addition, we identified the first splicing mutation in the GJB1 gene (c.-16-3C>G). In some cases histopathological analysis revealed distinctive features: giant axons in the MPZ mutant and reduced density of

axonal neurofilaments and increase in microtubules density in the S135C HSP27 patient. Ongoing histopathological characterization will allow to further improve genotype/ phenotype correlations.

Our results underline that the integration between clinical, histopathological and genetic data might lead to the identification of a consistent number of mutations, even in sporadic patients, and possibly to the correlation with patient phenotype.

Papers published within the project

Bolis A, Coviello S, Visigalli I, Taveggia C, Bachi A, Chishti A, Hanada T, Quattrini A, Previtali S, Biffi A, Bolino A. Dlg1, Sec8 and Mtmr2 regulate membrane homeostasis in Schwann cell myelination. *J Neurosci* 2009;29(27): 8858-70.

Nodari A, Previtali SC, Dati G, Occhi S, Court FA, Colombelli C, Zambroni D, Dina G, Del Carro U, Campbell KP, Quattrini A, Wrabetz L, Feltri ML. Alpha6beta4 integrin and dystroglycan cooperate to stabilize the myelin sheath. *J Neurosci* 2008;28(26):6714-9.

Previtali SC, Malaguti MC, Riva N, Scarlato M, Dacci P, Dina G, Triolo D, Porrello E, Lorenzetti I, Fazio R, Comi G, Bolino A, Quattrini A. The extracellular matrix affects axonal regeneration in peripheral neuropathies. *Neurology* 2008;71(5):322-31.

Previtali SC, Quattrini A, Bolino A. Charcot-Marie-Tooth type 4B demyelinating neuropathy: deciphering the role of MTMR phosphatases. *Expert Rev Mol Med* 2007;9(25):1-16. Review.

TYPE 1 NEUROFIBROMATOSIS: AN INTEGRATED PROGRAM OF CLINICAL AND MOLECULAR DIAGNOSTICS

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The project included the clinical characterization of a cohort of Neurofibromatosis type 1 (NF1) adult patients by the Unit of the IRCCS Policlinico, permitting us to obtain a detailed overview on the spectrum of the disease in adult life. The cohort included 100 adult patients, 63 women and 37 men, aged between 18 and 70 years. All the patients underwent a clinical and genetic evaluation, aimed at defining the basal health status and planning clinical or instrumental examination. Every patient accepted the diagnostic and follow up program with a complete compliance.

The diagnosis of NF1 was confirmed in 97 patients (61 females and 26 males), while a segmental NF1 was diagnosed in three patients (two female and one male).

The incidences of NF1 diagnostic criteria in our population are listed below:

– Cutaneous cafè au lait spots present in 96% of patients.
– Iris Lisch nodules present in 75% of patients.
– Axillary or groin freckling present in 66% of patients.
– Cutaneous or subcutaneous neurofibromas present in 93% of patients.
– Optic nerve glioma diagnosed in infancy in 6% of patients.
– Specific osseous lesion diagnosed in 2% of patients.
– A first degree relative affected by the disease present in 48% of patients.

These data are in accordance with those of the literature about adult patients affected by the disease.

All patients underwent to a neurologic, ophthalmologic, dermatologic and surgical evaluation, in order to evaluate the presence of additional signs or symptoms related to the disease: 54% of patients presented with neurological symptoms, while in 9% a brain tumor (astrocytoma) was diagnosed.

In our study, after the clinical genetic evaluation a genetic counselling session was carried out for all patients and their relatives at risk for the disease. Information about the disease, reproductive aspects, and molecular tests (availability, feasibility, significance of the results) was always discussed, helping patients to draw attention to important issues, such as the degree of acceptance of the disease, the understanding of the genetic basis and the transmission risk of the condition to the offspring. Additional genetic counselling sections were carried out when requested to deepen further questions or problems.

Part of these patients was included in a genetic screening of 81 unrelated patients, 52 adults and 29 children: 57 were diagnosed according to NIH criteria while 24 only showed one of the typical clinical features. We found 36 mutations of the NF1 gene: 32 were present in patients with complete NF1 features. All mutations were investigated at the genomic level using denaturing high pressure liquid chromatography (DHPLC) and sequencing DNA fragments that appeared different from controls. More than 50% of the mutations were unknown. To increase

the mutation detection we subsequently studied by multiplex ligation-dependent probe amplification (MLPA) genomic DNA of patients that scored negatively at DHPLC and sequencing analysis.

We used the MLPA SALSA P081/082 NF1 kit to look for deletion or insertions located inside the NF1 gene and the MLPA SALSA P122B kit to look for deletions encompassing the entire NF1 gene as well as flanking genes (microdeletions). Nine more mutations were detected with this technique: two deletions of a single exon, 3 duplications of a single exon and 4 microdeletions. These four microdeletions were validated by array-comparative genomic hybridization (array-CGH). Seven of the nine mutations were present in patients with complete diagnosis of NF1, bringing from 56% to 67% the fraction of patients with a genetic diagnosis of NF1.

CALLOSAL AGENESIS: A BRAIN MALFORMATION WITH POLYGENIC ORIGIN. IDENTIFICATION OF CANDIDATE GENES AND LOCI THROUGH A MULTIDISCIPLINARY APPROACH OF CLINICAL, CYTOGENETIC AND MOLECULAR STUDIES OF A LARGE SET OF PATIENT WITH CORPUS CALLOSUM ANOMALIES

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Introduction

The Corpus Callosum is the largest fibre in brain and connects neurons in the cerebral hemispheres. Its main cognitive function is to coordinate and transfer information between left and right brain. Different defects in corpus callosum morphogenesis led to partial, complete, isolated corpus callosum agenesis or hypoplasia. Moreover, Corpus Callosum Agenesis (CCA) or hypoplasia may occur as an isolated malformation or as a component of a more complex malformation syndrome. In this research work we focused on corpus callosum agenesis only. In particular we excluded patients with callosal hypoplasia because, in our opinion, it is more frequently caused by an abnormal event during cortical development such as in preterm infants or in cases of more or less severe perinatal asphyxia. For the same reasons also patients with CCA associated with cerebral lesion secondary to perinatal injury were excluded.

Until now over 50 syndromes with CCA and a different mode of inheritance (autosomal dominant, autosomal recessive, X-linked or unknown) have been described in humans.

Some individuals with CCA have intelligence quotients within the normal range; however recent evidence suggests that some of these individuals are more susceptible to behavioural and neuropsychiatric problems (in children), learning difficulties, sleep disorders, visuo-spatial attention deficits, language and social communication disorders.

The incidence of CCA varies as a function of diagnostic techniques and sample populations; it's more elevated in children with developmental disabilities.

Mutations in genes encoding guidance molecules/receptors, transcription factors, extracellular matrix, signalling/cytoplasmic molecules, growth factors, have been shown to cause CCA in mouse. So, it is reasonable to hypothesize that the same genes, or genes with the same evolutionary conserved functions, might be involved in callosal development and agenesis in humans.

Some instruments help researchers to find and study such genes and molecules. First of all, the identification of chromosomal breakpoints in association with variation in human phenotypes often leads to the discovery of novel genes or the characterization of the clinical importance of known genes. In particular, cytogenetic abnormalities in patients with CCA are useful to select chromosome regions with candidate genes responsible for Corpus Callosum

development (positional candidates). Array-CGH is a new useful method for this purpose. CCA animal models are also useful to characterize functional genes.

In 2006 we reported a clinical and genetic study regarding 63 CCA patients referred to our Institute. High-resolution karyotype and FISH using subtelomeric probes identified, respectively, 7 and 3 chromosomal rearrangements (involving loci 8p23, 4p15, 10p,10q11, 21 trisomy and loci 1p36, 1q44, 6q27, 13q32).

In 2007 we started a new 3-years study about genotype and phenotype of an enlarged cohort of CCA subjects referred to our Institute.

Aims and methods

To expand the previously collected cohort extending the analysis on new patients with any type of CCA referred to our Institute. All of them underwent a complete clinical, neuropsychiatric and dysmorphological evaluation.

Subjects were divided in subgroups on the basis of callosal malformation: partial or complete CCA; isolated CCA (isoCCA); CCA in association to other CNS (Central Nervous System) malformation (CCAplus); CCA in association to CNS and non-CNS malformation (syndromic CCA, CCAsyndr).

To perform cytogenetic analysis (high-resolution karyotype and FISH analysis using subtelomeric probes in all new collected subjects; array-CGH analysis in selected patients) in order to detect new chromosomal rearrangements.

To perform molecular analysis of CCA candidate genes selected on the basis of chromosomal location (detected in chromosomal rearrangements found both in our patients and in the literature) or by functional homology data with mouse CCA genes.

Results

The study was performed on 116 CCA subjects: 57 (49%) with complete CCA (iso-, plus-, syndr- CCA) and 59 (51%) with partial CCA (iso-, plus-, syndr- CCA).

We can reach the following conclusions based on the analysis of "clinical results": i) CCA can manifest as isolated malformation or in association to other CNS and/or no-CNS congenital anomalies: 70% of cases are "syndromic"; ii) Cranio-facial malformation is often associated to CCA (65%). Anomalies of posterior fossa and brainstem are associated to partial CCA, while cortex anomalies are more often associated to complete CCA; iii) Delay in developmental milestones or mental retardation is common (mainly associated with multiple malformations); iv) Epilepsy can also be present, more often when CCA is associated to other CNS malformations; v) The "cytogenetic workpakage" led to the detection of a chromosomal imbalance in 19% of patients: high resolution karyotype was positive in 14/114 (12%) subjects (14% partial CCA and 10% complete CCA); FISH using subtelomeric probes was positive in 5/100 (5%) subjects (4% partial CCA and 6% complete CCA); CGH-array detected an interstitial chromosomal imbalance in 3/29 (10%) complete CCA subjects: an interstitial deletion in 1p36; a deletion in 1q44 involving AKT3 gene and a microdeletion of 2,6Mb in 17q11.2 encompassing NF1 gene in a 3 years girl with cafè au lait spots and complete CCA.

As far as the "Molecular workpakage" is concerned, no mutation has been detected in the following genes: AKT3 (performed in 26 subjects), NRP1 (performed in 26 subjects), Netrin1

(performed in 26 subjects), HESX1 (performed in 26 subjects), MCPH1 (performed in 26 subjects), and EMX2 (performed in 26 subjects).

ARX analysis gave a positive result in 1/26 subjects with CCA, epilepsy and mild dysmorphic features.

Conclusions

Others studies are needed to clarify the role of our candidate genes in corpus callosum development and agenesis.

As far as AKT3 is concerned, we can reasonably conclude that it is not involved in callosal agenesis.

Molecular analysis in 26 patients and a recent collaborative study about 1q44 deletion syndrome denied a role for AKT3 in CCA.

Papers published within the project

Bedeschi MF, Bonaglia MC, Borgatti R. *et al.* Agenesis of the Corpus Callosum: clinical and genetic study in 63 young patients. *Pediatric Neurology* 2006;34(3):186-93.

Dobyns WB.Absence makes the search grow longer. *Am J Hum Genet* 1996;58:7-16.

Hines RJ, Paul LK, Brown WS. Spatial attention in agenesis of the corpus callosum: non literal language and affective prosody. *Brain Lang* 2003;85(2):313-24.

Richards LJ, Plachez C, Ren T. Mechanisms regulating the development of the corpus callosum and its agenesis in mouse and human. *Clin Genet* 2004;66:276-89.

Van Bon BW, Koolen DA, Borgatti R *et al.* Clinical and Molecular Characteristics of 1qter Syndrome: Delineating a Critical Region for corpus callosum agenesis/hypogenesis. *Med Genet.* 2008;45(6):346-54.

AUTOSOMAL RECESSIVE SPASTIC PARAPLEGIA WITH THINNING OF CORPUS CALLOSUM AND PERIVENTRICULAR WHITE MATTER CHANGES: CLINICAL, MOLECULAR AND NEUROIMAGING STUDIES

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Introduction

Hereditary Spastic Paraplegia (HSP) refers to a group of genetically heterogeneous neurodegenerative disorders characterized by insidiously progressive gait disturbance due to spasticity and weakness in the lower extremities. Although the estimated prevalence of HSP is about 1/100000 in Europe, these disorders represent important health problems because they cause progressive functional deterioration and handicap.

The single most common form of Autosomal Recessive Hereditary Spastic Paraplegia (ARHSP) appears to be SPG11 on chromosome 15. SPG11 is particularly prevalent in Japan, but also occurs in North America and Europe. Its typical clinical features consist of early-onset spastic paraparesis, urinary bladder dysfunction, deep sensory deficit in the legs, and cognitive impairment insidiously progressing to severe functional disability over a period of 10-20 years. Auxiliary studies reveal Thin Corpus Callosum (TCC) with variable cerebral cortical atrophy on magnetic Resonance Imaging (MRI), variable cortical and thalamic glucose hypometabolism on Positron Emission Tomography (PET), and predominantly axonal motor or sensorimotor peripheral neuropathy on nerve conduction studies.

The overall aim of this project was to study, clinically and genetically, families with ARHSP-TCC linked to the SPG11 locus and to gain insight into the function, in health and disease condition, of a new gene and its overall relationship to the clinical phenotype related to both cortico-motor and white matter degenerations.

Specific objectives were:
- identification of additional families with ARHSP-TCC;
- genetic analysis to prove or, eventually, exclude firmly linkage to the SPG11 locus; identification of additional ARHSP-TCC loci;
- functional neuroimaging in affected individuals;
- identification of array, type, frequency of mutations in a new gene associated with ARHSP-TCC;
- elucidation of the function of the gene and the effects of the mutations on this function;
- establishment of more complete genotype-phenotype correlations.

Methods

Selection of additional patients with ARHSP-TCC

We recruited 40 patients (21 men and 19 women) and 59 healthy relatives from 20 families meeting the clinical and neuroradiologic diagnostic criteria for ARHSP-TCC.

Linkage analysis and positional cloning of the SPG11 gene

We tested linkage to 15q13-q15, using a series of microsatellite markers spanning this chromosomal interval, in all the patients from the 20 families and in their unaffected relatives. Eight additional microsatellites covering the SPG11 were selected for linkage analysis and amplified by PCR in all sampled family members. Pairwise LOD scores were obtained using the FASTLINK version of the MLINK program, under the assumption of: equal male-female recombination rate, autosomal recessive inheritance, full penetrance, a gene frequency of 0.001, and equal allele frequencies for each marker. Multipoint LOD scores were calculated using the Allegro 1.2c program. To determine marker order and genetic distances, we consulted the Marshfield chromosome 15 genetic map and map positions were verified on the human genome sequence draft (http://www.ncbi.nlm.nih.gov, www.ensembl.org). Genes within the interval were directly sequenced used BigDye 3.1 chemistry.

SPG11 mutations: frequency and phenotype(s)

In the attempt to reveal the relative frequency of spatacsin variants, we performed molecular studies to estimate the frequency of SPG11 mutations in a large series of patients with ARHSP with or without TCC, mental retardation (MR) or cognitive impairment, to define the spectrum of the mutations in this gene and to describe the associated phenotypes

Search for a new locus/gene in ARHSP-TCC

We also identified a new highly-consanguineous family from Calabria where patients presented ARHSP-TCC but did not display linkage to chromosome 15q nor mutations in SPG11. We performed whole-genome scanning using highly spaced microsatellites. Refinement of the interval pinpointed candidate genes which were analyzed by direct sequencing.

Results

Selection of additional patients with ARHSP-TCC

Thirteen families were of Italian ancestry, two were Algerian, two were Portuguese, and one each came from Morocco, Turkey, and Germany. Consanguinity was present in 6 families. All but two patients were examined in our specialist centers. Onset occurred at the average age of 16.2±3.2 (range 1.5-21 years) and presented in all the cases through walking difficulties. Overall, peripheral neuropathy was detected in 75% of the cases, and mental retardation in 86%. Neuroradiologic evidence of TCC was obtained in nearly 90% of the cases, whereas periventricular white matter changes were found in 72%. Additional signs were occasionally

observed (e.g., cerebellar ataxia in 5 patients, amyotrophy in 12 cases). All metabolic causes of HSP had already been ruled out by appropriate laboratory investigations. Disease severity was ascertained adopting a modified Ashworth scale.

Linkage analysis and positional cloning of the SPG11 gene

Six families (6/10, 60%) showed pair wise LOD scores ranging from 0.6 to 3.8 at marker D15S659. Considering these data together with those of five previously reported SPG11-linked Italian families, a cumulative maximal multipoint LOD score of 5.97 was obtained in the D15S971-D15S123 marker interval. Through haplotype reconstruction, the telomeric boundary was defined at D15S123 by a recombination event found between D15S123 and D15S143.

A recombination event was also detected between markers D15S971 and D15S1044, and the centromeric boundary was defined at marker D15S1044. The new SPG11 interval partially overlaps the ALS5 locus and excludes the ACCPN locus. In four families linkage to the SPG11 region could be firmly excluded, confirming the genetic heterogeneity of ARHSP-TCC. The clinical picture and mean age at onset (16.1 ± 2.2 years) in the unlinked families were indistinguishable from those of the chromosome 15q-linked families. The newly defined interval of SPG11 contained ~40 genes, according to the National Center for Biotechnology Information and Ensembl databases. No mutations were found in 17 of them upon direct sequencing of all coding and non-coding exons as well as their splicing sites including at least 50 bp of intronic sequences.

When the KIAA1840 gene was analyzed in the whole group of SPG11-linked families, 10 different mutations were found in 11 kindred. All mutations (four nonsense, four small deletions and two small insertions) were in the coding sequence and were at the homozygous state, except in two kindred in whom the patients were compound heterozygous for two different variants.

SPG11 mutations: frequency and phenotype(s)

We collected and systematically evaluated a large number of families (n. 79), totalling 164 patients and 205 unaffected relatives, as well as 63 isolated cases. Two mutations in SPG11 were detected in 44 families (111 patients) and in 17 apparently isolated cases. Mutations were detected in patients with: HSP + mental retardation/cognitive impairment + TCC on MRI (34%); HSP and TCC without mental retardation/cognitive impairment (15%); HSP with mental retardation/cognitive impairment without TCC (29%); and HSP and mental retardation/cognitive impairment for whom MRI was not available (22%). A single heterozygous variant (nonsense= 1; splice-site= 1; small ins/del= 13) was detected in 8 families and 10 additional sporadic cases strengthening the possibility of unconventional variants (such as heterozygous large-scale deletions or deep intronic changes) in SPG11.

Another 11 families and 14 apparently sporadic subjects still need to be tested. With these numbers, we attempted to define the "typical" clinical phenotype of 44 SPG11 patients. Age at onset ranged from 2 to 27 with a mean of 14.0 ± 5.9 years. Onset was, in most cases, characterized by gait disorders (79%), or less frequently by mental retardation (16%), rarely dysarthria and tremor. After a mean disease duration of 14.9 ± 6.6 years (range: 2-35), all patients had a severe clinical picture that included progressive spastic paraplegia: most were at least wheelchair bound (53%) or needed assistance for walking (16%). Patients were wheelchair bound after a mean disease duration of 16.5 ± 5.8 years. Lower limb spasticity could be severe (67% of the cases) and dysarthria was frequently observed (42%). In addition, in 80% of the patients, cognitive decline was evident on examination and worsened with time. Interestingly, electroneuromyography detected lower motor neuron involvement in 81% of the cases after a mean disease duration of

14.4 ± 4.9 years. Brain MRI showed diffuse white matter hyperintensities on T2 images (69%) and cortical atrophy (81%), almost invariably associated with TCC (95%).

Leucoencephalopathy was periventricular and confluent, and its severity increased with disease duration. In mild cases, only frontal and occipital periventricular damage was seen. We also performed MR-Spectroscopy with Diffusion Tensor Imaging-MR in 4 patients having mutations in SPG11, 7 normal age-matched controls, and 1 case with a mutation in SPG4. Data are being processed.

Search for a new locus/gene in ARHSP-TCC

We identified a new highly-consanguineous family from Calabria with data compatible with linkage to the 14q-locus (SPG15). Our findings permitted to significantly refine the SPG15 locus and the identification of mutations in ZFYVE26 (SPG15), encoding spastizin. The relative frequency of mutations in ZFYVE26 in Italian patients with the classical ARHSP-TCC clinical phenotype showed that spastizin is a less frequent cause, accounting for about 5% of the cases.

Conclusion

Using a positional cloning and direct sequencing approach we identified the SPG11 gene as a major cause of ARHSP-TCC, and proved that mutations in SPG11 are a common cause of HSP in young patients from the Mediterranean basin. The presence of HSP and TCC is the best single indicator that SPG11 should be tested in patients with onset in the first to third decade, but the presence of one or more other signs, such as mental retardation and later cognitive deterioration, lower motor neuron involvement and white matter lesions, increases the chance of identifying SPG11 mutations.

Additionally, evidence of white matter abnormalities in the periventricular regions increases even further the probability that SPG11 is the cause of the disease, rather than other causes of leukodystrophy. We also identified a second gene (ZFYVE26) in ARHSP-TCC but its frequency is still to be fully determined. Also, the function of the ARHSP-TCC related gene products will need further research.

Papers published within the project

Denora PS, Muglia M, Casali C. Spastic paraplegia with thinning of the corpus callosum and white matter abnormalities: further mutations and relative frequency in ZFYVE26/SPG15 in the Italian population. *J Neurol Sci* 2009;277(1-2):22-5.

Denora PS, Schlesinger D, Casali C. Screening of ARHSP-TCC patients expands the spectrum of SPG11 mutations and includes a large scale gene deletion. *Hum Mutat*. 2009;30(3):E500-19.

Hanein S, Martin E, Boukhris A. Identification of the SPG15 gene, encoding spastizin, as a frequent cause of complicated autosomal-recessive spastic paraplegia, including Kjellin syndrome. *Am J Hum Genet* 2008;82(4):992-1002.

Stevanin G, Azzedine H, Denora P. Mutations in SPG11 are frequent in autosomal recessive spastic paraplegia with thin corpus callosum, cognitive decline and lower motor neuron degeneration. *Brain* 2008;131(Pt3):772-84.

Tessa A, Silvestri G, de Leva MF. A novel KIF5A/SPG10 mutation in spastic paraplegia associated with axonal neuropathy. *J Neurol* 2008;255(7):1090-2.

DEVELOPMENT OF NEW DIAGNOSTIC APPROACHES FOR TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHIES

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Transmissible Spongiform Encephalopathy (TSE) or prion disorders are rare neurodegenerative conditions affecting humans and other animal species. They are characterized by long incubation periods with death of affected individuals after a short clinical phase. Human TSE diseases are diffused worldwide with an incidence of about 1-2 cases per million people per year in humans. Most of them (about 90%) occur as sporadic Creutzfeldt-Jakob Disease (sCJD) with an unknown aetiology. The aetiology of the remaining cases is either genetic (associated with mutations in the PRNP gene encoding for the prion protein, PrP) or infectious (as for variant Creutzfeldt-Jakob disease, vCJD, caused by the consumption of meat infected with the bovine spongiform encephalopathy agent).

TSE diseases are characterized by the presence in the central nervous system and, at exceedingly lower levels, in other peripheral organs, of a host-derived amyloid protein termed PrPTSE. PrPTSE exists in a number of molecular subtypes that show differences in protease cleavage site, possibly reflecting a different conformation, and glycosylation pattern. Based on the electrophoretic mobility of the core fragments of PrPTSE after proteinase K digestion, two major human molecular PrPTSE types (type 1 and type 2) can be distinguished. PrPTSE is the only available marker of TSE diseases and its apparent absence in accessible tissues (like blood) hampers the identification of TSE infected individuals during the preclinical phase. The presence of infectivity traces in some body fluids (e.g., blood and cerebrospinal fluid) during this phase can only be demonstrated through expensive, poorly sensitive and time-consuming animal bioassays that have no practical utility in the current clinical practice or in surveillance activity. During the clinical phase of disease there are several laboratory and instrumental findings that can corroborate the clinical diagnosis such as the presence of the poorly specific, but relatively sensitive 14-3-3 proteins, which accumulates in the cerebrospinal fluid of patients with TSEs (as well as in non TSE patients where massive neural death occurs). However, a definite diagnosis of TSE is only obtained post-mortem by immunohistochemical or biochemical detection of PrPTSE in the brain.

The absence of an ante-mortem confirmatory test represents an obstacle for the design of targeted strategies for prevention, control, and treatment of these diseases. In this project we tried to improve the diagnosis and the prognosis of human TSEs through the optimization of available diagnostic tools and the development of new biochemical tests.

We dealt with this complex issue by multiple and complementary approaches.

The first goal was to improve the differential diagnosis between TSEs and other dementing illnesses by optimizing the available biochemical test for the widely used marker proteins 14-3-3 and PrPTSE.

We developed a specific protocol for the collection of body fluids from TSE patients, and from neurological and non-neurological patients. Mass spectrometry and 2D electrophoresis showed

that cerebrospinal fluids of sCJD contain γ, ε and ζ isoforms of 14-3-3 proteins, and that this pattern is not found in inflammatory and vascular disorders of the CNS (Central Nervous System). On the basis of these results an isoform-specific Western blot test will allow differential diagnosis with these disorders, if needed.

In parallel with 14-3-3 analyses, we performed Western blot analyses of PrPTSE types in a series of 225 CJD subjects. The goal was to understand the extent of codeposition of type 1 and type 2 PrPTSE conformers and to investigate the association, if any, of this codeposition with specific clinocopathological manifestations. In about 30% these CJD cases we observed the occurrence of PrPTSE types 1 and 2 with a significant association between disease phenotype and relative abundance of each type. These results allow formulating an updated classification of sCJD variants and may contribute to increase the accuracy of early differential diagnosis with other dementias.

The development of new biochemical tests was first performed by the application of the protein misfolding cyclic amplification (PMCA) to peripheral biological human fluids. PMCA amplifies minute amounts of PrPTSE (using normal brain containing PrPC as a substrate), and has been optimized for reproducibility, specificity and sensitivity on brain samples from an experimental TSE model (263K scrapie in hamsters). We have also adapted the conditions of amplification to human brain and obtained a 105-fold of PrPTSE amplification. To overcome the limited availability of non-CJD human brains as a substrate, we have also developed PMCA using brain from humanized mice (Tg Hu-PrP). We achieved an efficient amplification using substrate and template with the same genotype, and a minor amplification in the cross-experiments. This result will be now subjected to large-scale validation test to verify its application in the field.

In keeping with the goal to identify new diagnostic markers, we employed a multi-step proteomic approach for the identification of proteins that are recovered at the end of a purification protocol specific for the protease-resistant core of PrPTSE (PrP27-30). We identified ferritin, CaMKIIα, ApoE, and tubulin as the major components associated with PrP27-30 but also trace amounts of actin, cofilin, Hsp90α, the γ subunit of the T-complex protein 1, glyceraldehyde 3-phosphate dehydrogenase, histones, and keratins.

Whereas some of these proteins (tubulin and ferritin) are known to bind PrP, other proteins (CaMKIIα, Hsp90α) may associate with PrPTSE fibrils during disease. ApoE and actin have been previously observed in association with PrPTSE, whereas cofilin and actin were shown to form abnormal rods in the brain of patients with Alzheimer disease. The roles of these proteins in the development of the disease are still unclear and further work is needed to explain their involvement in the pathogenesis of TSEs and if they can be used for disease staging or as therapeutic targets.

In conclusion we underline that some of the results produced in this project are ready to be evaluated by ad-hoc international panels working to develop classification and diagnostic criteria for CJD, whereas other results (primarily those from proteomic analyses) have just started their way to contribute to CJD diagnosis and treatment.

Papers published within the project

Giorgi A, Di Francesco L, Principe S, Mignogna P, Sennels L, Mancone C, Alonzi T, Sbriccoli M, De Pascalis A, Rappsilber J, Cardone F, Pocchiari M, Maras B, Schinina ME. Proteomic profiling of PrP27-30-enriched preparations extracted from the brain of hamsters with experimental scrapie. *Proteomics* 2009;9(15):3802-14.

Pocchiari M, Poleggi A, Principe S, Graziano S, Cardone F. Genomic and post-genomic in human prion diseases. *Genome Medicine* 2009;1:63.

Principe S, Maras B, Schininà ME, Pocchiari M, Cardone F. Unraveling the details of prion (con)formation(s): recent advances by mass spectrometry. *Current Opinion in Drug Discovery and Development* 2008;11:697-707.

RELIABILITY AND EFFICACY OF THE CURRENT DIAGNOSTIC APPROACH IN NARCOLEPSY AND SEARCH FOR NEW GENETIC MARKERS

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Introduction

Narcolepsy is a chronic central nervous system disease characterized by Excessive Daytime Sleepiness (EDS), typically associated to cataplexy and other phenomena due to the abnormal occurrence of REM sleep elements during wakefulness and sleep/wake transition. Probably, its pathogenesis is due to a dysfunction in hypothalamic neurons which produce hypocretin (or orexin), a neurotransmitter involved in the complex interaction of neuron networks responsible for the regulation of the sleep/wake cycle. Some HLA system antigens seem to play a role as predisposing factors because they are present in at least 95% of patients.

Currently, the diagnosis is based on the International Classification of Sleep Disorders 2nd Ed. (ICSD-2) criteria which distinguish Narcolepsy with Cataplexy (Narcolepsy/Cataplexy) from Narcolepsy without Cataplexy.

We followed a complete clinical and laboratory diagnostic protocol in two groups of patients with EDS (one with Narcolepsy/Cataplexy and another with sleep apnea sindrome); the main scope was to determine the subset of diagnostic interventions able to discriminate these two groups from each other. At the same time, we planned to evaluate with an advanced molecular genetics methodology (gene microarrays) the eventual involvement of genes in the pathogenesis of Narcolepsy/Cataplexy because its genetic basis remains unknown. We describe here this second genetic part of the study.

Methods

In order to test the gene expression profile, microarray analysis was performed on RNA isolated from the blood of 10 patients with Narcolepsy/Cataplexy, 10 patients with sleep apnea syndrome and 10 healthy controls. Total RNA was extracted from frozen blood by "Paxgene" kit (Qiagen Inc.USA). RNA purity and integrity were assessed by spectophotometric analysis and by Agilent Bioanalyzer. Total RNA was then analyzed by Affymetrix Genechip microarray. The syntheses of cDNA and biotinylated cRNA were performed according to the protocols provided by the manufacturer (Affymetrix, Santa Clara, CA, USA). Biotinylated fragmented cRNA probes were hybridized to the GeneChip® Human Genome U133A 2.0), which contained probe sets for over 14500 known transcripts and expressed sequence tags. Hybridization was performed at 45°C for 16 h in a hybridization oven (Affymetrix).

The Genechips were then automatically washed and stained with streptavidin–phycoerythrin conjugate in an Affymetrix Genechip Fluidics Station. Fluorescence intensities were scanned

with a Affymetrix GeneChip Scanner 3000. To prevent overcorrelation, samples were processed eight at a time and arranged so that narcoleptic, sleep apnea, and control samples were all in each experimental session. Gene expression Affymetrix data were then analysied using Partek Genomics Suite software (version 6.4 Copyright c 2009 Partek Inc., St Louis, MO, USA). For this purpose CEL files were imported using the default Partek normalization parameters. Probe-level data was pre-processed, including background correction, normalization, and summarization, using robust multi-array average (RMA) analysis; subsequent data normalization was performed across all arrays using quantile normalization. The normalized probe intensity values were then compiled, or summarized, within each probe set, using the median polish technique, to generate a single measure of expression. These expression measures were then log transformed, base 2. Quality control on data set was performed using Principal Component Analysis (PCA) on all the genes in order to test the segregation efficiency.

The list resulting from the statistical analysis (ANOVA) of microarray data was annotated according to functional roles or biological processes according to the Gene Ontology Consortium directions.

Results

In patients with Narcolepsy/Cataplexy 173 probes were found to be expressed significantly different from normal controls; while the probes expressed significantly different from normal controls were 312 in sleep apnea patients. Table 1 shows the 10 probes with the biggest changes found in the two groups of subjects. It is interesting to note that only 17 probes were expressed significantly different from normal controls in both groups of patients and the direction of the changes (increase or decrease) was in all cases the same in the 2 patient groups.

Table 1. Probes with the biggest fold change with respect to normal controls in the two groups of patients

Probeset ID	Gene Symbol	Pathway	p-value	Fold change
Narcolepsy/Cataplexy				
208661_s_at	TTC3	---	0.006	1.56
215392_at	---	---	0.006	1.49
211547_s_at	PAFAH1B1	Wnt signaling	0.007	1.46
200602_at	APP	---	0.008	1.46
217523_at	CD44	---	0.002	1.45
39313_at	WNK1	---	0.002	1.44
202760_s_at	PALM2-AKAP2	G Protein Signaling	0.006	1.43
207223_s_at	ROD1	---	0.002	1.42
207186_s_at	BPTF	---	0.002	1.42
205483_s_at	ISG15	---	0.003	-1.78
Sleep Apnea Syndrome				
212509_s_at	MXRA7	---	0.011	1.87
215698_at	JARID1A	---	0.029	1.59
215121_x_at	IGLV2-14	---	0.017	-1.58
204019_s_at	SH3YL1	---	0.040	-1.61
209138_x_at	IGL	---	0.044	-1.72
209841_s_at	LRRN3	---	0.037	-1.78
209840_s_at	LRRN3	---	0.034	-1.85
212592_at	IGJ	---	0.027	-2.12
217022_s_at	LOC100126583	Inflammatory Response Pathway	0.048	-2.16

Conclusions

The complex results obtained are now under analysis for the identification of metabolic pathways involved by the gene patterns expressed differentially in the patient groups versus controls and also the common differentially expressed genes will probably provide important information on the common clinical feature of Narcolepsy/Cataplexy and sleep apnea syndrome, such as EDS, obesity, metabolic changes, etc.

Papers published within the project

Ferri R, Franceschini C, Zucconi M, Vandi S, Poli F, Bruni O, Cipolli C, Montagna P, Plazzi G. Searching for a marker of REM sleep behavior disorder: Submentalis muscle EMG amplitude analysis during sleep in patients with narcolepsy/cataplexy. *Sleep* 2008;31:1409-17.

Ferri R, Franceschini C, Zucconi M, Drago V, Manconi M, Vandi S, Poli F, Bruni O, Plazzi G. Sleep polygraphic study of children and adolescents with narcolepsy/cataplexy. *Developmental Neuropsychology* (in press).

EVALUATION AND REHABILITATION OF SWALLOWING DYSFUNCTION IN PATIENTS WITH RARE NEUROLOGICAL DISORDERS AND MOVEMENT DISORDERS: FIRST YEAR OF STUDY

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In recent years there has been increasing awareness of the feeding difficulties experienced by patients with neurological disorders children with neurodevelopmental disability and adults with movement disorders. The majority of the studies have been in children with cerebral palsy and in patients with Parkinsonism in particular Progressive Supranuclear Palsy (PSP). Many have been found to have major problems with eating and swallowing.

During the first year, we studied 15 PSP patients followed at the Neurological Unit of IRCCS San Raffaele in Rome (Table 1).

Table 1. Characteristics of the PSP patients (n. 15) analyzed within the project

#	Pts	Year of birth	Sex	Disease duration (years)	PSP scale	PSP stage	Apraxia	Cognitive impairment	Dysfagia	ORL
1	P-C	1938	F	1	22	2	N	Y	Y	
2	R-S	1929	F	1	28	2	N	N	N	
3	E-B	1926	M	5	52	4	N	Y	Y	Y
4	A-G	1930	M	5	63	4	Y	Y	Y	Y
5	C-M	1930	F	8	46	3	N	N	Y	Y
6	P-L	1948	M	7	62	3	Y	Y	Y	Y
7	L-L	1931	M	6	51	3	Y	N	Y	Y
8	ODE	1939	M	8	58	5	Y	Y	Y	
9	R-A	1935	M	4	35	3	N	N	Y	Y
10	MAP	1945	F	6	63	5	Y	N	Y	
11	E-C	1939	F	4	41	3	Y	Y	Y	
12	A-P	1929	M	9	44	4	N	Y	Y	
13	G-O	1937	M	3	48	3	N	Y	Y	
14	A-B	1938	F	6	48	3	Y	Y	Y	Y
15	R-G	1928	F	4	24	4	N	N	Y	

ORL: Validation by otorhinolaryngologists

Clinical evaluation was made using the "Progressive Supreanuclear Palsy Rating Scale and Staging System". Fourteen out of 15 patients developed an impairment of swallowing, also in early stage of disease (range of disease duration 1-9 years). 7 out 15 patients underwent to ORL assess by use of clinical and common instrumental tools: Videofluoroscopy (VFS), Flexible Endoscopic Evaluation of Swallow (FEES) and EMG activity of submental muscles. 10 patients were admitted to voice rehabilitation by LSVT method (Lee Silverman Voice Treatment), but we cannot find a good efficacy.

All patients with Duchenne Muscular Dystrophies (DMD), Spinal Muscular Atrophies (SMA), congenital myopathies, congenital muscular dystrophies followed at the Paediatric Neurology Unit of the Catholic University underwent to semi structural questionnaire to evaluate present the dysphagia, dietary manipulation, history of choking episodes or other chewing, swallowing or respiratory abnormalities. The questionnaire was conducted in 118 DMD and 105 SMA patients.

IDENTIFICATION OF GENETIC FACTORS RESPONSIBLE FOR RARE DISORDERS WITH CONGENITAL HEART DEFECTS

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Congenital Heart Defects (CHDs) are individually rare, but altogether affect 6-8/1000 newborns. Their aetiologies are largely unknown, although mutations in a number of genes have been identified in few sporadic and familial cases. A total of 238 patients affected by different conotruncal (CT) CHDs were collected. These patients were screened for mutations in cardiogenic transcription factors NKX2.5, GATA4, ISLET1, TBX20 and ZFPM2/FOG2, together with other candidate genes expressed in developing and adult heart (GJA5, GDF1 and BMP4). A subgroup of 49 patients with tetralogy of Fallot (ToF) was further analyzed for JAG1 mutations, the gene responsible for Alagille syndrome. Mutation screening was performed by dHPLC followed by bidirectional sequencing. The GATA4 gene was further investigated for copy-number-changes by MLPA. No clear pathogenic mutation was identified in the GATA4, ISLET1, BMP4, TBX20 and GDF1 genes. One NKX2.5 (Arg25Cys) change was detected in 2 patients with ToF. Mutation analysis of ZFPM2/FOG2 gene identified 3 novel (Ile227Val, Met544Ile and Lys1029Ile) and one (Glu30Gly) previously detected mutations. One GJA5 missense mutation was identified in two unrelated patients. This change was absent in more than 400 control chromosomes and was proven to alter connexin 40 cellular localization by immunohistochemistry. One splice site variant (1107+3InsGT) and 2 missense mutations (G309R and R937Q) were found in JAG1 gene in 3 ToF patients. These results i) confirm that mutations in NKX2.5, GATA4, FOG2, TBX20, and GDF1 are rare events in CT CHDs ii) indicate a role for GJA5 gene in CT CHDs; iii) provide evidence that mutations in JAG1 gene account for a significant proportion of isolated ToF.

Probands of 7 families with isolated transposition of great arteries (TGA) and family history of concordant or discordant CHDs and 20 sporadic patients with atrioventricular canal defect (AVCD: 17 with partial and 3 with complete AVCD) were screened for mutations in the ZIC3, ACVR2B, LEFTYA, CFC1, NODAL, FOXH1, GDF1, CRELD1, GATA4 and NKX2.5 genes. Mutation analysis allowed the identification of 5 sequence variations in 2 (28.6%) out of 7 TGA probands, IVS2-1G>C (NODAL), Asn21His and Arg47Gln (CFC1), Pro21Ser (FOXH1), and Gly17Cys (ZIC3). The Asn21His and Arg47G CFC1 changes were also detected in 2/3 (66.7%) cases with complete AVCD. No variation was found in patients with partial AVCD. None of these changes was found in control subjects (n. 300), excepting for CFC1 variations Asn21His and Arg47Gln (2/300; 0.7%). These results demonstrate that mutations in laterality genes could occur in a significant proportion of families with TGA and argue for an oligogenic or complex mode of inheritance in these pedigrees.

To evaluate whether the GJA5 gene mutations cause "criss-cross" heart development in humans, we screened the entire coding region of the GJA5 gene in a group of 6 well characterized patients with criss-cross heart. No pathogenic mutation was identified, suggesting that GJA5 mutations are not responsible for criss-cross heart in humans or are not a major cause for this defect. To evaluate the presence of a locus for non syndromic absent pulmonary valve (APV) on 18q and for Ebstein anomaly on chromosome 8p23, we screened for mutations the NFATC1 and GATA4 genes in 2 subjects affected by isolated non syndromic APV and 7 patients with Ebstein anomaly, respectively. Neither NFATC1 nor GATA4 mutations were identified in these CHDs. Furthermore, a total of 30 patients affected by AVCD and a group of 50 phenotypically normal subjects were analyzed for NFATC1 mutations. Two non-synonymous changes (Val210Met and Ala367Val) were detected in 3 AVCD patients, while no mutation was detected in unaffected subjects. Both mutations were found to be absent in additional 400 controls, suggesting that NFATC1 gene could be responsible for a number of AVCD patients.

To further evaluate the mutation spectrum associated to Noonan Syndrome (NS), Costello Syndrome (CS), Cardio-Facio-Cutaneous Syndrome (CFCS) and LEOPARD Syndrome (LS) causative genes encoding for members of the RAS-MAPK pathway were studied by dHPLC analysis followed by bidirectional sequencing. Mutation screening of HRAS gene identified a novel "bona-fide" mutation outside the classical HRAS "hot-spot" region for CS. Thirty-three patients with CFCS were screened for mutations in MEK1 and MEK2 genes. Three MEK1 and two MEK2 mutations were detected in six patients. To investigate the phenotypic spectrum and molecular diversity of germ line mutations affecting BRAF, which encodes a serine/threonine kinase functioning as a RAS effector frequently mutated in CFCS, subjects with a diagnosis of NS (n. 270), LS (n. 6), and CFCS (n. 33), and no mutation in PTPN11, SOS1, KRAS, RAF1, MEK1, or MEK2, were screened for the entire coding sequence of the gene. Besides the expected high prevalence of mutations observed among CFCS patients (52%), a de novo heterozygous missense change was identified in one subject with LS (17%) and five individuals with NS (1.9%). Our findings provide evidence for a wide phenotypic diversity associated with mutations affecting BRAF, and occurrence of a clinical continuum associated with these molecular lesions.

Papers published within the project

Calcagni G, Digilio MC, Sarkozy A, Dallapiccola B, Marino B. Familial recurrence of congenital heart disease: an overview and review of the literature. *Eur J Pediatr* 2007;166(2):111-6.

De Luca A, Sarkozy A, Consoli F, De Zorzi A, Mingarelli R, Digilio MC, Marino B, Dallapiccola B. Exclusion of Cx43 gene mutation as a major cause of criss-cross heart anomaly in man. *Int J Cardiol* 2009;16. [E-pub ahead of print].

Dentici ML, Sarkozy A, Pantaleoni F, Carta C, Lepri F, Ferese R, Cordeddu V, Martinelli S, Briuglia S, Digilio MC, Zampino G, Tartaglia M, Dallapiccola B. Spectrum of MEK1 and MEK2 gene mutations in cardio-facio-cutaneous syndrome and genotype-phenotype correlations. *Eur J Hum Genet* 2009;17(6):733-40.

Digilio MC, Sarkozy A, Capolino R, Chiarini Testa MB, Esposito G, de Zorzi A, Cutrera R, Marino B, Dallapiccola B. Costello syndrome: clinical diagnosis in the first year of life. *Eur J Pediatr* 2008;167(6):621-8.

Limongelli G, Pacileo G, Digilio MC, Calabro' P, Di Salvo G, Rea A, Miele T, Frigiola A, Sarkozy A, Dallapiccola B, Marino B, Calabro' R. Severe, obstructive biventricular hypertrophy in a patient with Costello syndrome: Clinical impact and management. *Int J Cardiol* 2008;130(3):108-10.

Limongelli G, Pacileo G, Marino B, Digilio MC, Sarkozy A, Elliott P, Versacci P, Calabro P, De Zorzi A, Di Salvo G, Syrris P, Patton M, McKenna WJ, Dallapiccola B, Calabro R. Prevalence and clinical significance of cardiovascular abnormalities in patients with the LEOPARD syndrome. *Am J Cardiol* 2007;100(4):736-41.

Limongelli G, Sarkozy A, Pacileo G, Calabrò P, Digilio MC, Maddaloni V, Gagliardi G, Di Salvo G, Iacomino M, Marino B, Dallapiccola B, Calabrò R. Genotype-phenotype analysis and natural history of left ventricular hypertrophy in LEOPARD syndrome. *Am J Med Genet A* 2008;146A(5):620-8.

Mascheroni E, Digilio MC, Cortis E, Devito R, Sarkozy A, Capolino R, Dallapiccola B, Ugazio AG. Pigmented villonodular synovitis in a patient with Noonan syndrome and SOS1 gene mutation. *Am J Med Genet A* 2008;146A(22):2966-7.

Piacentini G, Digilio MC, Sarkozy A, Placidi S, Dallapiccola B, Marino B. Genetics of congenital heart diseases in syndromic and non-syndromic patients: new advances and clinical implications. *J Cardiovasc Med (Hagerstown)* 2007;8(1):7-11.

Sarkozy A, Carta C, Moretti S, Zampino G, Digilio MC, Pantaleoni F, Scioletti AP, Esposito G, Cordeddu V, Lepri F, Petrangeli V, Dentici ML, Mancini GM, Selicorni A, Rossi C, Mazzanti L, Marino B, Ferrero GB, Silengo MC, Memo L, Stanzial F, Faravelli F, Stuppia L, Puxeddu E, Gelb BD, Dallapiccola B, Tartaglia M. Germline BRAF mutations in Noonan, LEOPARD, and cardiofaciocutaneous syndromes: molecular diversity and associated phenotypic spectrum. *Hum Mutat* 2009;30(4):695-702.

Sarkozy A, Digilio MC, Dallapiccola B. Leopard syndrome. *Orphanet J Rare Dis* 2008;3:13.

Sarkozy A, Schirinzi A, Lepri F, Bottillo I, De Luca A, Pizzuti A, Tartaglia M, Digilio MC, Dallapiccola B. Clinical lumping and molecular splitting of LEOPARD and NF1/NF1-Noonan syndromes. *Am J Med Genet A* 2007;143A(9):1009-11.

NEW GENETIC SYNDROMES WITH AORTIC TORTUOSITY AND DISSECTION

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Introduction

Mutations in the TGFBR1 and TGFBR2 genes cause the autosomal dominant Loeys-Dietz Syndromes (LDS) whose vascular phenotype is characterized by aortic and extra-aortic arterial aneurysms, dissections and arterial tortuosity. Based on clinical phenotypes, the LDS syndromes have been defined as Type I when prominent craniofacial involvement is present and Type II when craniofacial traits are minor or absent. Additional traits recurring in LDS, in particular LDS1, are skeletal, ocular, cutaneous and nervous. Although ectopia lentis (EL) is typically absent, it has been described in two carriers of TGFBR1 and TGFBR2 gene mutations.

The phenotypes may partly overlap Marfan Syndrome (MFS) diagnosed on Ghent criteria or Thoracic Aortic Aneurysm and dissection (TAAD). Existing evidences indicate that it is clinically useful to distinguish phenotypes causally linked to mutations of TGFBR1 and TGFBR2 genes from those caused by FBN1 gene: a) the severity of aortic aneurysms dissect at a smaller size than in Marfan syndrome and at an early age; b) the presence of arterial aneurysms in extra-aortic locations; c) the higher risk of unpredictable events as first clinical manifestation (especially in patients with LDS2 with minor or absent craniofacial traits) including not only aortic aneurysms and dissection but also cerebral hemorrhage.

We report vascular and clinical phenotypes, cardiovascular events and follow-up of a consecutive series of 150 members of 103 unrelated families with mutations of TGFBR1 and TGFBR2 genes, diagnosed with LDS.

Methods and results

The series consists of 103 probands diagnosed with LDS1 (n. 72; 57 de novo) and with LDS2 (n. 31; 12 de novo) and 47 affected relatives from two national referral centers (OSM and JHU). Probands and relatives underwent multidisciplinary clinical and imaging evaluations, genetic counseling and testing: 109 carried TGFBR2 and 41 TGFBR1 mutations.

LDS1 and LDS2 shared aortic aneurysm (91% *vs* 82%), dissections (23% *vs* 36%), arterial tortuosity (95% *vs* 93%) and, at a lower rate, aneurysms of other arteries (36% *vs* 23%), respectively. The mean age at first diagnosis was 14.73±14.23 *vs* 27.58±18.56 (p<0.0001) in

LDS1 and LDS2, respectively. The first diagnosis coincided with aortic dissection in 30 patients: 8 died of aortic rupture at surgery. During 35±51 months of follow-up in 142 patients, there were 30 re-interventions in the 24 patients who survived first dissection and 51 elective surgeries in 48 non-dissected patients. One of these latter had elective surgery at 20 years, developed type B dissection 4 years later and underwent endoprosthesis placement. Two patients died at 2nd and 7th intervention and one of cerebral hemorrhage one year after elective surgery. None of the non-dissected patients died.

Conclusions

LDS1 and LDS2 are malignant diseases affecting cardiovascular system with high rate of aortic dissection in undetected patients. Timely diagnosis prevents catastrophic events and allows preventive elective surgery.

Papers published within the project

Arbustini E, Grasso M, Ansaldi S, Malattia C, Pilotto A, Porcu E, Disabella E, Marziliano N, Pisani A, Lanzarini L, Mannarino S, Larizza D, Mosconi M, Antoniazzi E, Zoia MC, Meloni G, Magrassi L, Brega A, Bedeschi MF, Torrente I, Mari F, Tavazzi L. Identification of sixty-two novel and twelve known FBN1 mutations in eighty-one unrelated probands with Marfan syndrome and other fibrillinopathies. *Hum Mutat* 2005;26(5):494.

Arbustini E, Marziliano N, Magrassi L. Aneurysm syndromes and TGF-beta receptor mutations. *N Engl J Med* 2006;355(20):2155; author reply 2156.

Disabella E, Grasso M, Marziliano N, Ansaldi S, Lucchelli C, Porcu E, Tagliani M, Pilotto A, Diegoli M, Lanzarini L, Malattia C, Pelliccia A, Ficcadenti A, Gabrielli O, Arbustini E. Two novel and one known mutation of the TGFBR2 gene in Marfan syndrome not associated with FBN1 gene defects. *Eur J Hum Genet* 2006;14(1):34-8.

Faivre L, Collod-Beroud G, Callewaert B, Child A, Binquet C, Gautier E, Loeys BL, Arbustini E, Mayer K, Arslan-Kirchner M, Stheneur C, Kiotsekoglou A, Comeglio P, Marziliano N, Wolf JE, Bouchot O, Khau-Van-Kien P, Beroud C, Claustres M, Bonithon-Kopp C, Robinson PN, De Backer J, Coucke P, Francke U, De Paepe A, Jondeau G, Boileau C. Clinical and mutation-type analysis from an international series of 198 probands with a pathogenic FBN1 exons 24-32 mutation. *Eur J Hum Genet* 2009;17(4):491-501.

Faivre L, Collod-Beroud G, Callewaert B, Child A, Loeys BL, Binquet C, Gautier E, Arbustini E, Mayer K, Arslan-Kirchner M, Kiotsekoglou A, Comeglio P, Grasso M, Beroud C, Bonithon-Kopp C, Claustres M, Stheneur C, Bouchot O, Wolf JE, Robinson PN, De Backer J, Coucke P, Francke U, De Paepe A, Boileau C, Jondeau G. Pathogenic FBN1 mutations in 146 adults not meeting clinical diagnostic criteria for Marfan syndrome: further delineation of type 1 fibrillinopathies and focus on patients with an isolated major criterion. *Am J Med Genet A* 2009;149A(5):854-60.

Faivre L, Collod-Beroud G, Child A, Callewaert B, Loeys BL, Binquet C, Gautier E, Arbustini E, Mayer K, Arslan-Kirchner M, Stheneur C, Kiotsekoglou A, Comeglio P, Marziliano N, Halliday D, Beroud C, Bonithon-Kopp C, Claustres M, Plauchu H, Robinson PN, De Backer J, Coucke P, Francke U, De Paepe A, Boileau C, Jondeau G. Contribution of molecular analyses in diagnosing Marfan syndrome and type I fibrillinopathies: an international study of 1009 probands. *J Med Genet* 2008;45(6):384-90.

Faivre L, Collod-Beroud G, Loeys BL, Child A, Binquet C, Gautier E, Callewaert B, Arbustini E, Mayer K, Arslan-Kirchner M, Kiotsekoglou A, Comeglio P, Marziliano N, Dietz HC, Halliday D, Beroud C, Bonithon-Kopp C, Claustres M, Muti C, Plauchu H, Robinson PN, Biggin A, Benetts B, Brett M, Holman KJ, De Backer J, Coucke P, Francke U, De Paepe A, Jondeau G, Boileau C. Effect of

mutation type and location on clinical outcome in 1,013 probands with Marfan syndrome or related phenotypes and FBN1 mutations: an international study. *Am J Hum Genet* 2007;81(3):454-66.

Faivre L, Masurel-Paulet A, Collod-Beroud G, Callewaert BL, Child AH, Stheneur C, Binquet C, Gautier E, Chevallier B, Huet F, Loeys BL, Arbustini E, Mayer K, Arslan-Kirchner M, Kiotsekoglou A, Comeglio P, Grasso M, Halliday DJ, Bouroud C, Bonithon-Kopp C, Claustres M, Robinson PN, De Backer J, Coucke P, Francke U, De Paepe A, Boileau C, Jondeau G. Clinical and molecular study of 320 children with Marfan syndrome and related type I fibrillinopathies in a series of 1009 probands with pathogenic FBN1 mutations. *Pediatrics* 2009;123(1):391-8.

Fusar-Poli P, Klersy C, Stramesi F, Callegari A, Arbustini E, Politi P. Determinants of quality of life in Marfan syndrome. *Psychosomatics* 2008;49(3):243-8.

Gambarin FI, Favalli V, Serio A, Regazzi M, Pasotti M, Klersy C, Dore R, Mannarino S, Viganò M, Odero A, Amato S, Tavazzi L, Arbustini E. Rationale and design of a trial evaluating the effects of losartan vs. nebivolol vs. the association of both on the progression of aortic root dilation in Marfan syndrome with FBN1 gene mutations. *J Cardiovasc Med* 2009;10(4):354-62.

Marziliano N, Grasso M, Pilotto A, Porcu E, Tagliani M, Disabella E, Diegoli M, Pasotti M, Favalli V, Serio A, Gambarin F, Tavazzi L, Klersy C, Arbustini E. Transcriptomic and proteomic analysis in the cardiovascular setting: unravelling the disease? *J Cardiovasc Med* 2009;10(5):433-42.

SYSTEMATIC DIAGNOSIS OF RARE ERYTHROENZYMOPATHIES: GENERATION OF GUIDELINES AND STUDY OF THE GENOTYPE/PHENOTYPE CORRELATION

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Introduction

Over the past few years the inherited disorders of erythrocyte metabolism have been the object of intensive research which has resulted in a better understanding of their molecular basis; however red cell metabolism disorders remain a rare group of diseases of difficult diagnosis. The phenotypic heterogeneity of these diseases pinpoints to the need to correlate the molecular and the clinical phenotype. The most frequent red cell enzymopathies associated with chronic hemolytic anemia are pyruvate kinase (PK) (about 400 cases described), pyrimidine-5'nucleotidase (Pyr5'N) (about 100 cases), glucosephosphate isomerase (GPI) (50 cases), hexokinase (Hx), phosphoglycerate kinase (PGK), phosphofructokinase (PFK), adenylate kinase (AK) and triose phosphate isomerase (TPI) deficiency. At Fondazione IRCCS Ospedale Maggiore Policlinico, Mangiagalli, Regina Elena there is the unique opportunity to study these diseases because of the presence of renowned experts interested in prevention, molecular and prenatal diagnosis, and clinical care of these diseases during childhood and adult life.

The project was divided in two distinct objectives:

1) Study of the genotype/phenotype relationship to be used in prognosis and genetic counseling.

2) Elaboration of guidelines for diagnostic and therapeutic approach to rare chronic hemolytic anemias due to defects of red cell metabolism.

In the first year the project was focused on the characterization of the molecular variants of red cell enzyme deficiency previously diagnosed. In particular, we described a case of congenital red cell pyruvate kinase (PK) deficiency associated with hereditary stomatocytosis.

Moreover, we studied the clinical and molecular characteristics of 6 new patients with recessive hereditary methemoglobinemia due to cytochrome b5 reductase deficiency (Cytb5r), and we identified one new variant of triose phosphate isomerase deficiency (TPI) associated with a very severe clinical pattern. Finally, in collaboration with the Department of Biochemistry of Pavia University, we investigated Pyrimidine 5' nucleotidase deficiency (P5'N) and Phosphoglycerate mutase (PGK) deficiency, performing the functional analysis of highly purified mutant enzymes and comparing their stability, molecular, structural, and catalytic properties with those of corresponding wild-type proteins. In the second year we focused on the elaboration of guidelines, particularly we addressed the following practical aspects: Which individuals should be screened for red cell enzymopathies? Which are the best diagnostic tests?, When is it necessary to perform and what is the role of molecular genetic analysis?. As regards

the management of the disease we addressed the need of folate supplementation, the indications, timing and method of splenectomy, the post-splenectomy antibiotic prophylaxis, the necessity and frequency of booster vaccination, and, finally, the management of gallstones.

Results and conclusions

Pyruvate kinase deficiency and stomatocytosis

We described the first case of PK deficiency associated with hereditary stomatocytosis. The propositus is a 30 years-old Italian male with a β-thalassemia trait and a life-long history of moderate to severe chronic haemolytic anaemia of unknown origin, who underwent cholecistectomy and splenectomy at the age of 20 years; splenectomy resulted in a slight increase of haemoglobin and was followed by two episodes of portal vein thrombosis. The study of the most important red cell enzymes revealed reduced PK activity (43% of normal) and impaired thermal stability. Direct sequencing of PK-LR gene showed a compound heterozygosity for mutation c.1456T (Arg486Trp) and the new variant c.-73g>c in the R-type promoter region. The history of post splenectomy thrombosis and the presence of stomatocytes in peripheral blood smear prompted us to investigate for the coexistence of hereditary stomatocytosis. We found increased intracellular sodium and decreased potassium concentrations suggestive of dehydrated stomatocytosis (Marcello *et al.*, 2008).

Cytochrome b5 reductase deficiency

Among the 6 new patients with recessive hereditary methemoglobinemia due to Cytb5r deficiency, one was affected by Type-II disease with cyanosis and severe progressive neurological dysfunction, whereas the others displayed the benign Type-I phenotype. 8 different mutations in the DIA1 gene were detected and two of them (Gln27STOP and Arg45Trp) were new. Gln27STOP is the most upstream mutation so far detected and determines the absence of almost all the protein. Arg45Trp affects a residue located in the N terminal of Fβ1 strand in the FAD binding domain, in a region highly conserved among mammalian species; the analysis of 3-D structure of Cytb5r showed that the substitution of the positively charged Arg45 with a non-polar neutral tryptophan is likely to disrupt the secondary structure and the stability of the enzyme. The identification of molecular mutations within the DIA1 gene can contribute in understanding the clinical phenotype of recessive hereditary methemoglobinemia. In fact, missense mutations that reduce enzyme stability without affecting the catalytic function result in symptoms confined to red cells, which cannot replace the degraded protein. Conversely, disruptive mutations or mutations that markedly reduce catalytic activity affect all cells expressing Cytb5r resulting in Type-II disease.

Triose phosphate isomerase deficiency

The propositus was a 7 weeks old baby. Soon after birth he had respiratory distress and was noticed to be jaundiced. At 4 weeks of age he was investigated for jaundice and failure to thrive, and was found to have hemolysis. He had reduced muscle tone, with abnormal posturing, and required intubation and ventilation, and frequent blood transfusions. At the time of the study the hemoglobin was 7.4 g/dL, and reticulocyte count 400x109L. TPI activity was at the lower limit of normal (1332 IU/gHb, normal values 1317-2905) in spite of the presence of a large number

of transfused cells, whereas both parents displayed low activity in the heterozygote range. The sequence of the complete coding region of TPI gene showed the presence of the missense mutation GAG-GAC (nt 315, Glu104Asp) and the new variant c.28insG. When the diagnosis of TPI deficiency was made, the baby was extubated and died shortly afterwards at the age of 10 weeks from respiratory failure. c.28insG variant causes a frameshift and a premature stop codon at residue 71, resulting in a nonfunctional protein product; it the more upstream frameshift mutation so far reported in TPI gene and likely account for the very severe clinical pattern observed in the patient.

Pyrimidine-5'-nucleotidase deficiency

We characterized at the protein level three newly described missense mutations (c.187T>C, c.469G>C and c.740T>C). The mutant enzymes (C63R, G157R and I247T) were obtained as recombinant forms by means of heterologous expression systems and site-directed mutagenesis techniques, and purified to homogeneity. All enzymes were altered, although to a different extent, either in their catalytic efficiency or in thermal stability, the G157R being the most impaired enzyme. The G157R protein was highly heat unstable, halving the activity in about 23 min at 37°C, whereas C63R and I247T mutants at the same temperature maintained fully activity for more than 2 hours. However, at higher temperature also C63R and I247T mutants resulted less stable than the wild-type enzyme losing the activity in few minutes ($t_{1/2}$ at 46°C, about 5 min *vs* 2 hours of the wild-type enzyme). Therefore, although mutations targeted different regions of the P5'N-1 structure, they produced similar aberrant effects on the molecular properties of the enzyme providing evidence that all affected aminoacids are functionally and structurally important for preserving the enzyme activity during the red cell life span. The information obtained on mutant enzymes may serve as a valuable tool to make a definitive diagnosis of erythroenzymopathies. The knowledge of new molecular variants and the compared analysis with the corresponding clinical pattern will be useful for prognosis and genetic counseling.

Phosphoglycerate Kinase deficiency

To analyze the mutations at protein level and possibly to correlate the genotype to clinical phenotype, we started with the molecular characterization of the wild-type PGK1 enzyme and three mutants (I47N, D164 and S320N) obtained from E.coli as recombinant proteins. The corresponding mutations, i.e., c.140T>A, c.491A>T and c.959G>A, have been identified in patients with PGK deficiency and affected by severe hemolytic anemia and progressive mental retardation.

The wild-type enzyme was crystallized in both free form or complexed with 3-PG.The corresponding structures were solved to high resolution (1.8 and 1.6 A, respectively) and compared. Essentially, binding 3-PG caused a 6° rotation of the N-domain in respect to the C-domain The recombinant enzyme exhibited kinetic properties similar to those of the authentic enzyme, displaying *vs* 3-PG and ATP alike specific activities (about 1000 U/mg) and alike Km values (about 1mM). I47N and S320N mutant enzymes showed kcat values 3-fold lower than the wild-type enzyme. The D164V was characterized by a Km value *vs* 3-PG 15 times higher than that of the other enzymes studied and a catalytic efficiency 70 times lower. Finally, all mutant enzymes turned out to be highly heat unstable with respect to the wild-type enzyme, losing half of their activity after approximately 10 minutes of incubation at 37 °C. At higher temperatures, the wild-type enzyme was protected from heat inactivation by Mg-ATP or 3-PG. On the contrary, no one mutant was protect by Mg-ATP and the D164V and S320N mutants were not even protected by 3-PG. Therefore, these preliminary studies indicate that all

mutations target amino acid residues located in positions primarily important for preserving the protein stability during the red cell life span.

Suggested guidelines

A suggested diagnostic pathway of a patient presenting with hemolytic anemia includes family history, blood film examination and investigation of the most common causes of hemolysis, such as immune-mediated (direct antiglobulin test with different methods) and red cell membrane defects (osmotic fragility tests, EMA-binding and SDS-analysis of erythrocyte membrane proteins). Blood film examination should be performed by an expert and could give important information, for example the presence of typical elements: spherocytes, stomatocytes, ovalocytes, ellyptocytes point to a membrane defect and basophilic stippling to pyrimidine-5'-nucleotidase deficiency. Family history should be carefully recorded, considering the dominant or recessive transmission of these rare enzyme defects. Hemoglobin defects should be excluded by HPLC, and, if necessary, by molecular genetics.

In case of negative results of this first-step investigation, the enzyme activity should be tested by spectrophotometry, with particular attention to recent transfusion, incomplete leukocyte removal, and elevated reticulocyte counts that could influence the enzyme levels. The molecular characterization of the enzyme defect is useful in confirming the diagnosis and fundamental in genetic counselling.

Moderately and severely affected individuals are likely to benefit from splenectomy, which should be performed after the age of 6 years and with appropriate counselling about the infection risk, which is not completely eliminated by the currently recommended preoperative vaccinations and postsplenectomy antibiotic prophylaxis.

Laparoscopic surgery, when performed by experienced surgeons, can result in a shorter hospital stay and less pain. It is particularly important to rule out stomatocytosis, where splenectomy is contraindicated because of the thrombotic risk. The need for reimmunization and its frequency are unclear as are the optimal duration of post-splenectomy antibiotic prophylaxis and choice of drug. Folate therapy is recommended in severe and moderate forms of haemolytic anemia. Transfusion may be necessary during pregnancy, aplastic crisis and infection. Iron overload should be carefully monitored and treated.

Papers published within the project

Chiarelli LR, Morera SM, Galizzi A, Fermo E, Zanella A, Valentini G. Molecular basis of pyrimidine 5′-nucleotidase deficiency caused by 3 newly identified missense mutations (c.187T>C, c.469G>C and c.740T>C) and a tabulation of known mutations. *Blood Cells Mol Dis* 2008;40:295–301.

Fermo E, Bianchi P, Vercellati C, Marcello AP, Garatti M, Marangoni O, Barcellini W, Zanella A. Recessive hereditary methemoglobinemia: two novel mutations in the NADH-cytochrome b5 reductase gene. *Blood Cells Mol Dis* 2008;41:50-5.

Marcello AP, Vercellati C, Fermo E, Bianchi P, Zaninoni A, Barcellini W, Zanella A. A case of congenital red cell pyruvate kinase deficiency associated with hereditary stomatocytosis. *Blood Cells Mol Dis* 2008;41:261-2.

Morera S, Chiarelli LR, Rovida S, Bianchi P, Fermo E, Galizzi A, Zanella A, Valentini G. Phosphoglycerate Kinase deficiency: characterization of the wild-type enzyme and three pathological variants generated from C.140T>a, C.491A>T and C.959G>a mutations. *Blood* 2008;12 (suppl), 2875.

BOTH FAMILIAL AND SPORADIC ATYPICAL HEMOLYTIC UREMIC SYNDROME ARE ASSOCIATED WITH GENETIC COMPLEMENT ABNORMALITIES AND THE GENOTYPE GREATLY IMPACT OUTCOME AND RESPONSE TO TREATMENT

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Introduction

The Hemolytic Uremic Syndrome (HUS) is characterized by microangiopathic hemolytic anemia, thrombocytopenia, and renal impairment. Most childhood cases are caused by Shiga-toxin-producing bacteria. The other form, atypical HUS (aHUS), accounts for 10% of cases and has a poor prognosis.

Results

Genetic screening

The complete CFH, CFI and MCP sequences were analyzed in 272 patients with atypical HUS (76 with familial aHUS, 196 with sporadic aHUS).

Forty-five independent CFH mutational events were found in 73 patients (overall: 27%, familial aHUS: n. 41, sporadic aHUS:n. 32), all Caucasians. All mutations are heterozygous, with the exception of the Y899X and the 3675-3699del that were found in homozygosity in a sporadic patient and in 10 patients from a Bedouin pedigree, respectively. Of note, also 1 out of 5 heterozygous carriers from the latter family developed aHUS.

In 4 non-consanguineous patients of African origin 6 CFH variants were found. The 6 variants were detected in African controls (n. 11) but not in 120 Caucasian controls, indicating that Africans have peculiar CFH genotypes that should be carefully considered when screening aHUS patients of African origin.

To complete CFH genetic analysis, we also screened exon 10, that produces the splice variant CFHL1. No mutations and/or polymorphisms were found.

Eleven independent CFI mutational events were found in 13 Caucasian patients (overall 4.8%, familial aHUS n. 5, sporadic aHUS: n. 8), all heterozygous. Eighteen MCP mutational events were found in 25 Caucasian patients (overall: 9.2%, familial aHUS:n. 10, sporadic aHUS: n. 15); all but 2 mutations are heterozygous. Eight patients (2.9%) carried combined mutations in complement regulatory genes.

Forty-two patients without mutations in CFH, CFI or MCP were screened for CFH-CFHR1 rearrangements. Three patients from 2 families carried a CFH-CFHR1 hybrid gene (7%). CFH auto-antibodies were found in two out of 45 patients (4.4%) without mutations in CFH, CFI or MCP, studied during the acute phase.

One hundred fifty-three patients were screened for mutations in the thrombomodulin gene (THBD). Eight carried a mutation in THBD (overall 5.2%, familial aHUS: n. 3, sporadic aHUS: n. 5), none of whom had mutations in complement genes.

Among 167 patients without alterations in CFH (including mutations, hybrid gene and autoantibodies), CFI or MCP, 40 patients with low C3 and normal C4 serum levels and 8 patients with a very severe form of the disease (ESRD or dead at onset) were screened for C3 and CFB mutations. Ten C3 heterozygous mutations were found in 10 patients (6 sporadic and 4 familial; 1 patient carries 3 mutations; frequency in the selected panel: 21%); two of them had normal C3 serum levels. In CFB, a new heterozygous mutation was found in a patient with sporadic aHUS. Moreover 8 CFB polymorphic variants were identified of which the 32W variant was significantly associated with aHUS (genotype frequency, P=0.031; allele frequency, P=0.006 *vs* controls).

A panel of 48 patients, that did not carry mutations in all the above genes were screened for new candidate genes, namely CD59, CLU (clusterin), OPN (osteopontin), VSIG4 (CRIg), VTN (vitronectin), C1-inh (C1-inhibitor), all encoding complement inhibitors, and CFP encoding properdin that stabilizes the AP C3 convertase. We did not found any variation in CD59, CLU, OPN, VSIG4. In C1-inh we found 3 different mutations in 2 patients with sporadic aHUS (A1283T causing the K342N aminoacid change and C196T causing the A2V change) and in a healthy control (G1288A; R344H). These mutations do not alter C1-inhibitor levels and activity. A hemizygous CFP mutation (G895A, D299N) was found in another patient with sporadic aHUS.

In summary, mutations were identified in overall 130 out of 272 patients (overall: 48%, familial aHUS: 74%, sporadic aHUS:36%). Among sporadic cases, mutations were found in 39% of idiopathic forms but also in 14 to 44% of forms associated with malignant hypertension, pregnancy, transplantation and other renal and/or systemic diseases. Genetic analysis in relatives of 17 sporadic patients showed that in 15 cases the mutation was inherited from an unaffected parent, while in two the mutation (in CFH) was de novo. In 42% of patients with sporadic aHUS and no mutation in candidate genes we found aHUS predisposing polymorphisms, namely the E963D in SCR16 of CFH and /or the R32W in CFB.

Clinical findings

The clinical data of patients were analyzed after grouping on the basis of specific genetic defects. Patients with mutations in more than one gene were excluded from further analysis (manuscript in preparation).

The disease manifested mostly during childhood (≤18 years) in all groups with the exception of patients carrying CFI and C3 mutation. The earliest onset (0-1 year) was found in patients with CFH and THBD mutations. However disease onset varied even in subjects with the same mutation. Among five carriers of the R1215Q CFH mutation in one family, 3 siblings developed aHUS in childhood, their grandfather at 83 years, while their father is unaffected at 57 years of age.

Diarrhoea and/or gastroenteritis and upper respiratory tract infections were frequent triggers in all groups. Malignant hypertension complicated the disease in 17 patients (4 with CFH mutation). Pregnancy-related HUS was reported in 13 patients; 11 patients had de-novo post-

transplant aHUS, while 16 patients had other underlying renal diseases. Overall, triggering/underlying conditions were found in 71% of patients.

Extra-renal (central nervous system or multivisceral) involvement during HUS episodes was observed in 10-30% of patients with the exception of patients with mutations in MCP.

Low C3 levels were reported more frequently in patients with mutations in CFH than in patients without mutations. The median ADAMTS13 activity was lower in the patients without mutations than in the other groups taken together, with 19 out of 105 patients with ADAMTS13 activity ≤50% during remission, thus suggesting a possible role of defective protease activity in these cases.

About 50-60% of patients with CFH, CFI and C3 mutations and about 40% of patients with THBD mutations and without mutations developed ESRF or died at the first episode. The prognosis was better in patients with MCP mutations, complete or partial remission being the most frequent outcome of the presenting episode. Disease recurrences were recorded more frequently in patients with MCP mutations than in those with CFI mutations and without mutations. Despite of that, AATA at three years confirmed a worse outcome in patients with CFH, CFI, C3 and THBD mutations and in patients without mutations than in the group with MCP mutations. In all groups children had a trend to a better prognosis than adults both at onset and at 3-year follow-up.

Mutations affecting short consensus repeats (SCRs) 19-20 of CFH were associated with worse short and long-term prognosis than those affecting SCRs1-18.

The Kaplan-Meier cumulative estimates of the rates of first event (ESRF or death) during follow-up period, evidenced a significantly higher combined incidence of ESRF or death in patients with CFH, or CFI, or C3 or THBD mutations than in patients with MCP mutations. In addition the fractions of patients with CFH and THBD mutations still alive at any time points after HUS onset were significantly lower than all the other groups.

Complete or partial remission was achieved in 63%, 25%, 67% and 90% of plasma-treated episodes in patients with CFH, CFI, C3 and THBD mutations, respectively. Patients with MCP mutation underwent remission in 97% of plasma-treated episodes, but also in all the 14 episodes not treated with plasma.

Transplantation outcome in patients with CFH mutations was very poor: 12 out of 17 kidney grafts were lost for recurrence, rejection or thrombosis within one year. Of the 5 patients with good outcome, 3 received intensive plasma prophylaxis and the grafts are well functioning at 1, 2 years and 4 months. Patients with CFI mutations experienced recurrence in 4 out of 6 grafts within the first year. Kidney-liver transplantation was performed in 4 patients with CFH mutations and in 1 with combined CFH-CFI mutations. Three patients with CFH mutations died at few days (n. 2) and 4 years (n. 1) after transplant for severe thrombotic liver complications or sequelae of hepatic encephalopathy. The other 2 patients are well with preserved liver and kidney functions at 1 year. Seven kidneys were transplanted in four patients with C3 mutations, aHUS recurrence manifested in 3 grafts, 2 were lost whereas the third recovered following 4 plasma exchanges. One patient with THBD mutation lost the kidney graft for recurrence. In the non-mutated group 60% of the grafts were lost within 1 year, and one patient experienced disease recurrence after 3 years. Kidney transplant was performed in 3 patients with MCP mutations, all of them have good graft function at 13, 3 and 1 years.

Among patients without mutations who had received a kidney transplant, those carrying the 32W disease predisposing variant had a worse outcome (all grafts had failed at one years post-transplant) than patients with the wild-type genotype.

Conclusions

Both familial and sporadic aHUS are genetically determined. Genetic abnormalities predispose to complement over-activation and cause aHUS upon triggering/underlying conditions that activate complement. Individual genetic defects impact response to plasma and transplant outcome.

Papers published within the project

Delvaeye M, Noris M, DeVriese A, Esmon C, Esmon N, Ferrel G, Del-Favero J, Plaisance S, Claes B, Lambrechts D, Zoja C, Remuzzi G, Conway E. Mutations in thrombomodulin in hemolytic-uremic syndrome. *N Engl J Med* 2009;361(4):345-57

Fang CJ, Fremeaux-Bacchi V, Liszewski MK, Pianetti G, Noris M, Goodship TH, Atkinson JP. Membrane cofactor protein mutations in atypical hemolytic uremic syndrome (aHUS), fatal Stx-HUS, C3 glomerulonephritis and the HELLP syndrome. *Blood* 2008; 111:624-32.

Martinez-Barricarte R, Pianetti G, Gautard R, Misselwitz J, Strain L, Fremeaux-Bacchi V, Skerka C, Zipfel PF, Goodship T, Noris M, Remuzzi G, de Cordoba SR; European Working Party on the Genetics of HUS. The complement factor H R1210C mutation is associated with atypical hemolytic uremic syndrome. *J Am Soc Nephrol* 2008;19(3):639-46.

Noris M, Remuzzi G. Thrombotic microangiopathy. What not to learn from a meta-analysis. *Nature Rev Nephrol* 2009;5:1-3

Noris M, Remuzzi G. Translational mini-review series on complement factor H: Therapies of renal diseases associated with complement factor H abnormalities:atypical haemolytic uremic syndrome and membranoproliferative glomerulonephritis. *Clin Exp Immunol* 2008;151:199-209.

CIRCULATING MICROPARTICLES AND THROMBIN GENERATION CAPACITY IN SEVERE HEMOPHILIACS WITH MILD CLINICAL PHENOTYPE: DIAGNOSTIC AND PROGNOSTIC IMPLICATIONS

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Introduction

Some severe hemophiliacs (FVIII/FIX <1IU/dL) exhibit a mild bleeding tendency corresponding to 1-3 bleeds/year (mild bleeders) compared to other severe patients who bleed spontaneously two or more times per month (severe bleeders). The basis for this clinical heterogeneity is poorly understood. This study investigated the relationship between the values of Endogenous Thrombin Potential (ETP) and clinical phenotype in severe hemophiliacs, taking also into account the impact of FVIII/FIX gene mutation type and of thrombophilic polymorphisms. Moreover, the levels of circulating microparticles were measured.

Methods

Severe hemophiliacs older than 18 years without inhibitor history and treated on demand were eligible. Mild bleeders (MB) and severe bleeders (SB) were defined as follows: spontaneous bleeding episodes per year <2 (MB) or >25 (SB) and concentrate consumption <500 (MB) or >2000 (SB) IU/kg/year. Patients who did not fit these criteria were considered as intermediate bleeders (IB) and were analyzed as controls together with SB. Blood samples were drawn after a wash-out period of at least 5 days from the last concentrate infusion. FVIII was measured by one-stage clotting and chromogenic assay and ETP was measured in platelet-rich (PRP) and platelet-poor (PPP) plasma after addition of tissue factor (TF, 1pM). FVIII/FIX antigen (FVIII/FIX:Ag) were measured with an ELISA-based method with commercially available kits. Microparticles (MP) were enumerated by quantitative flow cytometry as total MP (Annexin V-PE-binding), platelet-derived MP (Annexin V-PE and antiGpIIbIIIa-FITC-binding events below 1μm) and TF-expressing MP (Annexin V-PE and anti-TF-FITC-binding events below 1 μm).

Results

Between June 2007 and June 2008, 175 adult patients with severe hemophilia A or B were evaluated at our center. Twenty-eight were judged not eligible because they have received

prophylaxis and 52 did not sign the written consent. Overall, 95 patients (84 with hemophilia A and 11 with hemophilia B) were included in the study, but only 72 were considered in the final analysis owing to the exclusion of 23 patients with hemophilia A in whom FVIII was detectable when measured with the chromogenic assay. According to the aforementioned criteria 22 MB and 50 controls (22 SB and 28 IB) were identified. Age at enrolment and prevalence of thrombophilic polymorphisms were similar comparing MB and controls. Hemophilia B was more frequent in MB (32%) than in controls (8%; p<0.05). MB had lower orthopedic (median 3, interquartile range, IQR: 0-7) and radiological scores (median 18, IQR: 9-25) when compared with both all controls (median orthopedic score: 15, IQR: 9-24; median radiological score: 35, range: 25-46) and SB (median orthopedic score: 18, range: 13-28; median radiological score: 44, range: 22-50; p<0.01). MB showed an older age at first bleed (median 3.5 yrs, IQR: 1-6) compared to controls (median 1 yr, IQR: 1-2) and to SB (median 1 yr, IQR: 1-1.2; p < 0.01). The prevalence of severe FVIII/FIX gene defects (referred to as null mutations) was lower (10%) and ETP values measured in PRP were higher (median: 850 nM x min) in MB compared with both controls (null mutations in 58%; median ETP: 460 nM x min) and SB (null mutations in 62%; median ETP: 414 nM x min; p<0.05). No difference was observed when ETP values measured in PPP were considered suggesting a role of platelets in the system. Levels of FVIII/FIX:Ag were associated to the type of FVIII/FIX gene mutation, being undetectable in 25/30 (83%) patients with null mutations and in 8/37 (22%) with non-null mutations (p<0.01). Similarly, FVIII/FIX:Ag levels were more frequently undetectable in patients with ETP < 597 nM x min (median value measured in PRP of the whole population) than in those with ETP > 597 nM x min (24/35, 69% *vs* 12/35, 34%; p<0.01). No association was found between ETP values and type of FVIII/FIX gene mutation.

In a multivariate model after adjustment for the other variables, non-null mutations were the only independent risk factor of mild bleeding tendency (adjusted OR: 8.9, 95%CI 1.3-60.6). With respect to MP evaluation, hemophiliacs had higher levels of circulating MP compared to healthy controls, however no difference was found when MB were compared with both all controls and SB (data not shown). Larger studies may be useful in order to clarify the role of MP in this setting.

Conclusions

Our results indicate that the type of FVIII/FIX gene mutation has a relevant impact on the modulation of clinical phenotype in patients with severe hemophilia, being the non-null mutations more prevalent in MB. The measurement of thrombin generation in platelet-rich plasma may allow to identify this subgroup of patients, not otherwise distinguishable by conventional functional assays, however further studies are warranted in order to better define the biological mechanism that enhances thrombin generation in these patients.

Papers published within the project

Artoni A, Santagostino E, Mancuso ME, Lecchi A, Mannucci PM. Microparticle levels are high in patients with hemophilia A and can be further increased by DDAVP administration. In: 49th ASH Annual Meeting, Atlanta, Georgia, 8-11 December 2007. *Blood* 2007; 110: 923a.

Artoni A, Santagostino E, Mancuso ME, Lecchi A, Mannucci PM. Microparticle levels are high in patients with hemophilia A and can be further increased by DDAVP administration. In: XXVIII

International Congress of the World Federation of Hemophilia, Istanbul, June 1-5, 2008. *Haemophilia* 2008;14(2):59.

Santagostino E, Mancuso ME, Tripodi A, Chantarangkul V, Clerici M, Mannucci PM. Endogenous thrombin potential (ETP) in severe hemophiliacs with mild phenotype. In: XXVIIth International Congress of The World Federation of Hemophilia. Vancouver, 21-25 May 2006. *Haemophilia* 2006;12(2):28.

Santagostino E, Mancuso ME, Tripodi A, Chantarangkul V, Clerici M, Sei C, Mannucci PM. Endogenous thrombin potential (ETP) in severe hemophiliacs with mild phenotype. In: XIX Congress of the Società Italiana per lo Studio dell'Emostasi e della Trombosi (SISET). Milano, 14-17 September 2006. *Haematologica* 2006;91(s2):9.

Santagostino E, Mancuso ME, Tripodi A, Chantarangkul V, Clerici M, Sei C, Mannucci PM. Thrombin generation in severe hemophiliacs with different clinical phenotype. In: 48th ASH Annual Meeting, Orlando, 9-12 December 2006. *Blood* 2006;108:298a.

Santagostino E, Mancuso ME, Tripodi A, Chantarangkul V, Clerici M, Sei C, Siboni SM, Mannucci PM. Thrombin generation in severe hemophiliacs with different clinical phenotype. In: XXI Congress of the International Society on Thrombosis and Haemostasis, Ginevra 2007, 6-12 July. *J Thromb Haemost* 2007;5(2):O-S-016.

Santagostino E, Mancuso ME, Tripodi A, Chantarangkul V, Clerici M, Sei C, Siboni SM, Mannucci PM. Thrombin generation in severe hemophiliacs with different clinical phenotype. In: 49th ASH Annual Meeting, Atlanta, Georgia, 8-11 December 2007. *Blood* 2007;110:350a.

Santagostino E, Mancuso ME, Tripodi A, Chantarangkul V, Pasta G, Mannucci PM. Low prevalence of null mutations of F8/F9 gene in severe hemophiliacs with mild clinical phenotype. *Blood Transfus* 2008;6(suppl 3):s15-6.

Santagostino E, Mancuso ME, Tripodi A, Chantarangkul V, Pasta G, Siboni SM, Mannucci PM. Increased thrombin generation in severe hemophiliacs with mild clinical phenotype. In: 50th ASH Annual Meeting, San Francisco, 6-9 December 2008. *Blood* 2008;112(11):194.

Santagostino E, Mancuso ME, Tripodi A, Chantarangkul V, Siboni S, Mannucci P. Increased thrombin generation in severe hemophiliacs with mild clinical phenotype. In: XXVIII International Congress of the World Federation of Hemophilia, Istanbul, June 1-5, 2008. *Haemophilia* 2008;14 (2):61.

Santagostino E, Mancuso ME, Tripodi A, Chantarangkul V, Siboni SM, Pasta G, Mannucci PM. Increased thrombin generation in severe hemophiliacs with mild clinical phenotype. *Haematologica* 2008;93(s3):96.

ESTABLISHMENT OF A EUROPEAN NETWORK OF RARE BLEEDING DISORDERS

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The aim of the project is to set up a European network among Treatment Centres dealing with Rare Bleeding Disorders (RBDs), to increase the collection of clinical, genetic and treatment data and to develop a computer tool with the purpose of managing, editing and viewing all entered data and make them readily available through database queries. Data on patients affected by RBDs were entered by 10 European partner centers. Italian centers were also involved in this project through the help of the Associazione Italiana Centri Emofilia (AICE), a reliable coordinating association of the Italian hemophilia treatment centers, to allow the creation of a National Registry in the frame of the European database.

The main step of the project was represented by the testing phase which was performed during the last year activity. This step was meant to verify the quality of entered data (correct setting of the online help, controls on guided choices, clarity of field-browsing and simplicity of database interrogation tools).

At the end of the testing phase, partners were asked to give their feedback on the database anomalies, logical, browsing and comprehension errors, possible improvements or modification proposals. A number of issues were raised, such as:

- a standardized definition for the phenotype and clinical severity for each type of coagulant factor deficiency;
- possibility to enter more then one option in different fields of the database (blood product treatment, dosage, trigger, complication, hemorrhagic site);
- duration of bleeding episodes;
- amelioration of the Bleeding Score through the insertion of missing fields;
- semplification of the Genetics analysis section.

Data on 521 patients have been entered up to now (May 2009).

The distribution of RBDs and their phenotype severity (severe deficiency was defined for factor levels <5% and fibrinogen level <10 mg/dL, moderate 5-10% and hypofibrinogenemia

<50mg/dL and mild >10% or dysfibrinogenemia >50mg/dL) were calculated on the entire group of patients, showing that FVII and FXI deficiencies (30%) were the most prevalent RBDs, while the rarest disorders were found to be FV+FVIII and FII deficiencies (2%).

The second part of analysis was based on the prevalence and severity of bleeding manifestations in 221 patients with complete data required by EN-RBD questionnaire. 260 cases coming from French registry contained only demographic information and annual amount of treatment with no detail clinical information; in additional 40 records have been removed after the first quality control. Further analyses were performed to compare clinical severity of patients and their lab phenotypes.

These results showed that severe bleeding episodes such as haematoma, haemarthrosis, umbilical cord, gastrointestinal tract (GI) and Central Nervous System (CNS) bleedings, were significantly more frequent in patients with severe phenotype (coagulant activity less than 5% and patient affected by afibrinogenemia) (p<0.0001). Mucocutaneous type of bleedings such as epistaxis, cutaneous and oral cavity, were present in patients affected with moderate or mild deficiency (more than 5% of coagulant activity and hypo- and dys-fibrinogenemia)(p<0.05). Menorrhagia and haematuria were present in all three groups of patients with different type of phenotype severities. A good correlation between lab phenotype results and clinical severity was observed in patients affected by afibrinogenemia and severe FXIII and FX deficiencies since most of these patients (>75%) had at least one severe bleeding. There was no report on patients with afibrinogenemia and severe FXIII and FX deficiencies who remain asymptomatic in their entire life. One third of patients with severe FXI deficiency and 5% of patients with severe FV deficiency remain asymptomatic. Additional data entry and improvements are required to answer question on: onset of spontaneous bleeding symptoms *vs* triggered, bleeding during surgery, efficacy of prophylaxis during surgery. These modifications are leading to a second version of data collection (EN-RBD 2.0), with new functions and page layout, scheduled to be ready in July 2009.

The Italian patients analyzed (a total of 117 subjects) came from the same centre (Angelo Bianchi Bonomi Hemophilia and Thrombosis Centre). We have already established a good communication with the Italian Registry of coagulation disorders to improve either Italian data collection, based on the experience obtained during the last year by the EN-RBD project and our European data collection by increasing the number of patients entered in our database. As soon as the link between EN-RBD and the AICE computer-based clinical data collection system, named EMOWEB will be established, we will proceed with the extraction of data as EMOWEB currently contains complete data on patients affected by haemophilia A, B and von Willebrand disease, while data on RBDs are very limited and focussed on demographic and laboratory phenotype severity.

RBDs patients have therefore not been analyzed with regards to clinical severity, genotype and treatment. A few important fields (e.g. parents' consanguinity, antigen level, bleeding score) indicated in the EN-RBD questionnaire are not in EMOWEB. In order to uniform the two data collection forms, these additional pages will be added to EMOWEB and information on RBDs patients will flow periodically from EMOWEB to EN-RBD.

In March 2009, Alekos, the computer managers of EN-RBD, started to collaborate with the EMOWEB informatics group to organize the exchange of the data between the two systems and to define the compatibility between the two applications. The amount and quality of data involved in the process, and the alignment of the databases fields will be analyzed in order to optimize the bilateral exchange of data in the future. There are several ongoing works on the implementation of the automated migration process as the first release of the EN-RBD registry is expected in September 2009.

DEVELOPMENT OF NOVEL MOLECULAR APPROACHES FOR THE DIAGNOSIS AND CHARACTERIZATION OF GENETIC LYMPHEDEMA

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Introduction

The lymphatic system plays a central role in maintaining fluid homeostasis in the body. Its network of blind-ended, thin-walled vessels composed of a monolayer of lymphatic endothelial cells collects extravasated fluids and immune cells from peripheral tissue for recirculation by the cardiovascular system. Defects in lymphatic system development or function underlie many disorders including lymphedema, lymphangiectasia, lymphangioma, and lymphatic dysplasia, and lymphatic function is thought to be one of the key determinants of tumor metastasis and hence cancer morbidity.

The homeodomain transcription factor PROX1 is known to act as a key regulator of lymphatic vascular development. Expression of Prox1 is first detected in the mouse at 9.5 days post coitum (dpc) in a dorsolaterally polarized subpopulation of cells budding from the cardinal vein that subsequently adopt a lymphatic endothelial phenotype. PROX1 over-expression in human vascular endothelial cells represses numerous vascular endothelial-specific genes and enhances the expression of lymphatic endothelial cell-specific markers, consistent with the concept that a subset of vascular endothelial cells is reprogrammed by PROX1 to a lymphatic fate during embryonic development. Targeted inactivation of Prox1 results in the complete absence of lymphatic vasculature. In Prox1-/- mutant mice, endothelial cells bud from the cardinal vein but fail to express lymphatic endothelial markers and retain a vascular endothelial phenotype. On the basis of these observations, Prox1 is widely considered to be a master switch for lymphatic endothelial cell specification.

Like other transcription factors of the SOX family, SOX18 selectively bind the heptameric consensus DNA sequence, 5'-A/TA/TCAAA/TG-3', by virtue of a 79 amino acid HMG domain. SOX18 also is able to activate transcription via a trans-activation domain adjacent and C-terminal to the HMG domain. During embryogenesis, Sox18 gene expression begins at 7.5 dpc in yolk sac blood islands and endothelial cells of the allantois. By 8.5 dpc, Sox18 is expressed in endothelial cells of the embryo proper; transient expression of Sox18 becomes a hallmark of endothelial cells undergoing both vasculogenesis and angiogenesis.

Hereditary lymphedema is a rare developmental disorder characterized by a chronic swelling of the extremities due to impaired lymphatic drainage which causes disability and predisposition to infection and chronic ulceration. More specifically, few families have been described with a hereditary condition in which hypotricosis, lymphedema and telangiectasia are associated (HLT syndrome). Genetic analysis of three families with HLT syndrome showed that the transcription factor Sox 18 was mutated and accounted for both recessive and dominant forms of the disease. Although these data are consistent with a dominant role of Sox 18 in lymphatic development the mechanism by which SOX 18 is involved in the pathophysiology of HLT was unknown when we started our work and constituted the focus of our research

By studying Sox18 mutant mouse models, we found that Sox18 plays a critical role in initiating lymphatic outgrowth from vascular precursors and in subsequent patterning of the lymphatic vessels. We found that SOX18 acts by directly activating transcription of Prox. In more detail, we studied lymphatic development and function in two mouse models. The first, ragged-opossum (RaOp), expresses a truncated form of Sox 18 which acts as a dominant negative mutant, with heterozygotes showing sparse hair, cyanosis and chylous ascites. The lymphatic system was essentially absent in homozygous RaOp/RaOp embryos, which die in utero within 13.5 dpc. In the second model we inactivated Sox18 gene. This revealed absence of lymphatics, gross subcutaneous edema and fetal lethality in all homozygotes.

We further found that SOX18 stimulates expression of Prox 1 and most lymphatic markers during the differentiation of ES cells along the endothelial lineage and may therefore promote commitment of multipotent embryonic endothelial cells to a lymphatic fate. These observations were confirmed by standard techniques such as CHIP showing that Sox 18 can indeed bind and activate Prox-1 promoter. These findings explain to a large extent the etiology of lymphatic defects in HLT, and place SOX18 upstream of Prox1 in the transcriptional hierarchy that determines lymphatic endothelial cell specification in the embryo. More recently, we investigated why the phenotype of Sox18-null mutant mice varies dramatically depending on the genetic background. Sox18 is a member of the Group F Sox subfamily, a group of three genes (Sox7, -17, and -18) encoding proteins with remarkably similar primary structure. As is often the case with different members of the same Sox subfamily, Sox7, -17 and -18 share some domains of expression that suggest functional overlap; for example, all three genes are known to be expressed during vascular development in endothelial cells. Functional redundancy between Group F SOX factors has been hypothesized to explain the reported lack of vascular and lymphatic phenotype of Sox18 knockout mice.

We found that SOX7 and SOX17, are upregulated in the absence of SOX18 during the genesis of the lymphatic vasculature, but only in some genetic backgrounds and not in others. Like SOX18, SOX7 and -17 are able to activate Prox1 transcription in cultured cells and in transgenic mice. Thus, when Sox 7 and 17 are upregulated, the effect of ablation of Sox 18 are almost undetectable, in contrast, when these two transcription factors remain low, the inactivation of Sox 18 leads to a dramatic alteration of lymphatic development. The extent of up-regulation differed with mouse strain, indicating that Sox7 and -17 are able to act as strain-specific modifiers of the Sox18 lymphatic phenotype.

These data may explain the variability of the symptoms in the different individuals affected by HLT. In conclusions, from a diagnostic point of view, alterations of expression or mutations of Sox18, 17 and 7 may help to identify patients with hereditary lymphedema.

Papers published within the project

Baluk P, Fuxe J, Hashizume H, Romano T, Lashnits E, Butz S, Vestweber D, Corada M, Molendini C, Dejana E, McDonald DM. Functionally specialized junctions between endothelial cells of lymphatic vessels. *J Exp Med* 2007;204(10):2349-62.

Birdsey GM, Dryden NH, Amsellem V, Gebhardt F, Sahnan K, Haskard DO, Dejana E, Mason JC, Randi AM. The transcription factor Erg regulates angiogenesis and endothelial apoptosis through VE-cadherin. *Blood* 2008; 111(7):3498-506.

Cermenati S, Moleri S, Cimbro S, Corti P, Del Giacco L, Amodeo R, Dejana E, Koopman P, Cotelli F, Beltrame M. Sox18 and Sox7 play redundant roles in vascular development. *Blood* 2008; 111:2657-66

Dejana E, Taddei A, Randi A. Foxs and Ets in the transcriptional regulation of endothelial cell differentiation and angiogenesis. *Biochim Biophys Acta. Cancer Reviews* 2007;1775(2):298-312.

Dejana E, Tournier-Lasserve E, Weinstein BM. The control of vascular integrity by endothelial cell junctions: molecular basis and pathological implications. *Dev Cell* 2009;16(2):209-21.

François M, Caprini A, Hosking B, Orsenigo F, Wilhelm D, Browne C, Paavonen K, Karnezis T, Shayan R, Downes M, Davidson T, Tutt D, Cheah KS, Stacker SA, Muscat GE, Achen MG, Dejana E, Koopman P. Sox18 induces development of the lymphatic vasculature in mice. *Nature* 2008;456(7222):643-7.

Hosking B, François M, Wilhelm D, Orsenigo F, Caprini A, Svingen T, Tutt D, Davidson T, Browne C, Dejana E, Koopman P. Sox7 and Sox17 are strain-specific modifiers of the lymphangiogenic defects caused by Sox18 dysfunction in mice. *Development* 2009;136(14):2385-91.

Liebner S, Corada M, Bangsow T, Babbage J, Taddei A, Czupalla CJ, Reis M, Felici A, Wolburg H, Fruttiger M, Taketo MM, von Melchner H, Plate KH, Gerhardt H, Dejana E. Wnt/beta-catenin signaling controls development of the blood-brain barrier. *J Cell Biol* 2008;183(3):409-17.

Liebner S, Corada M, Bangsow T, Babbage J, Taddei A, Czupalla CJ, Reis M, Felici A, Wolburg H, Fruttiger M, Taketo MM, von Melchner H, Plate KH, Gerhardt H, Dejana E. Wnt/beta-catenin signaling controls development of the blood-brain barrier. *J Cell Biol* 2008;183(3):409-17.

Montero-Balaguer M, Swirsding K, Orsenigo F, Cotelli F, Mione M, Dejana E. Stable vascular connections and remodeling require full expression of VE-cadherin in zebrafish embryos. *PLoS One* 2009;4(6):e5772

Potente M, Ghaeni L, Baldessari D, Mostoslavsky R, Rossig L, Dequiedt F, Haendeler J, Mione M, Dejana E, Alt FW, Zeiher AM, Dimmler S. SIRT1 controls endothelial angiogenic functions during vascular growth. *Genes Dev* 2007;21(20):2644-58.

Rudini N, Dejana E. Adherens junctions. *Curr Biol* 2008;18(23):R1080-2.

Rudini N, Felici A, Giampietro C, Lampugnani MG, Corada M, Swirsding K, Garre' M, Liebner S, Letarte M, ten Dijke P and Dejana E.VE-cadherin is a critical endothelial regulator of TGF-beta signalling. *EMBO J.* 2008;27(7):993-1004.

Taddei A, Giampietro C, Conti A, Orsenigo F, Breviario F, Pirazzoli V, Potente M, Daly C, Dimmeler S, Dejana E. Endothelial adherens junctions control tight junctions by VE-cadherin-mediated upregulation of claudin-5. *Nat Cell Biol* 2008;10(8):923-34.

Zovein AC, Hofmann JJ, Lynch M, French WJ, Turlo KA, Yang Y, Becker MS, Zanetta L, Dejana E, Gasson JC, Tallquist MD, Iruela-Arispe ML. Fate tracing reveals the endothelial origin of hematopoietic stem cells. *Cell Stem Cell* 2008;3(6):625-36.

INVESTIGATION OF GENETIC AND EPIGENETIC MECHANISMS UNDERLYING BECKWITH-WIEDEMANN SYNDROME (BWS) ON A LARGE COHORT OF ITALIAN PATIENTS

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Beckwith-Wiedemann Syndrome (BWS, OMIM 130650) is a congenital disorder with an incidence of 1:15.000, characterized by overgrowth, a variety of developmental anomalies and increased risk of pediatric tumors. Clinical diagnosis ranges with a continuum of clinical signs from complete BWS syndrome to incomplete BWS till to Isolated Hemihypertrophia (HI) The genetic basis is complex and involves genetic/epigenetic alterations of imprinted growth regulatory genes in the 11p15 region. Genetic defects include 11p15 chromosomal rearrangements (2-3%), segmental chromosome 11 paternal UPD (20%) and mutations of the CDKN1C gene. Epigenetic alterations affect either IC1 (~2-7%) associated with H19 and IGF2 or IC2 associated with KCNQ10T1, which rules the expression of KCNQ and CDKN1C (~ 50%). Familial microdeletions associated with methylation within IC1 have been demonstrated by our group to be a rare mechanism involved in the syndrome.

The two Working Unit (WU), WU1 and WU2, jointly collected a wide cohort of patients including 270 complete BWS, 98 incomplete BWS and 104 HI. All genetic defects were found in the complete BWS cohort confirming the clinical diagnosis in about 66% of the patients, while only about 16% of cases were identified to carry a defect at IC2/IC1 within the remaining two groups, so attesting that other unknown mechanisms may be causative for incomplete BWS or IH. Concerning genotype-phenotype correlation our study substantially confirms what reported in the literature, mainly revealing the occurrence of overgrowth and omphalocele, associated with IC2 defects. Tumours were developed in at least 26 patients, with a major frequency among patients with IH, 11/104, roughly 10%, and complete BWS, 13/207 corresponding to 4,8%, while only 2 were developed by incomplete BWS cases. Most were Wilms tumors, but one hepatoblastoma, two neuroblastomas, two pancreatoblastomas and one very precocious mammary fibroma were detected. Unfortunately tumor tissues have been most often unavailable.

As the analysis of methylation defects and of uniparental disomy needs the use of multiple techniques, WU2 validated the MS-MLPA technique as a useful tool to detect 11p15 number and methylation anomalies.

In order to reveal methylation defects at genomic regions different from 11p15, WU1 and WU2 investigated the cohort of BWS patients both without and with a known genetic defect. One ICR normally methylated on the paternal allele, GTL2-IG (14q32), and eight maternally methylated ICRs, PLAGL1 (6q24), IGF2R (6q25.3), GRB10 (7p21), MEST (7q32.2), SNRPN

(15q11), PEG3 (19q13), GNAS (20q13.32) and NESPAS (20q13.32), were investigated. Methylation analysis of the above 11 ICRs showed that hypomethylation affecting multiple imprinted loci was restricted to 17 patients with hypomethylation of the KCNQ1OT1 ICR, and involved only maternally methylated loci. Both partial and complete hypomethylation was demonstrated in these cases, suggesting a postzygotic origin of a mosaic imprinting error. The study was carried out with the contribution of other European groups and has been reported.

Furthermore WU3 and WU1 developed a strategy to investigate fetuses with ultrasonographic indication (omphalocele occurrence) for BWS testing. Among 20 cases four revealed hypomethylation of IC2 and one had a mutation of CDKN1C, thus proving that this feature can be considered a II trimester good indicator of BWS in prenatal diagnosis.

Papers published within the project

Bliek J, Verde G, Callaway J, Maas S, De Crescenzo A, Sparago A, Cerrato F, Russo S, Ferraiuolo S, Rinaldi MM, Fischetto R, Lalatta F, Giordano L, Ferrari P, Cubellis MV, Larizza L, Mannens M, Mackay DJG, Riccio A. Hypomethylation at multiple maternally methylated imprinted regions including TND and PHP1b loci in Beckwith-Wiedemann syndrome. *Eur J Hum Genet* 2009;17(5):611-9

Cerrato F, Sparago A, Verde G, De Crescenzo A, Citro V, Cubellis MV, Rinaldi MM, Boccuto L, Neri G, Magnani C, D'Angelo P, Collini P, Perotti D, Sebastio G, Maher E and Riccio A. Different mechanisms cause imprinting defects at the IGF2/H19 locus in Beckwith-Wiedemann Syndrome and Wilms' tumour. *Hum Mol Genet* 2008,17:1427-35.

Murrell A, Ito Y, Verde G, Huddleston J, Woodfine K, Silengo MC, Spreafico F, Perotti D, De Crescenzo A, Sparago A, Cerrato F, Riccio A. Distinct methylation changes at the IGF2-H19 locus in congenital growth disorders and cancer. *PLoS ONE* 2008;3(3):e1849.

Priolo M, Sparago A, Mammì C, Cerrato F, Laganà C, Riccio A. MS-MLPA is a specific and sensitive technique for detecting all chromosome 11p15.5 imprinting defects of BWS and SRS in a single-tube experiment. *Eur J Hum Genet* 2008,16:565-71.

Riccio A, Sparago A, Verde G, De Crescenzo A, Citro V, Cubellis MV, Ferrero GB, Silengo MC, Russo S, Larizza L, Cerrato F. Inherited and sporadic epimutations at the IGF2-H19 locus in Beckwith-Wiedemann syndrome and Wilms' tumor. *Endocr Dev.* 2009;14:1-9

Sparago A, Russo S, Cerrato F, Ferraiuolo S, Castorina P, Selicorni A, Schwienbacher C, Negrini M, Battista Ferrero G, Cirillo Silengo M, Anichini C, Larizza L, Riccio A. Mechanisms causing imprinting defects in familial Beckwith-Wiedemann syndrome with Wilms' tumour. *Hum Mol Genet* 2007;16:254-64.

GENETIC AND CLINICAL ASPECTS
OF RARE LYMPHOMAS

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Within this project we have investigated two types of rare diseases: primary Cutaneous Lymphomas (pCL) and HCV-related lymphomas.

PCLs are a heterogeneous group of neoplasias that are characterized by an accumulation of mononuclear, mostly lymphocytic cells in the skin. The estimated annual incidence of pCL of about 1 case per 100.000 defines them as a rare disease; the vast majority is represented by T-cell type lymphomas whereas about the 25% is made of primary Cutaneous B Cell Lymphomas (pCBCL). Mycosis Fungoides (MF) and Sézary Syndrome (SS), along with some less common forms of skin lymphomas are collectively referred to as Cutaneous T Cell Lymphoma (CTCL), as they each originate in a T lymphocyte of the skin that has undergone a malignant transformation. While MF shows a skin-restricted infiltration of the clonal T-cell population and an indolent course, SS is a leukemic and erythrodermic variant of CTCL characterised by the presence of tumour lymphocytes in the skin, lymph nodes and peripheral blood. CBCL with the histology of a diffuse large B-cell lymphoma are classified as either primary cutaneous follicle center lymphoma (pCFCL) or primary cutaneous large B-cell lymphoma, leg type (pCLBCL, leg type). pCFCLs are characterised by skin lesions confined to a limited area on the head or the trunk which rarely disseminate to extracutaneous sites and have an excellent prognosis (5-year survival>95%). pCLBCLs, leg type present with skin tumours on the (lower) legs, they differ from pCFCLs by a higher age of onset, more frequent dissemination to extracutaneous sites, a poorer prognosis (5-year survival $\approx$ 50%) and a strong expression of BCL-2 and MUM1 proteins that represent valuable diagnostic markers.

With the aim of better understanding the processes that govern lymphomagenesis in the skin, we have characterised several genetic events and immunological aspects occurring in these tumours. Concerning CTCL, we have performed a genome-wide analysis of DNA copy number (CN) changes in 28 SS samples using oligonucleotide microarray technologies (SNP and aCGH). Our findings identified 6 regions of highly recurrent CN aberrations affecting chromosomes 8, 9, 10 and 17. In the attempt to correlate CN data and clinical parameters we find a relationship between complex pattern of chromosomal aberrations, involving at least three recurrent CN alterations, and shorter survival. We have combined CN results with gene expression data from the same SS patients to generate a signature of genes differentially expressed and located within the chromosomal regions of interest that includes novel potential candidates of SS tumour development.

With reference to CBCL we have analysed the nucleotide sequence of clonal Ig heavy-chain Variable Gene (VH) rearrangements in 51 cases of pCBCL to investigate cellular origin and antigenic selection in these tumours: data indicate that neoplastic B cells of cutaneous lymphoma have experienced the germinal-center reaction and suggest also that the involvement

of VH genes is not completely random in pCBCL and that common antigen epitopes could be pathologically relevant in this cutaneous lymphomagenesis. Secondly, we have performed a mutation analysis of p16INK4A and p14ARF, in 20 samples of pCBCL (16 pCFCL and 4 pCLBCL, leg type); alterations in these genes have been associated with a more aggressive disease and a poorer prognosis. We have found two mutations in exon 2: a) A148T p16INK4A, an already described polymorphism, in 2 cases (1 pCFCL and 1 pCLBCL, leg type) and b) c151-1del4 (p16INK4A)/c316del4 (p14ARF), a deletion in the putative "splicing" junction, in 1 pCLBCL, leg type case, possibly affecting the mRNA processing of both p16INK4A and p14ARF. In exon 3 four cases (3 pCFCL and 1 pCLBCL, leg type) display g500c polymorphisms in the 3'UTR region. The p16INK4A promoter study revealed 4 cases of pCFCL and 1 pCLBCL, leg type showing total or partial hypermethylation. These data suggest that alteration of this genomic locus might correlate with this lymphoproliferative disease. Finally, we used the Genome-Wide Human SNP Array 6.0, recently released by Affymetrix, to investigate DNA CN changes and Loss of Heterozigosity (LOH) in an initial group of 10 selected pCBCL cases; seven of them satisfied the assay quality control parameters and could be analysed. The preliminary results showed that these tumours exhibit a different degree of DNA damage: we found one case affected by a particularly high level of CN changes involving large chromosomal regions or entire chromosomal arms, three samples with a reduced number/extension of CN alterations and three cases with only few and/or small regions of aberration. Interestingly, one pCLBCL, leg type showed an extended area of UPD (uniparental disomy) encompassing CDKN2A locus, thus reinforcing a possible pathogenetic involvement of this tumour suppressor gene. All the other represented pCFCL with a favourable prognosis, however correlation with other clinical data and follow-up studies may give new insights into this clinical entity.

In addition to this, as pCLs affect the quality of life (QoL) due to their impact on skin appearance and the occurrence of persistent symptoms, such as pruritus, we have evaluated patients' health-related QoL in relation to personal and clinical characteristics. Data were collected using a dermatology-specific questionnaire, the Skindex-29 (symptoms, emotions, and functioning scales), and an oncology-specific questionnaire, the EORTC QLQ-C30 (15 scales, concerning physical and emotional aspects). Complete data on QoL were obtained for 95 patients affected by CTCL (MF, SS) or CBCL. A decreasing QoL trend was observed for all the scales in patients with SS, followed by MF, and CBCL: the trend was particularly steep in the global health status scale, and for fatigue, pain and financial problems (EORTC QLQ-C30 questionnaire). Health related QoL impairment in all pCL types was higher in women than in men, in patients with probable anxiety or depression, and during worsening of the disease. The comparison of the mean values of the QoL scores in the different treatment groups showed that the patients treated with UVA or PUVA had the most impaired QoL, in all the scales except for cognitive function, nausea, appetite loss and diarrhoea. Symptoms were particularly reported by patients treated with interferon. In conclusion we showed that the detailed evaluation of QoL may help clinicians to identify aspects of patients' psychosocial well-being that may be overlooked, thus better managing the disease and its burden on patients' life. We also collected longitudinal data on more than 100 patients. The analysis of longitudinal data will yield important information on the course of the disease as well as on the possible effect of treatment.

Chronic HCV infection causes B-cell lymphoproliferative disorders that include type II mixed cryoglobulinemia (cryo-II) and, more rarely, B-cell non-Hodgkin's lymphomas (B-NHL). A number of studies indicate that cryo-II and B-NHL are sequential steps of a pathogenic B cell response to HCV. In fact, up to 15% of cryo-II patients develop B-NHL over time. Cryo-II is characterized by the benign monoclonal expansion of a selected B cell population expressing an antibody of the IgM class whose variable region is usually encoded by the VH1-

69 variable gene. During the course of this unconstrained monoclonal expansion, genetic anomalies may occur and lead to neoplastic transformation. The dependence of benign (cryo-II) and malignant B-cell proliferation on stimulation by HCV is further demonstrated by the regression of cryo-II and of splenic-type NHL following eradication of infection. We recruited 16 HCV-positive NHL patients and 19 cases of type II mixed cryoglobulinemia without lymphoma. We identified two distinct genetic events in HCV-driven lymphomagenesis. Gain of 3q was found to hallmark low-grade NHL or non-malignant B-cell clonal expansion, as it was observed in 4/6 cases of low-grade splenic marginal zone lymphoma (SMZL) as well as in the non-malignant lymphoproliferative phase of type II mixed cryoglobulinemia. Regression of lymphoma after eradication of HCV with antiviral therapy was observed in 3/3 cases of SMZL. Conversely, deletion at 2q22.3 was detected in 4/5 (80%) cases of clinically aggressive large B-cell lymphomas (LBCL). No response to antiviral treatment was observed in this subgroup.

Papers published within the project

Caprini E, Cristofoletti C, Arcelli D, Fadda P, Helmer Citterich M, Sampogna F, Magrelli A, Censi F, Torreri P, Frontani M, Russo G. Identification of key regions and genes important in the pathogenesis of Sézary syndrome by combining genomic and expression microarrays. *Cancer Res* 2009;69(21):8438-46.

Matteucci C, Bracci M, Barba G, Carbonari M, Casato M, Visentini M, Pulsoni A, Varasano E, Roti G, La Starza R. Different genomic imbalances in low- and high-grade HCV-related lymphomas. *Leukemia* 2008;22,219-22.

Perez M, Pacchiarotti A, Frontani M, Pescarmona E, Caprini E, Lombardo GA, Russo G, Faraggiana T. Primary cutaneous B-cell lymphoma is associated with somatically mutated immunoglobulin variable genes and frequent use of VH1-69 and VH4-59 segments. *Br J Dermatol* 2009 [E-pub ahead of print].

Sampogna F, Frontani M, Baliva G, Lombardo GA, Alvetreti G, Di Pietro C, Tabolli S, Russo G, Abeni D. Quality of life and psychological distress in patients with cutaneous lymphoma. *Br J Dermatol* 2009;160,815-22.

BIOMARKERS IN THYMIC EPITHELIAL TUMORS (TET). AN IMMUNOHISTOCHEMISTRY-BASED TISSUE MICRO ARRAY (TMA) MULTICENTER STUDY WITH PRELIMINARY GENETIC DATA ON THE EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR) GENE IN TET

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Introduction

Relapsing and/or metastatizing Thymic Epithelial Tumors (TET) constitute a therapeutic problem, as traditional chemotherapy is often uneffective and biologically-based, targeted therapies need to be validated thorough specific biomolecular investigations and clinical trials devoted to such a rare pathology. A Tissue Micro Array (TMA)-based study was designed in order to identify a statistically significant biopathological marker panel with prognostic and / or predictive value for TET management. Data from the literature point to the angiogenesis

relevance in malignant/relapsing TET. Therefore, an exhaustive exploration of the angiogenetic factors VEGF and their receptors and of Platelet Derived Growth Factor Receptorβ (PDGFRβ) distribution in TET was performed. Furthermore, the activation pathways of Epidermal Growth Factor Receptor (EGFR), involved in both TET growth and angiogenesis, requires to be investigated both at the protein expression distribution and genetic status. EGFR gene amplification and mutational status both have been related to EGFR hyperexpression and to sensitivity to different biologically-targeted therapies in different epithelial tumors. Moreover, recently, a novel EGFR transcriptional mechanism has been described, depending on the length of a CA repeat in intron 1 [CA simple sequence repeat 1 (CASSRI)] of the EGFR gene, where the number of CA repeats was inversely correlated to EGFR pre-mRNA synthesis.

Methods

Immunohistochemical study

Biomarkers of prognostic and/or predictive relevance in TET have been investigated by immunohistochemistry in a tissue-microarrays-based multicenter study established in Italy by a collaborative network among pathologists and clinicians. 350 cases of TET were collected and arrayed in TMA. As a training group, 200 cases were examined for histotype distribution and biomarker expression. Residual Thymus and some hyperplastic thymus samples were also examined. The cases were stained for angiogenesis factors (VEGFR-A, B and C) and their Receptors 1,2 and 3 and for Platelet Derived Growth Factor Receptor β (PDGFRβ), cell cycle and apoptosis biomarkers, as well as for membrane receptors involved in epithelial cell proliferation such as EGFR and for adhesion/signalling molecules such as E-Cadherin and Trop-1/Ep-CAM.

Furthermore, biothynilated commercial Avastin (bevacizumab), a VEGF inhibitor, has been tested on TMA. Statistical analyses were performed. As a validation group, the further 150 cases, provided from the Dept. of Biomedical Sciences and Human Oncology, University of Turin, will be analysed as a validation group.

Genetic study

Considering the central role played by the EGFR gene and EGFR-dependent proliferative pathways in several types of epithelial tumors, in parallel to the immunohistochemical study, we started a pilot study on the EGFR gene in TET by multiple approaches. TMA-Fluorescent In Situ Hybridization (TMA-FISH) was performed to analyse EGFR gene copy number by EGFR/CEN-7 FISH Probe mix. Furthermore, the EGFR mutational status in the exon 21 and the K-RAS (codon 12-13) mutational status have been investigated in a pilot study, as well as the EGFR microsatellite CASSRI. The gene fragments were amplified and directly sequenced and then analyzed using the ABI 3130 Genetic Analyzer (Applied Biosystems).

Results

Sample

The TET histotype distribution according to the World Health Organization classification in the training group was determined in 5% type A, 18.5% type AB, 15.5% type B1, 33.5% type

B2, 16.5% type B3 TET and 8% Thymic Carcinoma, as well as 3% of the rare Micronodular Thymoma (MNT) variant of TET. Immunohistochemical study- VEGF A,B and C and the 3 VEGF Receptors were observed in the different histotypes, as well as stainability with biothynilated AVASTIN and with antibody to PDGFRβ. Frequency of reactive Epithelial Cells was investigated as well as staining intensity. Immunohistochemical scores were compared among different histotypes and with different markers. Median % of positive tumor cells varied according to different angiogenetic factors and receptors considered, being higher for FLK-1 (VEGF-R2) (66%) and FLT-1 (VEGF-R1) (52%) in comparison to other markers. No significant histotype correlations were found, but statistical significancy was observed for several VEGF Receptor associations (as an example, for the association FLK-1 and FLT-4), or for biothynilated Avastin and FLT-4 (VEGF-R3) or PDGFRβ. Analysis with the other immunohistochemical markers applied is ongoing.

Genetic study

Preliminary data indicate that chromosome 7 polysomy is rarely detectable in TET by EGFR/CEN-7 FISH applied to TMA. Moreover, among the 17 TET cases tested so far, no mutations were found in the K-RAS (codon 12-13) and EGFR (exon 21) genes. Microsatellite analysis for CASSRI was performed on genomic DNA of 17 selected EGFR-positive (by immunohistochemistry) tumors. In 17 patients, the smaller allele had a length of 14 CA repeats (n. 1), the most prevalent bp length was 16 CA repeats (n. 11), and the longer allele had a length of 21 CA repeats (n. 2). The prevalent genotype showed omozygosity for 16 CA repeats (n. 4 cases) and the second most prevalent showed heterozygosity, with 16 and 20 CA repeats (n. 3 cases).

Conclusion

Immunohistochemical study

From the available data it appears that angiogenetic factors and their receptors are largely distributed among the several WHO TET histotypes; however, their associations appear to differ significantly in the different WHO histotypes, particularly in the B2, B3 groups. These histotypes, among Thymomas, have a more aggressive potential according to the literature data and own diagnostic and clinical experience. In our cases, it was possible to stain with the biothynilated commercial AVASTIN the TMA paraffin slides. Furthermore, comprehensive statistical analyses with the multiple biomarkers applied and correlations with the available clinical data will follow in order to identify a cluster of immunophenotypic characters with possible relevance to prognosis or predictive of targeted therapy sensibility.

Genetic study

Data from genetic study are very preliminary. However, it appears that in TET alternative mechanisms with respect to EGFR gene amplification and point mutations occur, and they could play a role in activating the EGFR proliferative pathways.

CYTOGENETIC AND MOLECULAR ANALYSIS OF RARE SALIVARY GLAND TUMORS

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Introduction

Salivary Gland Tumours (SGTs) are rare tumors that account approximately for < 0.5% of all malignancies whith an incidence of <1/100.000 in the World. SGTs are remarkable for their histopathologic and biologic diversity and, according to the 2005 WHO classification, there are 24 different histologic subtypes, some of them very rare.

Although exposure to ionizing radiation has been implicated as a cause of SGTs, the etiology of most of them cannot be determined. It's difficult to determine the prognosis and select the optimal therapeutic modality of SGTs since the rarity and the hystological growth pattern shared by different histotypes.

MucoEpidermoid Carcinoma (MEC) is the most common malignant salivary gland tumor which comprises about 10% of all SGTs and approximately 35% of malignant salivary gland neoplasma.

Adenoid Cystic Carcinoma (ACC) is the second most frequent SGT and it is subclassified in cribriform, tubular and solid according to the histological pattern. Dedifferentiated ACC (dACC) are a recently defined, rare variant of adenoid cystic carcinomas characterized histologically by two components: conventional low-grade adenoid cystic carcinoma and high-grade "dedifferentiated" carcinoma.

Polymorphous Low-Grade Adenocarcinoma (PLGA) is a low-grade rare tumor showing local invasion, often arising from minor salivary glands with palate being a common site

Hybrid tumors are very rare, and according to a definition by Seifert and Donath (1996) definition, are composed of two different tumor entities within an identical topographical area that have an identical origin in the same tissue.

An improved understanding of salivary gland cancer genetics can provide new markers for a better diagnosis and prognosis of these rare and complex malignancies.

Objectives and methods

Our aims are: i) to investigate genetic changes in salivary gland tumors with different histotypes, by using molecular cytogenetics and molecular genetics methods ii) to establish a correlation with clinico-pathological data.

Paraffine-embedded samples from salivary gland tumors with different hystotypes were obtained from Istituto di Anatomia Patologica, Bologna and from Istituto Regina Elena, IFO, Rome.

Comparative Genomic Hybridization was performed in five MECs, twelve ACCs, two hybrid tumors (ACC/epi-mioepithelial carcinoma), one PLGA in order to detected genomic imbalances, confirmed by Real-Time PCR.

Mutation analysis of genes involved in cell cycle and oncogenesis, as TP53, p14ARF, p16INK4a (exons 1β, 1α, and 2), HRAS (exons 2-3), KRAS (exons 2-3), NRAS (exons 2-3), PTEN (exon 7), BRAF (exons 11 and 15), MAP2K2 (exon 2), EGFR (exons 19 and 21), was performed in two dedifferentiated ACCs, one cribriform/tubular ACC, one hybrid tumor (ACC and epithelial-myoepithelial carcinoma) and one PLGA.

Microsatellite analysis with markers D9S1748, D9S942, D9S974 (gene locus CDKN2a/ARF) was performed in the dedifferentiated ACCs since normal tissue was available.

Results and conclusions

Molecular analysis

Mutational analysis performed in two dedifferentiated ACCa, one cribriform/tubular ACC, one hybrid tumor (adenoid cyctic carcinoma and epithelial-myoepithelial carcinoma) and one polymorphous low grade adenocarcinoma showed: i) snps missense in RAS genes and alterations with allelic instability in CDKN2A/ARF in two dedifferentiated ACCS; moreover, a double mutation in TP53 was detected in one of them; ii) snps missense in N-RAS genes and alterations in CDKN2A/ARF gene in one cribriform-tibular ACC; iii) CDKN2A/ARF alterations in the polymorphous low-grade adenocarcinoma.

Single Nucleotide Polymorphisms (SNPs) synonymous in coding regions of TP53, p14, p16, H-RAS and BRAF have been identified.

Our data suggest that p16INK4a-CD4/6/CycD-RB pathway might be involved in the pathogenesis of these histological subtypes of salivary gland tumors. Moreover a TP53 double mutation has been detected in a dedifferentiated tumour whith a very poor prognosis supporting the role of p53 as a potential prognostic predictor marker.

Molecular cytogenetic analysis

MECs: Comparative Genomic Hybridization performed in five mucoepidermoid carcinomas showed seventeen gains and seven losses, with a ratio 2.4:1.

Alterations were detected in four high-grade MECs, while no aberration was found in a low-grade MEC.

Gains were observed in 1q21-22; 1q41-42; 2p23-pter; 3q; 3q28; 4q34-35; 5p; 6q27; 8q21.3-22; 8q23; 10p15; 13p26; 16q22-23; 18q23.5; 21q22. Losses were detected in 3p; 11q24-25; 14q32; 18q23; X. High-level amplifications of 3q and 5q11.2-13 were observed in one case.

Real-Time PCR of selected genes mapping in altered chromosomal regions was performed in order to confirm genomic imbalances detected by CGH.

In literature classical cytogenetic studies show heterogeneity of imbalances; the only one rearrangement detected frequently is a balanced traslocation t(11;19), which has been associated with a positive prognosis.

To our knowledge only one CGH study has been reported in one SGT MEC cell line; in this report a 3q gain and a loss in 3p13-21 were detected suggesting that these two regions, involved in our study too, could have a role in the pathogenesis of MECs.

ACCs: CGH performed in twelve samples showed heterogeneity of chromosomal alterations; moreover small size chromosomal aberrations were identified suggesting that genomic instability is not a mechanism involved in these tumors.

Hybrid tumours: CGH showed i) in one case only gains in 2q37, 3p25-26, 11q24-25, 18p11.3, 18q23 ii) in a second case 3p26, 9p23-pter gains and 19 p loss. The finding of a common gain in 3p26 suggests a possible role of genes localised in this region in the pathogenesis of these tumors.

Papers published within the project

Floridia G, Censi F, Falbo V, Taruscio D. Alterazioni genetiche dei tumori delle ghiandole salivari. In: Spriano G (Ed.). *XCV Congresso Nazionale, Società Italiana di Otorinolaringoiatria e Chirurgia Cervico-Facciale, Torino, 21-24 maggio 2008. I Tumori delle ghiandole salivari maggiori.* p. 85-100 Disponibile all'indirizzo http://www.boffetta.it/tugh.pdf; ultima consultazione 18/1/2010.

Floridia G, Censi F, Foschini MP, Falbo V, Taruscio D. Characterization of chromosomal alterations in paraffin-embedded salivary gland tumors by comparative genomic hybridization. *European Journal of Human Genetics* 2007;15(Suppl 1):170.

Floridia G, Censi F, Foschini MP, Falbo V, Taruscio D. Comparative genomic hybridization in campioni paraffinati di tumori delle ghiandole salivari. In*: 10° Congresso nazionale SIGU. Abstracts*; 14-16 novembre 2007; Montecatini Terme.

Floridia G, Censi F, Foschini MP, Falbo V, Taruscio D. Molecular cytogenetic characterization of paraffin-embedded salivary gland tumors by Comparative Genomic Hybridization. *Eur J of Hum Gen* 2008;16(Suppl 2):236.

Floridia G, Censi F, Foschini MP, Falbo V, Taruscio D. Salivary gland tumours: comparative genomic hybridization in paraffine-embedded samples. In: Taruscio D, Salvatore M (Ed.). *Workshop. Rare Diseases and Orphan Drugs. Rome, November 7-8, 2007. Abstract book.* Roma: Istituto Superiore di Sanità; 2007. (ISTISAN Congressi 07/C8). p. 48.

Floridia G, Censi F, Marra M, Lanni S, Foschini MP, Falbo V, Taruscio D. Caratterizzazione genetica e citogenetico-molecolare di campioni paraffinati di tumori delle ghiandole salivari. In: *X Congresso SIGU*, Genova 23-25 novembre 2008.

Floridia G, Censi F, Marra M, Lanni S, Foschini MP, Falbo V, Taruscio D. Characterization of genetic and cytogenetic alterations in salivary gland tumors. In: Taruscio D, Salvatore M (Ed.). *Workshop. Projects on rare diseases funded within the bilateral agreement Italy (Istituto Superiore di Sanità) and USA (NIH, Office for Rare Diseases) on joint research and development of public health actions. Istituto Superiore di Sanità. Rome, October 29-31, 2008. Abstract book.* Roma: Istituto Superiore di Sanità; 2008. (ISTISAN Congressi 08/C10). p. 58.

CLINICAL AND GENETIC FEATURES
OF PARATHYROID CARCINOMAS

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The study entitled "Clinical and genetic features of parathyroid carcinoma" has begun in May 2007 and the following tasks have been accomplished:

1. Development of a database for the collection of clinical, biochemical or imaging data for the identification of diagnostic markers of parathyroid carcinoma and related HPT-JT syndrome. Four parathyroid carcinomas have been diagnosed up to now on the basis of the histological aspects of the parathyroid lesions from patients affected with primary hyperparathyroidism. Eight further cases of familial primary hyperparathyroidism with no mutations in MEN1, RET and calcium sensing receptor genes have been enrolled (Endocrine Unit, Fondazione Irccs Ospedale Maggiore Policlinico, Mangiagalli e Regina Elena, Milan).

2. Development of a clinical protocol for the diagnosis and the follow up of the recurrences of the parathyroid carcinomas and evaluation of the efficacy of the available medical therapy for recurrent parathyroid cancer, Cinacalcet HCl, in the control of PTH hypersecretion and hypercalcemia as well as in the control of the progression of the neoplastic disease (Endocrine Unit, Fondazione IRCCS, Ospedale Maggiore Policlinico, Mangiagalli e Regina Elena, Milan).

3. Collection and storage of DNA and cancer tissue samples from all patients with diagnosis of parathyroid carcinoma as well as of HPT-JT or familial primary HPT without HRPT2 gene mutations, enrolled in the 3 Medical Hospitals involved in the Project (Fondazione IRCCS Ospedale Maggiore Policlinico, Mangiagalli e Regina Elena, Milan; IRCCS Casa Sollievo della Sofferenza, San Giovanni Rotondo, Foggia; IRCCS Policlinico San Donato, S.Donato M.se, Milan) in order to identify new genes potentially involved in parathyroid tumorigenesis.

4. Analysis of DNA and paraffin-embedded tissue samples from patients with parathyroid carcinoma for germline or somatic mutations of the genes encoding for parafibromin and beta-catenin and for the loss of heterozygosity in the chromosomal regions where HRPT2, beta-catenin, Pygo1 and Pygo2 map (IRCCS Casa Sollievo della Sofferenza, San Giovanni Rotondo).

5. Preliminary data on *in vitro* investigation on the beta-catenin phosphorylation stimulated by the calcium sensing receptor activation in HEK293 cells stably transfected with the calcium sensing receptor (Endocrine Unit, Policlinico S.Donato and Endocrine Unit, Fondazione Ospedale Maggiore Policlinico, Mangiagalli e Regina Elena).

6. Identification of parathyroid cells positive for the haematopoietic/endothelial marker CD34 which express surface antigens typical of endothelial progenitors such as CD146 and CXCR4. These cells were more abundant in tumoral than in normal parathyroids. Purified CD34+ cells expressed parathyroid specific markers, such as glial cell missing B, PTH and CaRS, proliferated and differentiated into mature endothelial cells. Moreover, stem cells from tumors – but not from normal tissues – were immunopositive for nestin, a neural stem cell specific marker.

7. Identification of differentially expressed microRNAs in parathyroid cancers compared to normal tissues. TaqMan low-density array-based profiling of 4 parathyroid cancers detected the expression of 279 out of 362 human microRNAs assayed (77%), 14 and 3 microRNAs being significantly down- and over-expressed in parathyroid cancers, respectively. Of these, miR-296 and miR-139 were down-regulated and miR-503 and miR-222 were over-expressed with a null false discovery rate. Parathyroid adenomas could be discriminated from carcinomas by a computed score based on the expression levels of miR-296, miR-222 and miR-503 could discriminate between adenomas and carcinomas.

Papers published within the project

Corbetta S, Belicchi M, Pisati F, Meregalli M, Eller-Vainicher C, Vicentini L, Beck-Peccoz P, Spada A, Torrente Y. Expression of parathyroid specific genes in vascular endothelial progenitors of normal and tumoral parathyroids. *American Journal of Pathology,* 2009;175(3):1200-7.

Corbetta S, Vaira V, Scillitani A, Guarnieri V, Eller-Vainicher C, Vicentini L, Chiodini I, Beck-Peccoz P, Bosari S, Spada A. Differential expression of microRNAs in human parathyroid carcinomas compared with normal parathyroid tissue. *Endocrine Related Cancer.* Nov 19. [Epub ahead of print].

THERAPY-ORIENTED LARGE SCALE GENOMIC AND GENE EXPRESSION ANALYSIS IN THYMOMAS, MESOTHELIOMAS AND LUNG CARCINOIDS

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Introduction

Thymomas, mesotheliomas and lung carcinoids are rare tumors and common strategies for their cure are still missing. Indeed, due to the low incidence and prevalence of these tumors, clinical trials cannot give definitive results in terms of treatment strategies based on the commonly accepted criteria of "evidence based" medicine: the small number of patients that enter these trials prevents from obtaining a strong statistical power for any study.

We propose a genetic approach in order to identify genes that may play a role in tumorigenesis and, more importantly, that can become effective "drugable" targets, in these different tumor types.

Materials and methods

At the time of Video Assisted Thoracic Surgery (VATS) or tumor resection, neoplastic tissue samples were obtained. For each case, a portion of the sample was rapidly frozen and OCT embedded in liquid nitrogen, and suitably stored. These samples constituted a source for DNA and RNA isolation and analysis. Normal tissue samples were also obtained and stored as a source of normal DNA or RNA. Only samples with at least 70-80% of tumor cells content were considered suitable for the study.

The Affymetrix microarray technology, available at the IFOM-IEO campus, was chosen for expression profiling. The GeneChip HG-U133 Plus 2.0, allowing for the evaluation of the expression levels of over 54000 probe sets, was used. Raw data from Affymetrix were Background Corrected, Normalized and summarized with RMA algorithm as implemented in Partek Genomic Suite v6.4. Statistical analysis including ANOVA test, PCA, gene selection and visualization were all performed with Partek Genomic Suite v6.4. 10mm serial sections (about 10-20) from the collected OCT frozen samples, either tumor or normal, were obtained and total RNA was extracted using a RNA Spin mini Illustra (GE, Little Chalfont, UK). A qualitative and quantitative analysis of the RNA samples was performed prior to the microarray analysis, using an Agilent 2100 Bioanalyzer (Agilent Technologies).

Results

We concentrated our analysis on lung carcinoids since we had a good collection of samples, already available, and we were able to get new samples, in a few months. Six typical and 7 atypical lung carcinoids have been analyzed out of 13 samples. We also considered 8 normal samples (4 from patients with typical and 4 with atypical carcinoids). The results are showed in the PCA representation (Figure 1).
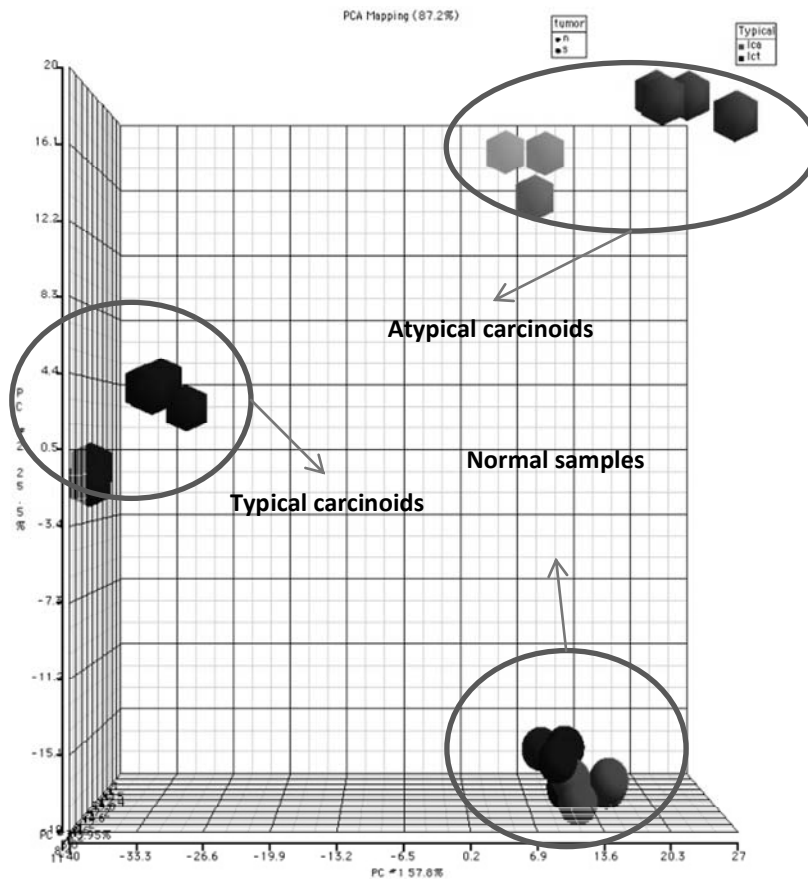
Figure 1. Typical and atypical carcinoids
(circles are for normal and hexagons for tumor)

Conclusions

Our results can be summarized as follows:

i)   the expression profile of normal lung tissue samples is homogenous and different from both typical and atypical carcinoids;

ii) the derived expression profiles are able to strikingly separate the typical versus atypical subtypes;

iii) it is possible to evidence a few markers, which specifically identify one of the two subtypes exclusively and could become new tools for diagnosis;

iv) by comparing atypical versus typical tumors, we identified specific gene expression profiles, suggesting resistance/sensitivity to standard chemotherapy regimens. Moreover, it was possible to point out putative targets for already available or newly-designed drugs.

We decided to further validate these intriguing and encouraging data on new sets of samples:

– paraffin embedded lung carcinoids samples, already available in our institute, for the immunohystochemical analysis of at least 3 diagnostic markers, as reported at point iii).

– 7 typical and 7 atypical additional lung carcinoid samples for real-time quantitative PCR analysis of 56 genes, which, from our expression profiles, resulted overexpressed in atypical versus typical tumors (point iv).

A MULTIDISCIPLINARY APPROACH FOR THE INVESTIGATION OF HYPERPARATHYROIDISM-JAW TUMOR SYNDROME

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The molecular basis of the HyperparaThyroidism-Jaw Tumor (HPT-JT) syndrome, a rare autosomal dominant disorder, has been recently identified. In fact, most of HPT-JT cases, and also some cases of Familial Isolated HyperparaThyroidism (FIHP), are related to mutations of the HRPT2 gene that encodes parafibromin. Mutations of HRPT2 are also detected in about 70% sporadic parathyroid carcinomas and, less frequently, in sporadic parathyroid adenomas/hyperplasia.

The aim of our research project was to investigate clinical features, molecular pathogenesis, and treatment modalities of hyperparathyroidism-jaw tumour syndrome (HPT–JT), by exploiting the multidisciplinary expertise of the collaborating research groups, which include a molecular genetics unit, an anatomy and pathology unit, and an endocrine-surgery unit. We extensively studied one large HPT-JT kindred, including 6 clinically symptomatic and 9 asymptomatic subjects, and 2 unrelated kindreds with FIHP, including a total of 10 symptomatic and 13 asymptomatic subjects. We showed that HPT-JT and FIHP patients had similar laboratory, clinical, and demographic features and shared primary hyperparathyroidism and other extra-parathyroid neoplasms, the most common of which was uterine polyposis. The kindreds had also the same genetic background, characterized by the occurrence of inactivating mutations of the HRPT2 gene. In addition, we detected a somatic HRPT2 mutation (probably, the "second hit", in agreement with the Knudson hypothesis) in a parathyroid tumor from a FIHP patient. All the mutations were predicted to result in truncated or inactive parafibromin, in agreement with its tumor suppressor activity. Detection of HRPT2 mutations in affected kindreds had also clinical impact, since we identified germline HRPT2 mutations not only in all affected patients of the three kindreds, but also in some young asymptomatic subjects, who have been submitted to a careful follow-up.

In order to identify other possible HRPT2 inactivation mechanisms in parathyroid tumors, we analyzed the methylation status of the HRPT2 promoter in parathyroid adenomas from HPT-JT patients without an identified second somatic mutation, but the methylation analysis did not demonstrate any methylated CpG site. We also evaluated parafibromin expression by immunohistochemistry, which demonstrated loss of parafibromin nuclear immunostaining in almost all tumor cells of parathyroid carcinomas and adenomas from HPT-JT and FIHP patients. At variance, intense nuclear immunostaining was present in all normal parathyroids obtained at surgery from both HPT-JT and FIHP patients and control subjects without hyperparathyroidism.

Anti-parafibromin immunohistochemistry was also performed on endometrial hyperplastic polyps from a HPT-JT patient and on sporadic endometrial hyperplastic polyps. In the HPT-JT-related uterine polyp, stromal cells did not show any anti-parafibromin reactivity, whereas epithelial cells had only moderate cytoplasmic immunoreactivity. In sporadic polyps, stromal

cells showed positive nuclear anti-parafibromin immunostaining and epithelial cells showed intense nuclear immunostaining and moderate cytoplasmic positivity. Loss of parafibromin nuclear immunostaining in both stromal and epithelial components of HPT-JT polyps, with respect to sporadic ones, supports the pathogenetic role for HRPT2 mutations in uterine polyposis associated with this syndrome.

Pathological evaluation showed that both HPT-JT and FIHP patients generally had single parathyroid gland involvement at bilateral neck exploration. Single-gland involvement was clinically confirmed by the postoperative course, since limited parathyroidectomy achieved a long-term cure (mean, 9.4 years) in all cases. HPT recurred in 25% of cases, after a long disease-free period (mean, 13.7 years); in every case, the recurrence involved a single gland, and cure was achieved at reoperation by selective parathyroidectomy, with acceptable rates of recurrences.

We are also investigating the role of parafibromin in the pathogenesis of sporadic parathyroid tumors. From June 2005 to date, we have collected a series of 60 sporadic parathyroid tumors, comprising 57 adenomas and 3 carcinomas. Sequencing of the HRPT2 gene detected a somatic nonsense HRPT2 mutation only in a parathyroid carcinoma. Analysis of HRPT2 expression was performed by quantitative RT-PCR analysis, Western blotting, and immunohistochemistry, which did not demonstrate significant differences in mRNA and parafibromin expression among normal parathyroid glands, parathyroid adenomas, and carcinomas, although there was wide variability in parafibromin expression and subcellular distribution among different samples. These findings are in agreement with other reports in the literature stating that the absence of nuclear parafibromin at immunoistochemistry, without other molecular markers, may not be considered a marker of malignancy for parathyroid tumors. We have now in progress the analysis of microRNA (miRNA) expression profile on parathyroid carcinomas with and without HRPT2 mutations and in parathyroid adenomas, in order to identify if HRPT2 mutations are associated with abnormal miRNA expression.

Expression of parafibromin in tissues other than the parathyroid glands has been rarely investigated. We extensively examined parafibromin expression and distribution in mouse and human tissues by immunohistochemistry. Parafibromin expression was widespread, without substantial differences in between mouse and human tissues. Parafibromin was highly expressed in hepatocytes, in cells of the base of gastric glands, in renal cortex tubules and in pars intermedia of the pituitary. At variance, no/poor parafibromin expression was demonstrated in connective tissue, smooth muscle, endothelium and some other types of epithelia (colonic, urinary, tubaric, uterine, thyroid). Heterogeneity of parafibromin immunostraining intensity and subcellular location was found between tissues and cell types, suggesting differential functional involvement of parafibromin.

The widespread expression of parafibromin in different tissues suggests it might be involved in the pathogenesis of sporadic tumors other than parathyroid neoplasms. Following our demonstration that parafibromin is highly expressed in the normal human and murine adrenal cortex, we have investigated parafibromin expression by western blotting and immunohistochemistry in a series of 60 sporadic adrenocortical tumors. Parafibromin was highly expressed in the nucleus of most normal adrenocortical cells, but significantly down-regulated in about 30% adrenocortical adenomas and 50% carcinomas. The mechanism of parafibromin dowregulation remains unknown, since no HRPT2 mutations nor methylation of the HRPT2 promoter were detected in tumors with low parafibromin expression and the full-length protein was detected at western blot analysis. Functional *in vitro* studies to assess if parafibromin has tumor suppressor activity in adrenocortical tumors are ongoing.

In conclusion, we performed a multidisciplinary study on HPT-JT and FIHP patients and in HRPT2-associated tumors. We demonstrated that patients with germline HRPT2 inactivating

mutations frequently develop primary hyperparathyroidism due to parathyroid adenoma/hyperplasia or, less frequently, parathyroid carcinoma, and other tumors, especially uterine polyposis. HPT-JT and FIHP patients with HRPT2 mutations and hyperparathyroidism generally have single parathyroid gland involvement, so a conservative surgical approach with limited parathyroidectomy allow to achieved a long-term cure with low recurrence rate. We also demonstrated widespread expression of parafibromin in tissues other than parathyroid gland, such as the adrenal cortex. Parafibromin inactivation could be involved in the pathogenesis of tumors arising form these tissues, as suggested by the frequent loss of parafibromin expression in adrenocortical adenomas and carcinomas.

Papers published within the project

Iacobone M, Barzon L, Porzionato A, Masi G, Macchi V, Marino F, Viel G, Favia G. Parafibromin expression, single gland involvement, and limited parathyroidectomy in familial isolated hyperparathyroidism. *Surgery* 2007;142:984-91.

Iacobone M, Barzon L, Porzionato A, Masi G, Macchi V, Viel G, Favia G. The extent of parathyroidectomy for HRPT2-related hyperparathyroidism. *Surgery* 2009;145(2):250-1.

Iacobone M, Masi G, Barzon L, Porzionato A, Macchi V, Ciarleglio FA, Palù G, De Caro R, Viel G, Favia G. Hyperparathyroidism-jaw tumor syndrome: a report of three large kindred. *Langenbecks Arch Surg* 2009;394(5):817-25.

Masi G, Barzon L, Iacobone M, Viel G, Porzionato A, Macchi V, De Caro R, Favia G, Palù G. Clinical, genetic, and histopathologic investigation of HRPT2-related familial hyperparathyroidism. *Endocr Relat Cancer* 2008;15(4):1115-26.

Porzionato A, Macchi V, Barzon L, Masi G, Iacobone M, Parenti A, Palù G, De Caro R. Immunohistochemical assessment of parafibromin in mouse and human tissues. *J Anat* 2006;209:817-27.

DIAGNOSTIC AND THERAPEUTIC TARGET OF SYSTEMIC AMYLOIDOSIS: VALIDATION OF NEW DIAGNOSTIC TOOLS AND DEVELOPMENT OF NEW DISEASE MODELS

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Introduction

Two main objectives were at the basis of this research project on systemic amyloidosis:
1. development of new diagnostic tools;
2. assessment of new models of the disease mimicking, at different levels of complexity, the pathophysiology of systemic amyloidosis.

Development of new diagnostic tools

We have applied the proteomic technology to dissect the composition of bioptical fat tissue specimens from patients affected by systemic amyloidosis. Through a combination of two-dimensional electrophoresis (2D-PAGE) followed by mass spectrometry (MS) and of MS-coupled two-dimensional liquid chromatography, we have accomplished an initial description of the proteome of adipose tissue. Using gel-free proteomics, hundreds of unique proteins (about 1200), including matrix and membrane ones, have been identified in the specimens, many of which (about 350) are common most patients. The proteomes of affected and control adipose tissue have been compared with the specific aim of defining changes in the expression of resident proteins in relation to amyloid deposition. This task has been exploited through a combined gel-based and gel-free proteomic approach, which allows an efficient global analysis of soluble as well as large extracellular and structural proteins, and membrane-bound ones. Preliminary semi-quantitative data have been obtained. Differences in expression between patients and controls have been observed for a wide array of proteins, including some involved in cell energetics and metabolism, cellular redox equilibrium, signal transduction, cell adhesion, and various proteins with regulatory functions.

Moreover, these preliminary results suggest the opportunity of exploring differences in the expression of extracellular matrix and chaperone proteins, which may be related to the onset and development of the disease. Congo red-positive fat specimens from patients with different types of amyloidosis (AL κ and λ, ATTR and AA amyloidosis) were analyzed by 2D-PAGE, with the aim of detecting and identifying spots related to amyloid deposits. In all patients, comparisons with control maps allowed locating groups of prominent novel spots, with migration patterns consistent with those of the expected amyloidogenic proteins.

These spots have been analyzed and identified by MS; in all cases, they corresponded to the predicted amyloidogenic protein, thus allowing direct biochemical assessment of disease type. Particularly in AL and ATTR amyloidosis, the proteins derived from amyloid-deposits were shown to be constituted by a heterogeneous population of intact precursor, fragments and post-translationally modified isoforms. Additionally, spots corresponding to amyloid-associated proteins have been located and identified, such as Serum Amyloid P (SAP). This represents a major step forward in the diagnosis of amyloidosis since proteomics allows the unequivocal typing of amyloid deposits which is the prerequisite for effective therapy. In the case of amyloidosis caused by β2-microglobulin (β2-m), associated with chronic hemodialysis, the proteomic approach has provided quite conclusive information about the constitutive presence of the N-terminal truncated form of β2-m (ΔN6β2-m) in the fibrils. In the same study we have excluded the role of β2-m cleaved in position 58 in the amyloidogenic process.

A second experimental task is focused on the optimisation of the usage of aprotinin and its fibril-specific sequence as a tracer targeting amyloid deposits. Experimental data indicate that a central part of the aprotinin sequence, isolated as a polypeptide cycled through a disulfide bridge, can recognize a general conformational motif of amyloid fibrils. Aprotinin is an amyloid-specific tracer able to detect cardiac involvement. This tracer is now being validated in the unique mouse model of ApoA-II systemic amyloidosis that presents important heart involvement.

The discovery of molecules ubiquitously associated with the amyloid fibrils and active on the fibril metabolism provide important information for the assessment of *in vitro* model of fibrillogenesis that mimic the pathophysiological environment. Discovery of new molecules able to inhibit protein fibrillogenesis *in vitro* and *in vivo* represents a challenging demand for treatment of amyloid disease. Recent advances in the elucidation of the mechanism of amylodogenesis of β2 microglobulin (β2-m) are offering the unique opportunity to discover new anti-amyloidogenic compounds.

Small polycyclic molecules like tetracyclines and high affinity antibodies raised against the amyloidogenic protein precursor represent two prototypic classes of interactors for potential pharmaceutical exploitation. Two methods of fibrillogenesis and one cell toxicity test have been used for the identification of active compounds. From a series of ten analogues of tetracyclines we have singled out the two best anti-amyloidogenic compounds (doxycycline and 4 epi-oxytetracycline), able to inhibit the fibrillogenesis of β2-m with an IC50 of approximately 50 micromolar and capable of solubilising preformed fibrils at an IC50 of 250 micromolar. Both compounds abrogate the cytotoxicity of oligomeric β2-m. While tetracyclines exert their activity through a low affinity interaction with the amyloidogenic conformers, the second class of compounds we have tested (monovalent nanobodies) displays an anti-amyloidogenic capacity through a nanomolar affinity against β2-m and its highly amyloidogenic species. For this class of compounds we have been able to identify how the contact of the nanobody with specific regions of β2-m can switch off its amyloidogenic propensity. Comparative analyses of the effectiveness of these molecules in the inhibition or disorganization of amyloid fibrils are essential preliminary steps towards the identification of therapeutic strategies in patients affected by dialysis related amyloidosis.

Assessment of new models of the disease

For diseases of high complexity such as systemic amyloidosis, animal models mimicking the pathology are essential tools for the elucidation of pathogenesis and for testing new anti-amyloidogenic drugs. The severity of the disease is highly dependent from the tissue

localization of the deposits and heart involvement is certainly the event that mostly affects prognosis and survival.

Therefore the availability of an animal model with significant heart involvement is extremely important. We have investigated at the hystogical, molecular, and functional level the mouse model of ApoA-II systemic amyloidosis and we have demonstrated that heavy amyloid infiltration in the heart occurs in the aged animal carrying the ApoA-IIb allele. The amyloid in the heart follows the earlier deposition of amyloid in spleen, liver and kidney. The kinetics deposition of the amyloid in the interstitial space of the heart is extremely slow in the first year, but progresses quickly thereafter, and 90% of the animals older than 16 months display an heavy amyloid infiltration in the heart. The echocardiographic scans confirm the histopathology and structural parameters such as septum size, ventricular mass and size of the left ventricular wall are consistent with the level of amyloid infiltration.

However, the functional parameters such as the ejection fraction do not strictly correlate with the structural/histological findings. This discrepancy is perfectly consistent with the clinical observation of patients with cardiac amyloidosis, where the amount of amyloid deposit does not strictly correlate with the functional impairment. Therefore the pathology we observe in these mice represents a real model of the disease in humans and will become an excellent system for studying physiopathology of amyloid in the heart and the response to new drugs.

Papers published within the project

Bellotti V, Nuvolone M, Giorgetti S, Obici L, Palladini G, Russo P, Lavatelli F, Perfetti V, Merlini G. The workings of the amyloid diseases. *Ann. Med* 2007;39(3):200-7.

Esposito G, Ricagno S, Corazza A, Rennella E, Gümral D, Mimmi MC, Betto E, Pucillo CE, Fogolari F, Viglino P, Raimondi S, Giorgetti S, Bolognesi B, Merlini G, Stoppini M, Bolognesi M, Bellotti V. The controlling roles of Trp60 and Trp95 in beta2-microglobulin function, folding and amyloid aggregation properties. *J Mol Biol* 2008;378(4):885-95.

Giorgetti S, Stoppini M, Tennent GA, Relini A, Marchese L, Raimondi S, Monti M, Marini S, Østergaard O, Heegaard NH, Pucci P, Esposito G, Merlini G, Bellotti V. Lysine 58-cleaved beta2-microglobulin is not detectable by 2D electrophoresis in *ex vivo* amyloid fibrils of two patients affected by dialysis-related amyloidosis. *Protein Sci* 2007; 16(2):343-9.

Lavatelli F, Perlman DH, Spencer B, Prokaeva T, McComb ME, Théberge R, Connors LH, Bellotti V, Seldin DC, Merlini G, Skinner M, Costello CE Amyloidogenic and associated proteins in systemic amyloidosis proteome of adipose tissue. *Mol Cell Proteomics* 2008;7(8):1570-83.

Montagna G, Raimondi S, Moro G, Uggetti C, Relini A, Magrini U, Esposito C, Giorgetti S, Congi L, Mosconi M, Galli G, Villa G, Segagni S, Casadei S, Obici L, Merlini G, Stoppini M, Bellotti V. Clinical, radiological and biochemical features of a bilateral buttock amyloidoma emerging after 27 years of hemodialysis. *Amyloid* 2009, Jul 15:1-7. [Epub ahead of print]

Palladini G, Russo P, Bosoni T, Verga L, Sarais G, Lavatelli F, Nuvolone M, Obici L, Casarini S, Donadei S, Albertini R, Righetti G, Marini M, Graziani MS, Melzi D'Eril GV, Moratti R, Merlini G. Identification of amyloidogenic light chains requires the combination of serum-free light chain assay with immunofixation of serum and urine. *Clin Chem* 2009;55(3):499-504.

Relini A, De Stefano S, Torrassa S, Cavalleri O, Rolandi R, Gliozzi A, Giorgetti S, Raimondi S, Marchese L, Verga L, Rossi A, Stoppini M, Bellotti V. Heparin strongly enhances the formation of beta2-microglobulin amyloid fibrils in the presence of type I collagen. *J Biol Chem* 2008;283(8):4912-20.

Stoppini M, Obici L, Lavatelli F, Giorgetti S, Marchese L, Moratti R, Bellotti V, Merlini G. Proteomics in protein misfolding diseases. *Clin Chem Lab Med* 2009;47(6):627-35.

INHERITED EPITHELIAL ADHESION DISEASES: DEVELOPMENT AND VALIDATION OF DIAGNOSTIC TOOLS AND GUIDELINES, AND ASSESSMENT OF QUALITY OF LIFE

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The project has dealt with the molecular diagnostics and genotype/phenotype correlations, definition of diagnostic guidelines and assessment of quality of life in Epidermolysis Bullosa (EB), a clinically and genetically heterogeneous group of rare blistering disorders due to fragility of skin and mucous membranes. Classically, three major EB types are defined based on the level of blister formation within the skin: EB Simplex (EBS), Junctional EB (JEB), and Dystrophic EB (DEB). A recently revised classification includes an additional major EB type, Kindler Syndrome (KS), which is also associated with mechanical fragility of the skin and characterized by an intriguing complex phenotype, comprising sun sensitivity and progressive development of skin poikiloderma and atrophy.

Seventy-eight EB cases, 43 of whom affected with DEB, 16 with JEB, 14 with EBS and 5 with Kindler syndrome were studied during the project. Severe EB subtypes and unusual phenotypes were also analyzed by mutational screening.

Mutation profile was established for the LAMA3, LAMB3, LAMC2, COL7A1 and FERMT1/KIND1 genes causing JEB with laminin-332 deficiency (LAMA3, LAMB3, LAMC2), DEB (COL7A1) and KS (FERMT1/KIND1), respectively. Mutational screening was performed by either direct sequencing or DHPLC scanning of genomic PCR fragments. These approaches allowed the definition of a constellation of recurrent (detected in more than 3 unrelated families) and private (family-specific) mutations affecting Italian EB families. Mutational profile comprised Alu/Alu recombination, splice site, nonsense, missense, silent and frameshift mutations, and the large majority of recurrent changes were due to propagation of ancestral alleles. An efficient screening strategy tailored to the Italian population was set up for the FERMT1 gene. With this approach, pathogenic mutations were identified in the entire cohort of Italian KS patients studied. Molecular testing of all lethal Herlitz JEB (HJEB) cases referred to the Italian Registry of hereditary EB, together with disease incidence data, allowed us to calculate the population carrier risk for HJEB, which is one in 375.

We also developed a novel approach for the prenatal diagnosis in kindred at risk for EB with pyloric atresia (EB-PA), which is caused by null mutations in either the α6β4 integrin genes or the plectin gene. The procedure was based on the original observation that chorionic villi express both integrin α6β4 and plectin. This finding was exploited to implement 25 prenatal diagnoses in kindred at risk for EB-PA by immunofluorescence analysis for expression of these proteins on chorionic villi frozen sections. Such analysis identified three EB-PA-affected

fetuses and 22 healthy ones. In 19 cases, including the three EB-PA pregnancies that were prematurely terminated, the results were confirmed by chorionic villous DNA-based tests. Our prediction was further sustained by the birth of 22 healthy babies. These results validate chorionic villi immunofluorescence examination as a novel tool for early prenatal diagnosis of EB-PA in kindred carrying unidentified genetic mutations.

With the aim to delineate new determinants of clinical severity in EB, we then characterized monozygotic adult male twins affected with recessive DEB (RDEB) and presenting markedly different phenotypic manifestations. We demonstrated that their phenotypic variations do not correlate with type VII collagen synthesis and matrix deposition. As monozygotic twins share a common genotype, phenotypic discordance in this model hints at a different epigenetic profile that imposes different gene expression patterns. Therefore, a microarrays analysis of the transcriptome expressed in the twins' fibroblasts was performed using the Human HG-U133A array GeneChip platform (Affimetrix). Data analysis confirmed the presence of genes differentially expressed between the two twins. When comparing the less affected twin to the more affected one, 110 genes were up-regulated and 113 down-regulated. The large majority of the differentially expressed genes showed changes less than 6 folds, but differences as high as 60 folds were measured. Modulated genes of interest code for protein components of the extracellular matrix, for molecules participating in matrix assembly and remodeling or related to cell signaling pathways involved in differentiation and development. A selection of identified genes has been validated by real time RT-PCR assays on total RNA from monozygotic twin fibroblasts, confirming the differences observed by hybridization on chips. These results support the notion that in RDEB individuals with identical genetic background, epigenetics can have an impact on clinical outcomes, by affecting the global transcriptome.

A multidisciplinary, multispecialty task force of experts, comprising dermatologists, pediatricians, geneticists, molecular biologists, ethicists and a representative of patients' association (DebRA), was convened in July 2007 under the coordination of the National Centre for Rare Diseases (Director Dr. D. Taruscio) with the aim to develop and validate national guidelines for EB diagnosis. The task force agreed to develop consensus statements in stages using a modified Delphi methodology, and in June 2009, elaborated final consensus documents (Delphi2) on specific topics (classification, molecular genetic analysis, genetic counseling, differential diagnosis, etc.). The final document is available at http://www.iss.it/site/cnmr/privato/lgebe/SD_01.html website.

Quality of Life (QoL) was assessed in 125 patients with EB.11 The following questionnaires were sent by mail: the Medical Outcome Study 36-item short form health survey (SF-36, employed to assess the general health), the Skindex-29 (to evaluate the impact of dermatology-specific aspects), the 12-item General Health Questionnaire (GHQ-12, to detect patients with psychological problems), and the EuroQoL 5 dimensions (EQ-5D, to measure health status both in adults and children). The Family Strain Questionnaire (FSQ) was addressed to patients' caregivers, in order to assess the burden of the disease on them. Patients with EB showed lower values in physical components of the SF-36 compared to the normative score for the general Italian population, while the mental components were not significantly impaired. Among EB types, patients with JEB and severe generalized recessive DEB reported lower scores for the SF-36 physical components and their GHQ-12 scores were significantly different from those of patients with EB simplex. There were no significant differences among EB types/subtypes for Skindex-29 values, however, the highest values (i.e. worst QoL) were consistently observed in patients with JEB. Women had a worse QoL compared with men in all Skindex-29 and SF-36 scales (P < 0.05). GHQ-positive cases were more frequent among women (48%) compared with men (16%) (P = 0.003); GHQ-positive cases had a worse QoL compared with GHQ-negative patients. The EQ-5D showed different median values in adults and children. A different

prevalence of problem presence was reported for "mobility" (33% *vs* 59%), "self care" (29% *vs* 82%), "usual activities" (52% *vs* 76%), "pain discomfort" (76% *vs* 82%), and "anxiety/depression" (52% *vs* 41%). The family burden increased with increasing patient's perceived disease severity and increasing patient's body surface involved, on all the scales and especially in the "emotional burden", "problems in social involvement" and "thoughts about death" scales.

Family burden was greater when the patients had probable depression or anxiety (GHQ-positive cases), for patients with a worse health status, as measured by the SF-36, and in patients whose Skindex-29 values were above the median value used as cut-off. No differences were seen among the different EB types. The impact of EB on QoL is more severe than that of other dermatological conditions, and imposes a heavy burden on the caregivers. Psychological support and close monitoring with QoL measurements may be of help to patients and their care providers. Moreover, such information could also help health care policy makers in the allocation of funds, when health care resources are scarce, while rare and severe diseases are more frequently and easily diagnosed.

Papers published within the project

Castiglia D, Zambruno G. Epidermolysis bullosa care in Italy. *Dermatol Clin* 2009 (in press).

Castiglia D, Zambruno G. Molecular testing in epidermolysis bullosa. *Dermatol Clin* 2009 (in press).

Castiglia D, Zambruno G. Mutation mechanisms in epidermolysis bullosa. *Dermatol Clin* 2009,28(1):17-22.

Castori M, Floriddia G, De Luca N, Pascucci M, Ghirri P, Boccaletti V, El Hachem M, Zambruno G, Castiglia D. Herlitz junctional epidermolysis bullosa: laminin-5 mutational profile and carrier frequency in the Italian population. *Br J Dermatol* 2008;158(1):38-44.

Castori M, Floriddia G, Pisaneschi E, Covaciu C, Paradisi M, Torrente I, Castiglia D. Complete maternal isodisomy causing reduction to homozygosity for a novel LAMB3 mutation in Herlitz junctional epidermolysis bullosa. *J Dermatol Sci* 2008;51(1):58-61.

D'Alessio M, Zambruno G, Charlesworth A, Lacour JP, Meneguzzi G. Immunofluorescence analysis of villous trophoblasts: a new tool for prenatal diagnosis of inherited epidermolysis bullosa with pyloric atresia. *J Invest Dermatol* 2008;128(12):2815-9.

Drera B, Floriddia G, Forzano F, Barlati S, Zambruno G, Colombi M, Castiglia D. Branch point and donor splice-site COL7A1 mutations in mild recessive dystrophic epidermolysis bullosa. *Br J Dermatol* 2009; Epub Mar 26.

Eady RA, Bauer EA, Bauer JW, Bruckner-Tuderman L, Heagerty A, Hintner H, Hovnanian A, Jonkman MF, Leigh I, McGrath JA, Mellerio JE, Murrell DF, Shimizu H, Uitto J, Vahlquist A, Woodley D, Zambruno G. The classification of inherited epidermolysis bullosa (EB): Report of the Third International Consensus Meeting on Diagnosis and Classification of EB. *J Am Acad Dermatol* 2008;58(6):931-50.

Kaçar N, Semerci N, Ergin S, Pascucci M, Zambruno G, Castiglia D. A novel frameshift mutation in the KIND1 gene in Turkish siblings with Kindler syndrome. *Br J Dermatol* 2008;158(6):1375-7.

Ouragini H, Cherif F, Kassar S, Floriddia G, Pascucci M, Daoud W, Osman-Dhahri AB, Boubaker S, Castiglia D, Abdelhak S. Dystrophic epidermolysis bullosa phenotypes in a large consanguineous Tunisian family. *J Dermatol Sci* 2009;54(2):114-20.

Tabolli S, Sampogna F, Di Pietro C, Paradisi A, Uras C, Zotti P, Castiglia D, Zambruno G, Abeni D. Quality of life in patients with epidermolysis bullosa. *Br J Dermatol* 2009 (in press).

BIOCHEMICAL AND CELLULAR REAL-TIME BIOMARKERS OF DIAGNOSTIC AND PROGNOSTIC VALUE IN THE MANAGEMENT OF KAWASAKI'S DISEASE

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Introduction

Three different points have been analyzed as valuable biomarkers of diagnosis and progression in blood samples from patients with Kawasaki's Disease (KD): oxidative stress, blood cell integrity and function and immunological profile.

Persistent arterial dysfunction in patients with a history of KD and an integral role of oxidative stress in the development of cardiovascular disease are increasingly recognized. We sought to test the hypothesis that oxidative stress is increased in KD patients by evaluating different possible plasmatic and cellular biomarkers. In addition, platelets are increased in their number in KD and their alterations have been suggested to exert a pathogenetic role.

Moreover, it has been reported that the dysregulation of immune system plays a critical role in the pathogenesis of KD. Therefore, we evaluate the phenotype and the functional competence of peripheral blood mononuclear cells (PBMC) from KD patients to establish the relationship between the functional markers and disease progression.

Methods

We measured by Electron Paramagnetic Resonance spin trapping with 1-hydroxy-3-carboxy-2,2,5,5-tetramethylpirrolydine (CPH) the production of free radicals in whole blood of KD patients compared to control health subjects. As concerns blood cells, erythrocytes and platelets were analyzed by static and flow cytometry. Regarding erythrocytes, redox imbalance and expression of proteins (glycophorin A and CD47) involved in cell aging and death were evaluated. Regarding platelets, molecules involved in cell adhesion (CD47) and death (phosphatidylserine externalization) have been evaluated.

PBMC obtained from KD patients at different disease onset as well as before and after therapy were isolated by Ficoll-Hypaque gradient separation. The expression of activation markers such as CD25 and CD86 on T, B and natural Killer (NK) cells were evaluated by flow cytometry. Antibody and cytokine production were measured by ELISPOT assay.

Results

Compared with controls, patients with KD had significantly higher rate of free radical production as demonstrated by the increase (+86% p<0.001) of the rate of CP• radical formation. The rate of CP• formation further increased about 4-5 times after addition of the transition-metal chelator EDTA, leading to the hypothesis that the increased radical formation may be mediated by catalytically active Iron. After therapy (aspirin 2mg/kg/die) the rate of CP• formation was decreased, but still significantly higher than controls (+30% p<0.001). As concerns erythrocytes, a decreased expression of glycophorin A and CD47 has been detected in KD patients with respect to healthy donors. For platelets, main finding deals with their increased aggregability and, more importantly, the increased Phosphatydilserine (PS) externalization without further sign of cell death (e.g. no caspase activation).

We found that NK cells of KD patients in the acute stage before treatment were activated as assessed by the increased number of CD56+ cells expressing CD25 (IL-2Rα) molecule. In the convalescent stage of KD subjects the number of CD56+/CD25+ is comparable to age-matched controls. We found that the absolute number of CD19+ B cells significantly increased in acute stage of KD patients respect age-matched controls, while it decreased during the convalescent stage of KD subjects. The increase of the absolute number of CD19+ B cells was not associated to an increase of CD86 activation marker expression both in acute and in convalescent stage of KD patients. Moreover, we found that after CpG-ODN polyclonal stimulation of CD19+ B cells, the number of Ig-secerning cells (Ig-SC) and IL-6 production were significantly decreased both in acute and in convalescent stage of KD patients. As concerns cytokine production, we found a drastic increase of the number of IL-17 SC during the acute phase of KD patients respect to age-matched controls, while it decreased during the convalescent stage of KD subjects.

Conclusions

Our findings suggest: i) oxidative stress is increased in KD patients at the onset of the disease. We suggest the presence of an inflammatory condition perhaps mediated by Iron overload and/or decompartmentalization; ii) the increased platelet counts in these patients could be due to a defective death pathway whereas PS externalization could be associated with an increased vascular risk; iii) the dysregulation of immune responses could be a consequence of an inflammatory state and of a down-regulation of B cell functional competence.

Altogether these findings suggest that further studies should be performed in KD blood samples in order to better assess these hypotheses that could help in the clinical management of this disease.

Papers published within the project

Matarrese P, Straface E, Palumbo G, Anselmi M, Gambardella L, Ascione B, Del Principe D, Malorni W. Mitochondria regulate platelet metamorphosis induced by opsonized zymosan A--activation and long-term commitment to cell death. *FEBS J* 2009;276(3):845-56.

GENETIC ANALYSIS OF ARRHYTHMOGENIC INHERITED DISEASES

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Familial arrhythmogenic diseases, such as Brugada Syndrome (BS), are cardiac disorders characterized by electrical ventricular instability that can lead to sudden death at young age. Diagnosis is based on a typical ECG sign: ST segment elevation in the right precordial leads. Since no anti-arrhythmic drug is effective in preventing from life-threatening arrhythmias, the only available treatment is Implantation of a Cardioverter Defibrillator (ICD). Actual guidelines select higher risk patients based on presence of syncope, family history for sudden death and spontaneous ECG pattern, however, the predisposition of these patients to develop ventricular arrhythmias cannot be predicted and the usefulness of programmed electrical stimulation is debated. New criteria for risk stratification of these patients are therefore needed. Genetic bases have been only partially understood, with six genes covering less than 30% of BS cases identified. According to the emerging concept of "arrhythmia genomics", the co-segregation of different variants and polymorphisms may modulate the presentation of the clinical phenotype. Our main goal is therefore to develop a molecular analysis approach of genes associated with arrhythmogenic syndromes both to provide a diagnostic tool for affected patients and their families and to correlate the genotype with the clinical phenotype, in order to improve risk stratification for ICD implant and the management of asymptomatic patients.

In the past three years, thanks also to the support of ISS, we enrolled 85 BS patients, who were carefully characterized from the clinical and electrophysiological point of view: collection of medical and family history; 24h Holter-ECG to characterize possible rhythm disturbances and to evaluate Heart Rate Variability; echocardiogram (ECHO) to verify the absence of structural defects; heart magnetic resonance imaging (MRI) to assess the composition of the cardiac muscle and the occurrence of fibrofatty substitution or trabeculation; exercise test to assess changes in ECG at increased heart rate. In addition, provocative pharmacological test based on flecainide injection and endocavitary electrophysiological study (EPS) were performed to confirm clinical diagnosis and assess cardiac electric vulnerability. Therapeutic options and cardioverter defibrillator (ICD) implantation were evaluated according to currently available guidelines. Patients were followed every six months by routine examination (clinical assessment, ECG, 24h Holter-ECG, ECHO). ICD registrations in implanted patients were also monitored to evaluate the occurrence of arrhythmic events.

Patients underwent molecular analysis of the SCN5A gene, leading to the identification of 16 mutations (in 19% of patients), nine of which were novel: 13 missense and 3 frameshifts causing an early truncation of the putative protein. The mutations are evenly distributed along the gene. This enabled us to extend the screening to first-degree relatives and diagnose BS in 16 asymptomatic relatives who may be at risk of sudden death and would not have been recognized otherwise. All relevant clinical and genetic data were collected in an electronic database shared among the participants to the project and managing a significant number of data for each

patient, including follow-up. Preliminary data analysis suggests that SCN5A-positive patients are more likely to experience arrhythmic events. Further statistical elaboration is currently underway to identify other genotype/phenotype correlations and risk stratification criteria. Our observations will have to be confirmed on a larger cohort of patients and during a longer follow up period.

In vitro functional studies were performed to evaluate variations in Na current associated with some SCN5A mutants. Despite the low prevalence of the disease among Caucasian subjects we identified two heterozygous SCN5A mutations on separate alleles in an occasionally-diagnosed asymptomatic child.

The variants were inherited from the asymptomatic parents: a new deletion causing frameshift and premature truncation of the putative protein and a missense variation. The effects of the two mutations on the Na channel function were evaluated by electrophysiology studies in transfected NIH-3T3 and HEK-293 cells.

Whole-cell sodium current density was significantly reduced by 60% in the deletion mutant, 40% in the missense, and an additive effect of the two mutations was observed. In addition, the missense mutation was found to rightward shift the voltage-dependency of the steady-state inactivation. Despite the mutations compromised the functionality of both alleles, Brugada-like ECG was observed only in the compound heterozygous child and not in his parents.

In vitro characterization was performed also for the SCN5A heterozygous missense mutation S216L identified in a 10-years old boy symptomatic for episodes of tachycardia displaying at ECG right bundle branch block with ST segment elevation compatible with BS. Subsequent analysis of the asymptomatic relatives detected the same mutation in the mother, displaying a normal ECG with no signs of ST elevation, even after flecainide provocative test. In a prior study S216L mutation had been identified in a case of sudden infant death syndrome and associated with a gain of function of the Na channel compatible with Long QT Syndrome (LQTS) and not BS, but no signs of LQTS were evident in our patients. To understand how the S216L mutation may result in the BS phenotype observed in our proband, we performed an electrophysiological evaluation of the mutant channel expressed in HEK293 cells. Our preliminary data confirm a small enhancement of persistent Na+ current, apparently inadequate to account for the clinical phenotype. Analysis of gating under non-equilibrium conditions and in silico modelling is currently in progress to detect kinetic abnormalities suitable to account for the BS phenotype.

These observations underline the difficulty of genotype/phenotype correlations in Brugada syndrome patients and support the idea of a complex disorder, where different mutations and variants can contribute to the clinical phenotype. The role of genetics is for the moment restricted to the identification of pre-symptomatic mutation carriers. Although this role is pivotal, allowing to diagnose BS in 16 hidden, but potentially at risk, relatives, further studies are still required to include genetic data in risk stratification guidelines. To expand genetic characterization of our BS patients, we therefore set up the molecular analysis of other three genes associated with arrhythmogenic syndromes: GPD1L, KCNQ1 and KCNH2.

The analysis of BS patients to identify possible causing mutations and modifier variants in these genes is actually ongoing. In addition, we set up a panel of candidate genes, including genes implied in other arrhythmogenic syndromes and genes selected on the basis of function reported in the literature, in order to identify variants with possible pathogenetic relevance. BS patients will be screened with high throuput simultaneous sequencing based on 454 pyrosequencing preceded by sequence capture microarray in collaboration with ITB-CNR. Preliminary studies confirm the feasibility of this approach.

This will lead to identification of new BS causing genes and putative modifier alleles segregating with the affected phenotypes. Genetic data obtained by high throughput sequencing

will be then correlated to the clinical outcome. This will allow to identify variants associated with a worse phenotype and their effectiveness as predictors of major arrhythmic events during patient follow-up.

These studies will allow to evaluate the contribution of genetic variants to the expression of the pathological phenotype and the predisposition to malignant arrhythmias. Our project will therefore allow to establish new genotype/ phenotype correlations. This information will increase diagnostic power, improving familial counselling and knowledge about the pathogenetic mechanisms, thus ameliorating therapeutic options and clinical management. The strict interaction between geneticists and clinicians will facilitate the implementation of existing guidelines for ICD implant and the follow-up of asymptomatic patients.

DEVELOPMENT OF AN EPIDEMIOLOGICAL AND MOLECULAR INTEGRATED APPROACH FOR THE PREVENTION OF CONGENITAL HYPOTHYROIDISM

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Congenital Hypothyroidism (CH) is a multifactorial disease in which genetic and environmental risk factors contribute to its aetiology. It represents the most frequent endocrinopathy in infancy (1:2500 live borns in Italy) and the most common cause of preventable mental retardation. In fact, an early treatment with the replacement therapy (L-thyroxine) prevents severe neuropsychological sequelae due to thyroid deficit in the neonatal period. CH is considered a rare disease given a prevalence of 3.5 cases/10,000 citizens in Italy. By measuring thyroid hormone and Thyroid Stimulating Hormone (TSH) in all babies shortly after birth, newborn screening programs are able to identify biochemically infants who may have CH even before there are any signs or symptoms of hypothyroidism. The majority of babies with CH are characterized by dysgenesis of the thyroid (agenetic, ectopic or hypoplastic gland) while inherited defects of thyroid hormone biosynthesis, secretion and utilization represent a minor fraction of all cases. It has been also demonstrated a high risk of extrathyroidal congenital malformations associated with CH. As we previously demonstrated, this risk is particularly high for malformations concerning heart, neural tube, eye and multiple malformations. This suggests that a very precocious molecular defect may occur during embryo development to explain the association between CH and malformations in organs that, like the thyroid, represent precocious structures in the developing embryo.

At present the availability of effective screening procedures, the efficient network composed of the 26 Screening Centres for CH active in Italy and the surveillance of the disease performed by the Italian National Register of Infants with CH (INRICH), have allowed an efficient secondary prevention for CH. This represents one of the most important results in the field of preventive medicine in our country and can be a model of intervention for other rare diseases. However, improvements in primary and tertiary prevention of CH should be made. In fact, as the occurrence of mutations in genes known to be involved in the development of the disease have been observed only in a small proportion of the patients and the role of specific environmental risk factors has not yet completely elucidated, the aetiology of CH is still largely unknown. Moreover, although early diagnosis and treatment of CH has led to the disappearance of severe mental retardation, it has been clearly recognised that persistent selective impairments may still occur in these children such as language delay, minor motor problems, visuospatial

defects, and attention problems. The understanding of why some children with CH show development delay is still a controversial issue.

The main objectives of this project were: 1) to use the almost 30-year Italian experience of screening for CH to evaluate the possible impact of "extended newborn screening program" in the Liguria Region; 2) to investigate possible spatial variations in CH risk (spatial clusters) and to verify a possible overlapping of spatial clusters of CH with spatial clusters of specific congenital malformations in our country; 3) to study the molecular basis of CH with eutopic gland and thyroid disgenesis; 4) to identify possible risk factors (familial, maternal, neonatal, environmental) associated with a poor neuropsychological outcome in CH children diagnosed by screening.

Some important preliminary results have been obtained. Specifically, for what concerns the first aim of the project data of the extended newborn screening program collected in Liguria Region between June 2005 and May 2008 were analyzed. Six positive cases were identified (argininosuccinate lyase deficiency 1, organic acidemia 2, fatty acid oxidation disorders 3) and an incidence of 1: 6098 live borns was estimated in this Region.

As regards the second aim of the project, a spatial analysis of CH incidence was conducted per municipalities on the data collected by the INRICH between 1995 and 2003. The presence of hot spots with high CH incidence (>1: 1000 live borns) were found spread all over the country suggesting the important role of environmental risk factors in the aetiology of CH. A Bayesian analysis conducted on the same data confirmed the presence of spatial cluster for CH in our country. Moreover, in collaboration with the Italian National Register of Rare Diseases, to which several regional Registries of Congenital Malformations contribute, an overlapping of spatial clusters of CH with spatial clusters of congenital malformations has been verified. This approach can help to identify possible common environmental risk factors. Specifically, data of the Regional Register of Congenital Malformations of the Toscana Region and the Regional Register of Congenital Malformations of the Emilia Romagna Region were analyzed. A specific clustering analysis (Kulldorff's Spatial Scan Statistic) showed an overlapping of a CH cluster with a cluster of congenital malformations with a significant excess of congenital heart defects (234 observed *vs* 175 expected cases, p=0.007) in the Toscana Region. Also in Emilia Romagna Region an overlapping of a CH cluster with a cluster of congenital malformations, with an excess of cleft palat (12 observed *vs* 1.48 expected cases, p=0.006) and malformation of Central Nervous System (10 observed *vs* 1.8 expected cases, p>0.05), was observed.

For what concerns the third aim of the project, studies aimed at characterizing the molecular basis of CH were performed on children with CH and eutopic thyroid and in a polygenic mouse model for CH with thyroid dysgenesis. As concerns CH with eutopic thyroid, 21 unrelated children with this form of CH and a Partial Iodide Organification Defect (PIOD) were studied. In these patients genes coding for dual oxidases (DUOX1 and DUOX2) and DUOX maturation factors (DUOXA1 and DUOXA2) were screened and for the first time a DUOXA2 defect in a Chinese girl with CH, PIOD and mild permanent hypothyroidism was found. Moreover, the analysis of the coding-region of DUOX2 gene in further 10 CH children with eutopic thyroid, identified 6 new mutations involving exon 22, exon 17, exon 23, exon 24, and exon 21. Finally, for what concerns molecular basis of thyroid disgenesis, 143 DHTP/bc mice were genotyped using 235 Single Nucleotide Polymorphisms (SNPs). Analysis of genotyping data revealed two chromosomal regions associated to CH: one on Chr 2 (with a LOD score of 11.2) and another on Chr 5 (with a LOD score of 2.5). 800 genes have been mapped on region of Chr 2 associated to the CH phenotype; about 400 of these are expressed in the thyroid. By using SNPs analysis 2 new candidate genes have been identified: calpain 3 and Dnajc17.

About the fourth aim of the project, we planned to assess the behavioural and neuropsychological consequence of CH in a sample of Italian children with a confirmed

diagnosis of CH and to compare the results with a sample of control children (without CH) born in the same week and hospital of cases. The enrolment of cases and controls eligible for this study was already carried out between 1997 and 2000 with the aim of identifying the most important risk factors for permanent and transient CH. Therefore baseline information of each child are already available and will be used for the identification of the most important risk factors (familial, maternal, neonatal and environmental) involved in auxological and neuropsychological outcomes in infants with congenital hypothyroidism. To this end we started recalling the children who were recruited between 1997 and 2000 and in all of them neuropsychological outcome will be evaluated by standardized tests (intellective abilities test, linguistic functions tests, learning abilities tests, attention and executive functions). The influence of blood TSH concentration at the time of screening, age at the start of thyroxine replacement therapy, severity of thyroid deficit and socio-cultural level of family, will be investigated.

In conclusion, the results obtained with this project allowed us: 1) to perform a positive cost-benefit evaluation of the "expanded newborn screening program" in the Liguria Region and an epidemiological evaluation of about 30 metabolic rare diseases in this Region, giving a model for the other Italian Regions; 2) to identify spatial clusters of CH in our country and to demonstrate an overlapping of spatial clusters of CH with spatial clusters of congenital malformations, suggesting possible common environmental risk factors such as iodine deficiency and/or endocrine disruptors exposure; 3) to demonstrate that more genes than those already known are involved in the aetiology of CH with both disgenesis and in situ thyroid. On the basis of the above mentioned results, we are confident that the findings deriving from this project will contribute to improve nationwide prevention strategies for CH and that the multidisciplinary approach to the disease that has been proposed here can represent a model for other rare diseases.

Papers published within the project

Muzza M, Persani L, de Filippis T, Gastaldi R, Vigone MC, Sala D, Weber G, Lorini R, Beck-Peccoz P, Fugazzola L. Absence of sonic hedgehog (Shh) germline mutations in patients with thyroid dysgenesis. *Clin Endocrinol* 2008;68:828-9.

Olivieri A and The Study Group for Congenital Hypothyroidism. The Italian National Register of Infants with Congenital Hypothyroidism: twenty years of surveillance and study of congenital hypothyroidism. *I J Ped* 2009;35:2

Olivieri A, Medda E, De Angelis S, Valensise H, De Felice M, C. Fazzini, I. Cascino, V. Cordeddu, M. Sorcini, M.A. Stazi, and The Study Group for Congenital Hypothyroidism. High risk of congenital hypothyroidism in multiple pregnancies. *J Clin Endocrinol Metab* 2007;92(8):3141-7.

Zamproni I, Grasberger H, Cortinovis F, Vigone MC, Chiumello G, Mora S, Onigata K, Fugazzola L, Refetoff S, Persani L, Weber G. Biallelic Inactivation of the Dual Oxidase Maturation Factor 2 (DUOXA2) Gene as a Novel Cause of Congenital Hypothyroidism. *J Clin Endocrinol Metab* 2008,93: 605-10.

INFANT BOTULISM

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Infant Botulism (IB) is a rare disease that results from absorption of Botulinum NeuroToxins (BoNTs) produced in situ by BoNT-producing clostridia colonizing the intestinal lumen of infants aged less than one year. Less than 3500 cases were worldwide recognized from 1976 to date. Recognition depends on awareness of IB by front-line pediatricians and specialists. An early clinical suspicion is essential for prompt diagnosis, to a rapid treatment and to avoid unuseful and unadvisable therapies.

The diagnosis is made on clinical grounds and confirmed in laboratory. Standard laboratory method includes the mouse bioassay and may require several days for definitive response.

Meticulous supportive care and antitoxin administration, only for serious cases, represent the meanly measures for management. Equine antitoxin used for adult patients, theoretically, has limited role mainly because of the short-life and the side effects. A specifically human-derived antitoxin (BIG-IV) is produced and distributed by the California Department if Public Health (CDPH) in collaboration with the FDA and CDC. Treatment of patients with BIG-IV reduces, the length of the hospital stay and intensive care unit stay, the duration of mechanical ventilation and tube or intravenuos feeding.

Although Italy report the high number of cases of IB in Europe, and represent the fourth country in the world for IB cases after USA, Argentina, Australia, the illness results still unknown by parents, physicians, and hospital workers. To study in depth the different aspects of IB and to improve medical knowledge of the disease, a research project has been developed by National Reference Centre for Botulism (NRCB) in collaboration with Pavia Poison Centre of IRCCS Foundation Salvatore Maugeri of Pavia and in close collaboration with IBTPP (Infant Botulism Treatment and Prevention Program, California).

The main objectives of this project were: to improve medical knowledge of the illness through a specific educational program for physicians; to create a 24 hours/day Reference Centre for Clinical Diagnosis and Treatment (RCCDT) of IB; to create a specific database for collecting all clinical data (history, clinical effects) regarding suspected and/or confirmed cases; to develop rapid molecular methods for detecting BoNT-producing clostridia in clinical specimens to obtain a rapid diagnosis of the disease; to disseminate collected data on clinical and microbiological features of the cases through a web-site, reports and peer reviewed papers, meetings, workshops.

These entire objectives were successfully met within the project timeframe.

To evaluate the knowledge of the illness among medical doctors, simulated IB case and a questionnaire were submitted to 116 physicians attending to 6 scientific events. Only 6% of interviewed physicians considered IB as first diagnosis. To improve medical knowledge, an *ad hoc* brochure has been distributed to the members of 16 Scientific Societies. Moreover, some scientific courses involving a total of 276 physicians have been realized. This research project has been presented and discussed during several scientific events involving more than 3000 scientists. Several abstracts, full papers, press articles have been published to disseminate information on infant botulism.

Nearby NRCB, that offers an expert advice available 24 hours a day every day, the RCCDT has been started to support physicians for early clinical diagnosis, therapeutic approach and to guide the antidotal treatment by Pavia Poison Centre.

All epidemiological, clinical and microbiological data on IB cases occurred in Italy from 1984 to date, have been collected and inserted in an electronically, password protected data-base that will be available (only to authorized personnel) in IB-web site.

About rapid molecular diagnostic methods, the extraction of nucleic acids method has been defined comparing different methods described in scientific literatures. Moreover a gel-based multiplex PCR, and a multiplex Real Time PCR protocol using Taqman®, LNA™ probes have been developed. These molecular methods have been in house-validated according ISO 16140 against the reference cultural method associated to the gold standard mouse bioassay. Developed methods have been successful utilized during the laboratory confirmation of botulism, particularly IB, cases occurred in Italy in the timeframe of the project.

A website on IB has been planed, providing separate sections for public and parent general informations, medical and technical consultation, global occurred epidemiology, and most considerable published literature. Links to National Center for Rare Diseases, IBTPP, and Pavia Poison Center will be inserted.

Papers published within the project

De Medici D, Anniballi F, Wyatt GM, Lindström M, Messelhäußer U, Aldus CF, Delibato E, Korkeala H, Peck MV, Fenicia L. Multiplex PCR to detect botulinum neurotoxin-producing clostridia in naturally contaminated samples. *Appl Environ Microbiol.* 2009;75(20):6457-61.

Fenicia L, Anniballi F, Aureli P. Human botulism in Italy, 1984-2007. In: *Proceeding of Clostridium botulinum – epidemiology, diagnosis, genetics, control and prevention.* Helsinki, Finland, June 16-19, 2008.

Fenicia L, Anniballi F, Di Giovangiulio M, De Medici D, Delibato E, Auricchio B. PFGE analysis of *Clostridium botulinum* and neurotoxigenic *Clostridium butyricum* isolated in Italy, and associated epidemiological findings – preliminary results. In: *Proceeding of: 45th IBRCC meeting.* Philadelphia, Pennsylvania, USA Sept. 14-18, 2008.

Fenicia L, Anniballi F. Infant Botulism. *Ann Ist Super Sanità* 2009;45:134-46.

Fenicia L, Di Pietro P. Il botulismo infantile. *Area Pediatrica* 2009;75(20):6457-61.

Lonati D, Locatelli C, Bigi S, Vecchio S, Petrolini V, Giampreti A, Fenicia L, De Medici D, Manzo L. An Italian network to ameliorate the diagnosis and treatment of Infant Botulism. *Emerg Care J* 2008;6.

Lonati D, Rossetto O, Fenicia L, Locatelli C. Botulism. In: Ballantyne B, Marrs TC, Syversen T (Ed.). *General and applied toxicology.* Vol. 6. Chapter 148. 3nd Ed. Chichester, UK: John Wiley & Sons, Ltd.; 2009. p. 3555-79.

SECTION 3
Treatment and clinical management

ELECTROPHYSIOLOGICAL AND PHARMACOLOGICAL ANALYSIS OF STRIATAL NEURONS FROM MICE EXPRESSING TORSIN A WITH THE DYT1 DYSTONIA MUTATION

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Dystonia is a rare neurological syndrome characterized by sustained, repetitive muscle contractions and abnormal postures, usually with a progressive course. Primary early-onset generalized torsion dystonia (Oppenheim's Dystonia) is an autosomal dominant disease linked to a GAG deletion in the DYT1 gene on chromosome 9q34, resulting in the loss of a glutamic acid residue near the carboxyl terminus of the protein torsinA. No definite evidence for neurodegeneration has been observed in neuropathological studies in DYT1 dystonia and it is thought that the protein mutation produces dystonia through an aberrant neuronal signalling within the basal ganglia. In particular, the symptoms of the disease are thought to arise through abnormalities in cholinergic and dopaminergic striatal signalling. In collaboration with David G. Standaert and colleagues at Harvard Medical School we established a colony of transgenic mice that express either human Mutant TorsinA (hMT) or human wild-type (hWT) torsinA, as an animal model of DYT1 dystonia. We performed electrophysiological recordings from striatal Medium Spiny Neurons (MSNs) from 2 month-old hWT and hMT transgenic mice. We first tested the intrinsic membrane properties of MSNs in hWT and hMT, in particular membrane resistance, resting membrane potential and current-voltage relationship. No significant difference was observed between the two groups of mice. To address possible changes in transmitter release probability, we recorded spontaneous GABA- and glutamate-mediated synaptic currents from MSNs. In from hMT mice we observed a significantly higher frequency, but not amplitude, of GABAergic spontaneous inhibitory postsynaptic currents (sIPSCs) and miniature currents (mIPSCs), whereas glutamatergic activity was normal. No alterations were found in hWT with respect to control mice. To identify the possible sources of the increased GABAergic tone, we recorded GABAergic Fast-Spiking (FS) interneurons that exert a feed-forward inhibition on MSNs. Both sEPSC and sIPSC recorded from hMT FS interneurons were comparable to hWT and controls. In physiological conditions, dopamine (DA) D2 receptor act presynaptically to reduce striatal GABA release. Notably, application of the D2-like receptor agonist quinpirole failed to reduce the frequency of sIPSCs in MSNs from hMT as compared to hWT and controls. Likewise, the inhibitory effect of quinpirole was lost on evoked IPSCs both in MSNs and FS interneurons from hMT mice. Our findings demonstrate a disinhibition of GABAergic synaptic activity, which can be partially attributed to a D2 DA receptor dysregulation. A rise in GABA transmission would result in a profound alteration of striatal output, which might be relevant to the pathogenesis of dystonia.

Clinically unaffected DYT1 gene carriers exhibit subtle abnormalities in motor behavior with impaired sequence learning. Corticostriatal synaptic plasticity is believed to play a central role in motor learning. We investigated possible changes in synaptic plasticity in either hWT or hMT transgenic mice. High-Frequency Stimulation (HFS) induced a Long-Term Depression

(LTD) in MSNs recorded from control and hWT mice, but failed to cause LTD in hMT littermates. Pretreatment with D1 or D2 dopamine receptor agonists was unable to restore LTD, whereas amphetamine, which increases endogenous dopamine availability partially rescued LTD. Because a paradoxical, excitatory D2-dependent effect has been shown in cholinergic interneurons from hMT mice (Pisani *et al.*, 2006), we tested the possibility that an excess in acetylcholine (ACh) striatal levels could impair LTD. Pre-incubation with either hemicholinium, which depletes endogenous ACh, or the M1-preferring muscarinic receptor antagonists, pirenzepine and trihexyphenidyl respectively, restored LTD. In the absence of magnesium, HFS was able to induce a long-term potentiation (LTP) in MSNs recorded from either controls or hWT mice. In hMT mice the magnitude of LTP was significantly higher than in hWT and controls. Once obtained a stable LTP, a low-frequency stimulation (LFS) protocol was able to induce a synaptic depotentiation (SD) both in controls and hWT mice. However, LFS failed to determine SD in hMT mice. Similarly to what observed for LTD, both hemicholinium and pirenzepine rescued SD when applied after LTP induction. Likewise, in mice deficient for the muscarinic autoreceptor M4, we did not observe SD. These results suggest that a dysregulation in the dopaminergic control over cholinergic signaling impairs synaptic plasticity in the striatum of DYT1 transgenic mice. These functional alterations might represent the cellular bases for the motor abnormalities observed in non-manifesting DYT1 carriers.

In this transgenic mouse model, we had previously identified a profound alteration of striatal cholinergic function in 9 month-old hMT mice since we observed a paradoxical excitatory response of cholinergic interneurons to activation of dopamine D2 receptors (D2Rs), instead of the inhibitory response observed in controls and hWT mice, which likely results in an enhanced acetylcholine release (Pisani *et al.*, 2006). We extended these observations to 3 and 5 month-old transgenic mice, and performed a detailed characterization of the mechanisms underlying the paradoxical response to D2R activation. No significant differences in the intrinsic membrane properties were observed among genotypes. Similarly, muscarinic M2-M4 autoreceptor function was unaffected, suggesting that the mechanisms regulating the autonomous pacemaking activity of cholinergic interneurons are preserved. However, both in 3- and 5 month-old hMT mice, bath-application of the D2R agonist quinpirole caused a membrane depolarization coupled to an increase in firing rate, which was blocked by the D2R antagonist sulpiride. Such a paradoxical effect was confirmed by voltage-clamp experiments performed at both ages. This response to D2R activation was prevented by loading recording pipettes with GDP-β-S, as well as by adding the calcium chelator BAPTA to the internal solution, suggesting that the effect of quinpirole requires G-protein activation and involves calcium currents. Patch-clamp recordings from dissociated interneurons showed that the quinpirole-mediated inhibition of N-type currents was significantly larger in hMT mice at both ages. To further characterize the quinpirole effect, we studied the effect of D2R activation on two main conductances that regulate firing activity of cholinergic interneurons, Ih and afterhyperpolararization (AHP) currents. Bath-application of ZD7288, an Ih inhibitor, failed to reduce the quinpirole effect, whereas preliminary pharmacological studies suggest the involvement of AHP in the aberrant response to D2R activation. These results confirm that an imbalance between striatal dopaminergic and cholinergic systems might play a pivotal role in the pathophysiology of dystonia, providing a clue to understand the ability of anticholinergic agents to restore motor deficits in generalized dystonia.

The adenosinergic neurotransmission is also known to exert a pivotal role in regulating basal ganglia function. We therefore performed motor behavioural tests to assess the role of A2A receptors in motor activity in both hWT and hMT mice. Spontaneous motor activity was measured by automated cages (43 cm x 43 cm x 22 cm; model Automex II; Columbus

Instruments, Columbus, OH), which allow to measure "total" motor activity as well as to discriminate among horizontal, vertical and stereotyped activity. The animals were individually placed in animal motor activity cages in a sound proof experimental room. The motor activity of each rat was evaluated for 30 min, stored and analyzed by a counter software (version 3.3; Columbus Instruments). There was no significant difference in spontaneous motor activity among the three genotypes (control, hWT, and hMT mice). We then treated both transgenic and control mice with a selective A2A receptor agonist (CGS 21680, 0.5-0.2 mg/kg i.p.), a selective A2A receptor antagonist (SCH 58261, 1-2 mg/kg i.p.) or vehicle. Mice treated with the A2A antagonist were subjected to an habituation period before treatment; habituated mice were then injected with SCH 58261, immediately returned to the motility cages and motor activity was recorded for 30 min. SCH 58261 dose-dependently stimulated motor activity in habituated rats, as expected by the block of A2A receptors. The activation of A2A receptors normally reduces spontaneous motility and, under our experimental conditions, the administration of 0.5 mg/kg of CGS 21680 in mice non-habituated to the open field, dramatically reduced motor activity of transgenic and control mice. At the 0.2 mg/kg dose, activity was significantly decreased 15 min after injection in hMT and hWT *vs* control mice and this effect became more evident after 30 min. No differences were observed in control mice, suggesting that the expression of both wildtype and mutant human TorsinA in mice increases the sensitivity to CGS motor depressant effects. Although our experiments suggest that the mutation of torsinA is not correlated with a A2A dysfunction in motor behaviour, they indicate a direct interaction between TorsinA protein and A2A receptors.

Papers published within the project

Bonsi P, Martella G, Cuomo D, Platania P, Sciamanna G, Bernardi G, Wess J, Pisani A. Loss of muscarinic autoreceptor function impairs long-term depression but not long-term potentiation in the striatum. *J Neurosci* 2008;28(24):6258-63.

Martella G, Tassone A, Sciamanna G, Platania P, Cuomo D, Viscomi MT, Bonsi P, Cacci E, Biagioni S, Usiello A, Bernardi G, Sharma N, Standaert DG, Pisani A. Impairment of bidirectional synaptic plasticity in the striatum of a mouse model of DYT1 dystonia: role of endogenous acetylcholine. *Brain* 2009;132(Pt 9):2336-49.

Pisani A, Bernardi G, Ding J, Surmeier DJ. Re-emergence of striatal cholinergic interneurons in movement disorders. *Trends Neurosci* 2007;30(10):545-53.

Sciamanna G, Bonsi P, Tassone A, Cuomo D, Tscherter A, Viscomi MT, Martella G, Sharma N, Bernardi G, Standaert DG, Pisani A. Impaired striatal D2 receptor function leads to enhanced GABA transmission in a mouse model of DYT1 dystonia. *Neurobiol Dis* 2009;34(1):133-45.

TESTING *IN VITRO* AND *VIVO* TREATMENTS FOR INCLUSION BODY MYOSITIS

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In vitro studies

In inclusion body myopathies, characterized by presence of autophagic vacuoles in skeletal muscle, abnormal proteins typical of neurodegenerative diseases such as Alzheimer, or Parkinson accumulate into the muscle fibers. The sporadic form called Inclusion Body Myositis (sIBM), because of presence of inflammation, is the most frequent, lacks effective treatment and is considered a disease of muscle aging. Both oxidative stress and altered proteolytic mechanisms appear to contribute to the pathology.

Our studies were concerned with characterization of the molecular defect in some hereditary forms of inclusion body myopathy; and with investigation of aspects concerning oxidative stress in muscle cell cultures from sIBM patients and controls. Our studies allowed us to identify mutations in the GNE gene in 2 patients and in the LAMP2 gene in another patient. The *in vitro* study has required more time than anticipated due to low proliferation rate of IBM cells probably due to increased aging.

We have analysed 5 control myoblast lines (age: 55-60 yrs) and 7 sIBM myoblast lines (age: 50-67 years, subdivided in group A and group B due to different response to treatment), either in basal conditions or after H_2O_2 treatment (16 µM per 30' a 37 °C in culture medium) to induce oxidative stress.

In basal conditions we found a significant increase in nitric oxide production, as measured by the Griess test, in IBM myoblasts compared to control cells. Furthermore, we found increased heme oxigenase-1 (HO-1) mRNA levels in 3 IBM patients (group A) and reduced levels in 4 IBM patients (group B); while Glutathione Synthetase (GS) mRNA levels were similar to levels of control myoblasts in group A and reduced in group B. In oxidative stress conditions (H2O2 treatment), HO-1 mRNA levels increased both in group A and B, with higher values in group A; and GS mRNA levels also increased with higher values in group B.

Based on these data we treated patient and control myoblasts with L-methionine, which is able to protect cells and tissues from free radicals and to activate HO-1. Activated HO-1 exerts a triple detoxifying action: due to production of biliverdin that, after conversion to bilirubin, exerts a strong antioxidant effect by reacting with ROS and RNS and making them soluble into lipids; due to the carbon monoxide produced by the heme catabolism induced by HO-1 activity, that has anti-apoptotic effect; and due to the release from the heme core of Fe ions that up regulate ferritin. Ferritin acts as antioxidant by sequestering Fe ions from the cytosol and by supporting glutathione in transforming hydrogen peroxide in H2O molecules.

Muscle cells from IBM patients and controls have been treated at 37°C for 24 h with 10 mM L-methionine both before and after induction of oxidative stress by H_2O_2.

The evaluation of nitric oxide production, of HO-1 and GS transcript levels and of HO-1 protein expression are ongoing. The study will be reported in a manuscript to be submitted for publication.

Another *in vitro* study has allowed to Dr. Mirabella's group to find an increased aging in myoblasts from IBM patients compared to control myoblasts.

In vivo study

Main aim of the clinical trial was to evaluate the safety and tolerability and the clinical efficacy of oral simvastatin treatment in IBM patients; the effect of this treatment has been evaluated by clinical and quality of life measures, by imaging, and by histopathology. The primary outcome measure of the study was the improved functional index; and secondary outcomes were the effect on muscle as verified by imaging and, when possible, by evaluation of a second muscle biopsy taken after 12 months therapy. Fourteen patients have been enrolled for the simvastatin treatment and 2 for intravenous IgG (IVIG) treatment.

Patients treated with simvastatin

All the simvastatin treated patients gradually increased the drug dosage by 10 mg every 4 weeks to a maximum of 4 mg/die. In the 14 patients who assumed simvastatin there have been 3 drop outs, due to personal reasons not related to the study. One patient had a significant increase in CK levels (about 4-5 times normal values) during the first months of treatment, without any other symptom neither subjective nor clinically proven by neurological examination. In this patient the simvastatin treatment was suspended and a tight clinical and laboratory monitoring during several weeks showed gradual decrease of CK levels to the values before therapy (about 2-3 times normal values), with no other clinical sign. Clinical findings and, when available, radiological and laboratory data, have therefore been investigated in 10 patients.

Patients treated with IVIG

One of 2 patients treated with IVIG, developed a tumour (not related to the treatment or to the disease) and died; the second patient has undergone bimestrial cycles of IVIG infusions without side effects or significant clinical or instrumental changes. Both patients were also taking steroids.

Clinical data

None of the 10 patients had side effects due to the treatment. Out of the 6 patients evaluated, 6 were also treated with steroids orally (20-50 mg of prednisone every other day) during the study; one patient suspended the steroids due to non efficacy/side effect appearing. Four patients in the past had assumed immunomodulatory/immunosuppressive therapies (IVIG, azatioprin, etanercept), with no clinical efficacy. Regarding clinical evolution no variations have been observed to be considered as "improvement" as defined by the IMACS group (improvement consists of at least 20% improvement in three of six core set measures, with no more than two worse by at least 25% nor including manual muscle testing).

Summarizing, of the 10 patients evaluated: 4 patients showed a mild clinical improvement (1-2 point in the score sets IMAC 2 and IMACS 9, and/or 4-5 points in the score set IMACS 4), confirmed in 3 cases over 4 also by subjective evaluation (score sets IMACS 3 and IMACS 10); 2 patients referred to be improved generally and in daily activities, however with no improvement in clinical status; 2 patients remained stationary (with only minimal variations); 2 patients showed slightly worsened clinical condition (confirmed also subjectively)

Radiological and laboratory investigations

Of the 10 evaluated patients 4 had dysphagia and therefore underwent an oro-pharingo-esophageal scintigraphy, and 9 patients out of 10 underwent a muscle MRI of the lower limbs (one patient could not have the MRI done because of a metallic prosthesis in his knee) before and after one year of treatment. Five out of 10 patients agreed to have another muscle biopsy taken after 1 year of therapy.

The muscle MRI of lower limbs at 12 months did not show any significant variation in all patients examined.

The evaluation of swallowing at 12 months after the first oro-pharingo-esophageal scintigraphy did not reveal worsening of dysphagia in none of the 4 patients examined.

The morphologic evaluation of histopathological findings in the second muscle biopsy in 5 patients did not show evident changes compared to the previous biopsy. To this regard, however, the difficulty to compare different muscles (sometimes also differently compromised) must be pointed out, although the basic features (amount of inflammation, vacuolar degeneration, fibro-adipose infiltration) tend to remain constant even after long intervals of time in a certain patient.

In conclusion, at 2 years from the start of the study we can assert that, although there was no significant clinical improvement, 80% of the patients treated with simvastatin had no significant clinical worsening either. The study will be reported in a manuscript to be submitted for publication.

Papers published within the project

Di Blasi C, Jarre L, Dassi P, Blasevich F, Mora M. Danon disease: a novel LAMP2 mutation affecting the pre-mRNA splicing and causing aberrant transcripts and partial protein expression. *Neuromuscul Disord* 2008;18:962-6.

Morosetti R, Broccolini A, Sancricca C, Gliubizzi C, Gidaro T, Tonali PA, Ricci E, Mirabella M. Increased aging in primary muscle cultures of sporadic inclusion-body myositis. *Neurobiol Aging* 2008 Sep 25. [E-pub ahead of print]

ENZYME REPLACEMENT THERAPY IN LATE-ONSET PHENOTYPES OF GLYCOGENOSIS TYPE 2

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Glycogenosis 2 (G2), also known as Pompe disease, is a lysosomal storage disorder caused by acid α-glucosidase (GAA) deficiency. Clinical trials in infants have demonstrated a positive response to human recombinant (rhGAA) enzyme replacement therapy (ERT). To date, no large studies have been published on late-onset G2 phenotypes.

Twenty-three patients with late onset G2 (9 females, 14 males) were enrolled in an observational, prospective, non-randomized, open-label study. They received bi-weekly infusions of 20 mg/kg of rhGAA. Clinical conditions, muscular function (6-minute walk test, 6MWT; Walton scale, WS), respiratory function, and biochemical parameters (including CK, LDH, AST, ALT, arterial blood pCO_2) were assessed every 6 months for 2 years.

After 24 months of ERT, muscular function improved (6MWT: $p<0.0001$; WS: $p=0.0002$) independently of patient age and clinical severity at baseline. Median daily duration of ventilation decreased from 14 to 8 hours. Tracheostomy was removed in 3 of the 4 carrying patients, and no further ventilatory support was necessary in 2 of them. Arterial pCO_2 decreased significantly ($p=0.0189$). Headache and muscle pain, which affected 34.8% and 47.8% patients respectively at baseline, persisted in 8.7% and 13.0% of them. Biochemical parameters (CK, LDH, AST and ALT) decreased significantly, $p<0.0001$. No severe secondary events were observed.

ERT with rhGAA was shown to be safe and effective in improving clinical symptoms and biochemical parameters independently of patient age and disease severity at baseline.

ADIPOSE TISSUE-DERIVED STEM CELLS FOR THE TREATMENT OF MUSCULAR DYSTROPHY

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Objectives

Duchenne Muscular Dystrophy (DMD) is a progressive X-linked muscle wasting disease, leading to early disability and death. DMD is caused by a mutation in the dystrophin gene that precludes the production of a stable protein, causing disruption of muscle contractile structures.

Therapeutic approaches to DMD aim at rescuing muscle damage by delivery of cells able to differentiate into skeletal muscle. Skeletal muscle-derived progenitor cells represent one choice because of their intrinsic myogenic potential.

Unfortunately, these cells are recovered in low number from DMD muscle biopsies and are poorly expandable *in vitro*. Stem cells of different origin may also restore dystrophin expression in several mouse models of muscular dystrophy. However, the frequency of stem cells incorporation into skeletal muscle is often too low to result in an important amelioration of the dystrophic phenotype. Thus it is important to identify alternative stem cell sources and to establish which population better contributes to skeletal muscle regeneration.

Adipose tissue (AT) provides a uniquely abundant and accessible source of multipotent cells. We have recently shown that in addition to mesenchymal stem cells, adipose tissue contains a subpopulation of cells, referred to as Adipose Tissue-derived–Autonomously Myogenic Cells (AT-AMCs), which are able to spontaneously differentiate into contractile skeletal myotubes.

Aim of this research proposal was to identify and characterize the myogenic precursors in both mouse and human AT and to establish their therapeutic potential in dystrophic muscle disorders.

Results

AT-derived AMCs amplified from single cell and expanded *in vitro*

AT-derived AMCs can be amplified from single cell and expanded in vitroBy seeding AT-derived cells at low density in a specific growth medium containing FGF2 we have been able to isolate clones of AT-AMCs from both subcutaneous (inguinal) and visceral (periaortic, omental) adipose tissue. Among several growth factors tested, only FGF2 has the ability to support AMCs clones formation from single cell. Myogenic AT-derived clones express markers characteristic of muscle progenitor cells such as Pax3, Pax7, c-Met and Flk-1. *In vitro* amplified clones display a homogeneous morphology and the ability to readily convert to multinucleated contractile myotubes upon switching to low serum conditions even after extended passages in culture.

AMCs in adult mouse tissues of endodermal and mesodermal origin

AMCs are not restricted to adipose tissue but can be found in a variety of adult mouse tissues of both endodermal and mesodermal origin. Tissues of both endodermal (i.e., pancreas, stomach, liver and lung) and mesodermal (i.e. spleen and aorta) origin were screened for the ability to generate skeletal myotubes. Single cell suspensions obtained by collagenase digestion of the different organs were plated on fibronectin coated dishes, kept in Growth Medium for 1 week and then switched to Differentiation Medium for additional 2 days. Contractile myotubes were visible in pancreas, spleen- and stomach-derived cell cultures while they were never detected in culture from heart, liver or lungs. AMCs were not detected in aorta-derived cells. However, the periaortic adipose tissue was particularly enriched for such cells.

By applying the same protocol used for adipose tissue, it has been possible to isolate several clones of myogenic cells from most of the tested sources, that is pancreas, spleen, stomach, and periaortic tissue while, as expected, no clones have been obtained from heart, liver or lungs. All clones have similar morphology and gene expression pattern.

These results show that clonable AMCs, totally undistinguishable from skeletal muscle satellite cells, can be isolated from various non-muscle tissues of adult mice and amplified *in vitro*.

Electrophysiological properties of AT-derived and skeletal muscle-derived myotubes

AT-derived and skeletal muscle-derived myotubes have comparable electrophysiological propertiesIn order to test the electrophysiological properties of AT-derived myotubes patch-clamp recordings were performed on multinucleated myotubes obtained from AT-derived cultures. Myotubes generated from skeletal-muscle derived primary satellite cells isolated from age-matched mice were used for comparison. Results show that AT-derived myotubes express functional ACh receptors and that their electrophysiological behaviour is very similar to that of satellite-cells derived-myotubes, thus demonstrating that AT-associated cells can give rise to terminally differentiated, mature and functional skeletal muscle cells. Analogous results have been obtained with pancreas-derived AMCs.

AT-AMCs from Flk-1 positive progenitors

AT-AMCs derive from Flk-1 positive progenitors. FACS analysis of uncultured cells samples obtained from digestion of various adipose tissue depots revealed that the Flk-1 antigen is expressed in all tested samples. In order to test whether AMCs derive from Flk-1+ progenitors, Flk-1+ cells were purified by immuno-magnetic sorting of cellular fractions obtained from subcutaneous or periaortic adipose tissue of adult mice. The expression of Flk-1 in sorted cells was checked by FACS. Sorted cells were plated in GM supplemented with bFGF on fibronectin-coated dishes. Plating was performed at low density to allow the formation of single cell derived clones. Equal numbers of unsorted cells, as well as of cells from the antigen depleted fractions were used as controls.

After 1 week, several clones of proliferating cells were visible. Although myogenic cells were not the only cell type obtained with this procedure, cell fractions sorted for Flk-1 were always enriched for AMCs clones and the enrichment factor was directly proportional to the Flk-1 content. The presence of AMCs was easily detected due to their characteristic morphology, and was always confirmed by the ability to form contractile, multinucleated

myotubes upon switching to low serum. Phenotypical analysis of non-myogenic clones identified most of them as endothelial, since they express endothelial markers such as Flk-1, CD31 and VE-cadherin while they do not express myogenic markers such as TnT and result positive to an Ac-LDL uptake assay. AMCs also express endothelial markers but differently from endothelial cells, do not uptake Ac-LDL. As established by both immunostaining and RT-PCR, endothelial and myogenic clones are both negative for smooth muscle markers such as SM-actin and SM-MHC.

AT-AMCs cells and skeletal muscle regeneration *in vivo*

AT-AMCs cells participate to skeletal muscle regeneration *in vivo*. To establish their in vivo myogenic potential, AMCs were assayed for the capacity to contribute to muscle regeneration invivo. AT-derived AMCs cell clones were isolated from GFP positive mice, expanded *in vitro* and then injected into the tibialis anterior (TA) muscle of GFP-negative syngenic mice where damage was induced by Cardiotoxin injection. Engrafted GFP-expressing cells in the injected muscle were visualized by an anti-GFP antibody 7 days after injection.

GFP positive fibers covered up to 20% of the total area of muscle sections from treated hindlimbs while no GFP staining was observed in control sections from PBS injected limbs. These data demonstrate that AT-AMCs cells can be efficiently incorporated into skeletal muscle fibers *in vivo* and may effectively contribute to skeletal muscle regeneration.

GFP donor cells can be observed in a sublaminal position characteristic of satellite cells. In addition, donor-derived Pax7+ mononucleated cells able to differentiate into multinucleated myotubes can be re-isolated from previously injected, regenerated muscle. Such results indicate that in addition to participate in the formation of new muscle fibers, transplanted AT-AMCs are retained as myogenic progenitors and may contribute to the replenishment of the muscle progenitor pool.

Flk-1+ AT-derived cells a muscle regeneration

Freshly sorted, Flk-1+ AT-derived cells enriched for myogenic progenitor participate to muscle regeneration with much higher efficiency compared to unsorted cells. As demonstrated by recent literature data, *in vitro* amplified committed myogenic precursors, such as satellite cells, have a reduced regenerative ability compared to freshly harvested ones. AMCs become committed to the myogenic phenotype only after isolation from AT and culture *in vitro*. Therefore it is not possible to extract committed myogenic progenitors from AT without passage in culture.

Ad described above, immuno-magnetic selection procedures indicate that AMCs from adipose tissue derive from Flk1+ progenitors and can be enriched in the purified Flk1 fraction. In order to test whether freshly isolated Flk1+ cell population, although not yet committed to the myogenic phenotype could posses as well myogenic regenerative properties *in vivo*, we injected the uncultured, sorted Flk1-population into damaged muscles.

Cells from GFP positive mice were injected 1 day after cardiotoxin treatment into the Tibialis Anterior (TA) of syngenic GFP negative mice and immunohistochemistry on muscle sections was performed after 1 week. Results show that when injected as freshly purified uncultured samples, Flk1 cells have an enhanced regenerative ability compared to cultured committed AMCs. In particular, with only 50.000 injected in the TA muscle, we found muscle section where 70% of the regenerating area is donor derived (GFP positive).

AMCs in human adipose tissue

AMCs can be identified in human adipose tissue. Human proliferating AT- AMCs are committed to the myogenic phenotype and express the satellite cell marker Pax7. Our subsequent step was to characterize the myogenic potential of human AT-derived cells. We first analyzed several samples from different sources of adipose tissue (visceral and subcutaneous, age of donor from 15 to 80 years) and tested them for the presence of spontaneously differentiating AMCs cell. We could detect AMCs from subcutaneous adipose tissue from individuals under 40 years of age. Human AMCs (hAMCs) are committed to the myogenic phenotype, express the satellite cell marker Pax7, and differentiate into multinucleated myotubes.

A phenotypic caharcterization of the adipose tissue-derived cell suspension showed that, an Flk1 fraction is present in all AT samples. As in mice, many Flk-1 cells are CD31 and CD146 negative and therefore are cellular subtypes distinct from differentiated vascular cells such as endothelium and pericytes. Experiment testing the *in vivo* regenerative potential of human AT-derived cells (unsorted or Flk1, CD31, CD146 sorted) in immuno-suppressed dystrophic mdx mice are currently ongoing.

Papers published within the project

Di Rocco G, Tritarelli A, Toietta G, Gatto I, Iachininoto MG, Pagani F, Mangoni A, Straino S, Capogrossi MC. Spontaneous myogenic differentiation of Flk-1-positive cells from adult pancreas and other non-muscle tissues. *Am J Physiol Cell Physiol* 2008;294:C604-12

EVALUATION OF THE PATHOGENETIC ROLE AND PHARMACOLOGY OF ISOLATED HUMAN TSC2 SMOOTH MUSCLE CELLS. NOVEL THERAPEUTIC PERSPECTIVES

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Introduction

Tuberous Sclerosis Complex (TSC) is characterized by multiorgan development of tumors affecting the kidneys, brain, heart and lungs. TSC is related to lymphangioleiomyomatosis (LAM), a rare disease characterized by widespread pulmonary proliferation of abnormal smooth muscle cells leading to cystic destruction of the lung parenchyma. In LAM patients somatic mutations in TSC1 or, more frequently, TSC2 have been identified in abnormal lung tissue and renal angiomyolipomas (AML). The original contribution of our laboratory has been the description of the biological and pharmacological characteristics of TSC 2 smooth muscle cells purified from the angiomyolipoma of a TSC patient. These cultured cells has so far represented an optimal means of studying and developing appropriate pharmacological strategies aimed at blocking the life-threatening growth of these smooth muscle cells in tuberose sclerosis (TSC) and limphangioleiomyomatosis (LAM). It has been suggested that LAM may develop by the lung metastatization of angiomyolipoma (AML) TSC2 -/- cells. We have been able to isolate and grow *in vitro* pure smooth muscle angiomyolipoma-derived TSC2 -/- cells (Lesma *et al.*, 2005) bearing an LOH lesion on TSC2 gene, their growth and survival depends on the availability of EGF and functionality of EGR receptor (EGFR).

Identification of the genetic abnormality and regulation of growth of purified TSC2 cells

TSC2-/- ASM cells cells express the apoptosis inhibitor protein survivin when exposed to IGF-1. Survivin expression is also triggered whenever culture conditions perturb normal TSC2-/- cell function, such as the omission of EGF from the growth medium, the supplementation of anti-EGFR, blockade of PI3K and ERK, or inhibition of mTOR. Interestingly, single or simultaneous inhibition of PI3K by LY294002 and ERK by PD98059 does not prevent IGF-1-mediated survivin expression. Apoptogenic Smac/DIABLO, which is constitutively expressed by TSC2-/- A+ cells, is down-regulated by IGF-1 even in the presence of LY294002 and PD98059. These cells release IGF-1 by means of a negative feedback-regulated mechanism that

is overrun when they are exposed to antibodies to IGF-1R, which increases the released amount by more than 400%. The autocrine release of IGF-1 may therefore be a powerful mechanism of survival of the tightly packed cells in the thick-walled vessels of TSC angiomyolipoma and in lymphangioleiomyomatosis (LAM) nodules. The effects of EGF on proliferation of TSC2-/- ASM cells and TSC2-/- ASM cells transfected with TSC2 gene were determined. In contrast to TSC2-/- ASM cells, growth of TSC2-transfected cells was not dependent on EGF. Moreover, phosphorylation of Akt, PTEN, Erk and S6 was significantly decreased. EGF is a proliferative factor of TSC2-/- ASM cells. Exposure of TSC2-/- ASM cells to anti-EGFR antibodies significantly inhibited their proliferation, reverted reactivity to HMB45 antibody, a marker of TSC2-/- cell phenotype, and inhibited constitutive phosphorylation of S6 and ERK. Exposure of TSC2-/- ASM cells to rapamycin reduced the proliferation rate, but only when added at plating time. Although rapamycin efficiently inhibited S6 phosphorylation, it was less efficient than anti-EGFR antibody in reverting HMB45 reactivity and blocking ERK phosphorylation. In TSC2-/- ASM cells specific PI3K inhibitors (e.g. LY294002, wortmannin) and Akt1 siRNA had little effect on S6 and ERK phosphorylation. Following TSC2-gene transfection, Akt inhibitor sensitivity was observed. Thus, our results show that an EGF independent pathway is more important than that involving IGF-I for growth and survival of TSC-/- ASM cells, and such EGF-dependency is the result of the lack of tuberin.

Pure smooth muscle-like cells (ASM) were also isolated from an angiomyolipoma of another TSC patient.

These cells lacked tuberin and labeled by HMB45 and CD44v6 antibodies, and carried constitutive S6 phosphorylation. They bear a germline TSC2 intron 8-exon 9 junction mutation, but DNA analysis and PCR amplification failed to demonstrated loss of heterozigosity. Testing for an epigenetic alteration as second hit, we detected the methylation of the TSC2 promoter. The biological relevance of this was confirmed by tuberin expression with reduction of HMB45 labeling and S6 constitutive phosphorylation following exposure to the chromatin remodelling agents, trichostatin A and 5-azacytidine. These cells were, named TSC2-/meth ASM cells. Their proliferation required epidermal growth factor (EGF) in the medium as we have previously described for TSC2-/- ASM cells. The blockade of EGF with monoclonal antibody caused the loss of TSC2-/meth ASM cells. Also rapamycin effectively blocked the proliferation of these cells. Our data show for the first time that methylation of the TSC2 promoter might cause complete loss of tuberin in TSC2 cells, and that the AML pathogenesis might also be originated from epigenetic defects in smooth muscle cells. In addition the effect of chromatin remodeling agents in these cells suggests a further avenue for the treatment of TSC and lymphangioleiomyomatosis.

We have also purified from the angiomyolipoma of a TSC2 patient a pure subpopulation of TSC2-/- ASM cells that co- express α-actin and keratin; these cells in spite of the constitutive phosphorylation of Akt and S6, show slow reduplication rate. They were named TSC2-/- AK+cells, the major aim is to determine whether this cell is a progenitor in angiomyolipoma formation. We shall investigate their role in the development of angiomyolipomas, and the role of constitutive phosphorylation of Akt as a key factor in the proliferation of smooth muscle TSC2 -/- cells.

Development of a LAM model

If the theory of metastasis in LAM development is true, the TSC2-/- ASM cells should invade, live and proliferate in lungs. The effects on lung structure and function of TSC2-/- ASM cells was studied in immunodeficient nude mice after endonasal administration. Cells were

applied in anesthetized mice by endonasal application of a droplet containing 250,000 cells. TSC2-/- cells survived and proliferated in lung parenchyma creating a condition that somewhat resembled LAM. Within 4 weeks of cell administration the lungs are invaded by lymphatics and in 6 months the extent of diffusion of the lymphatic system in the lungs is 10 fold higher than normal. The penetration and expansion of the lymphatic vessels driven by the TSC2-/- cells correlate with a destruction of lung parenchyma. Treatment with rapamycin and anti-EGFR was begun 6 months after cell administration and lasted 6 weeks. Both agents reduced by 80% the extent of lymphatics, while the number of proliferating S6-positive cells was reduced from 65% to control level by anti-EGFR and to 30% by rapamycin. The number of TSC2-/- cells per mm^3 drops from 197,1±58,27 to 32,17 ± 12,74*** after anti-EGFR and to 134,18±53,94* after rapamycin treatments. Thus, also *in vivo*, anti-EGFR is superior to rapamycin in eliminating TSC2-/- cells.

Isolation of pure LAM cells

We have, most recently, isolated and grown in culture from the chilus of a LAM English patient a pure population of smooth muscle TSC2 cells lacking tuberin, that require, similarly to angiomyolipoma-derived TSC2 cells, EGF supplementation for growth and proliferation. Thus, these cells are, thereafter, named LAM cells. The genetic alteration underlying the dysfunction of the TSC2 gene is not LOH and apparently of epigenetic origin, but the methylation of the TSC2 promoter is rather low and does not justify the gene blockade. In addition to the quest for the genetic defect, we shall concentrate also on another great pharmacological difference with the above described cells. The blockade of the EGF receptor in angiomyolipoma-derived TSC2 cells leads to the rapid counteraction of the constitutive phosphorylation of S6 and ERK in TSC2 cells with loss of proliferative activity and progressive cell death. Differently, the purified LAM cells maintain transiently (7-10 days) normal proliferation rate and normal levels of S6 and ERK constitutive phosphorylation when placed in a medium containing anti-EGFR and lacking EGF, later S6 and ERK dephosphorylation takes place and cell die within the following 10-14 days. Thus anti-EGFR shows transient agonist properties, and this suggests that the EGF receptor may also be genetically modified in LAM cells.

Conclusion

Our study suggests that TSC genetic lesions in TSC and LAM may be of different kinds, and different types TSC2 smooth muscle cells may contribute to the development of hamartomas and LAM. On the other hand, all types of TSC cells require EGF as a growth factor and are sensitive to EGFR blockade, while the cytostatic effects of mTOR blockade is mostly evident in TSC2-/meth ASM cells rather than in LOH TSC2-/- ASM cells.

Papers published within the project

Carelli S, Lesma E, Zadra G, Grande V, Bosari S, di Giulio AM, Gorio A. Survivin expression in Tuberous Sclerosis Complex cells. *Molecular Medicine* 2007;13:166-77.

Lesma E, Grande V, Ancona S, Carelli S, Di Giulio AM, Gorio A. Anti-EGFR Antibody efficiently and specifically inhibits human TSC2$^{-/-}$ Smooth muscle cell proliferation. Possible treatment options for TSC and LAM. *PLoS ONE,* 2008;3(10):e3558.

Lesma E, Sirchia SM, Ancona S, Carelli S, Bosari S, Ghelma F, Montanari E, Di Giulio AM, Gorio A. The methylation of the *TSC2* promoter underlies the abnormal growth of TSC2 angiomyolipoma-derived smooth muscle cells. *The American Journal of Pathology* 2009;174:2150-9.

MESENCHYMAL STEM CELLS FOR THE TREATMENT OF TIBIAL CONGENITAL PSEUDARTHROSIS ASSOCIATED WITH TYPE I NEUROFIBROMATOSIS

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Introduction

Type 1 NeuroFibromatosis (NF1), the most common single gene disorder found in humans, is the main cause of tibial Congenital Pseudarthrosis of the Tibia (CPT). The treatment of CPT consists on repeated surgical procedures which often fail with the inevitable outcome of severe disability. The use of autologous bone Marrow Stromal Cells (MSC) has been recently proposed, because the MSC pool contains osteogenic precursors, that in turn may enhance bone repair. The current study was designed to establish the biological basis for the employment of autologous MSC in order to improve the curing opportunities of CPT. The investigation was planned by taking into account that the pathophysiology of bone healing mainly depends on the osteogenic potential of the resident cells, although several factors play a crucial role in restoring the normal bone structure. We analyzed the osteogenic ability of MSC cultures obtained from bone marrow aspirates of subjects affected or not by CPT, associated or not with NF1. Biochemical, functional and molecular features of cell cultures were tested to determine whether the source, i.e. lesion site or iliac crest, and the underlying disease could influence the osteogenic properties of MSC. In addition, we evaluated whether the autologous serum and the microenviroment close to the lesion could affect the osteoblast differentiation of transplanted MSC.

Patients and Methods

The study design was approved by the institutional ethical committee on human research and signed informed consent was obtained from the parents of 17 patients enrolled in the study. 7 patients were affected by NF1 and CPT (CPT_NF1+; 6 males and 1 female, aged between 7 months and 18 years) and 6 patients affected by CPT without NF1 (CPT_NF1-; 4 males and 2 female, aged between 2.5 and 18 years). The control group was recruited from hospitalized children who underwent surgical treatment for congenital dysplasia of the hip without any other coexisting pathology ('No CPT'; 4 patients, 3 males and 1 female, aged between 3.3 and 10 years).

Bone marrow samples were collected from tibia, near to the pseudarthrosis site, (P) and from iliac crest (IC). Peripheral venous blood were collected from each patient to obtain the autologous serum (AUT) used in the cell cultures. The MonoNuclear Cell fraction (MNC) were maintained in MEM supplemented with 10% Fetal Bovine Serum (FBS) or AUT serum, and 100 μmol/L ascorbic acid-2 phosphate. After four days (T_0), nonadherent cells were removed, and 10^{-8} M dexamethasone was added to the culture medium. After the second confluence the culture medium

was added with 10mM β-glycerophosphate to induce the mineral nodules formation. Osteoblasts (OB) were obtained from bone fragments collected close to the surgical site in both CPT patients and control group. At the first confluence, the supernatant was collected and stored frozen at -20°C until the execution of the co-culture experiments. The MNC from IC stored in liquid nitrogen were thawed and cultured with a conditioned medium (OB-CM) containing 50% of the autologous OB supernatant to analyze the paracrine activity of OB on MSC. Two control cultures were used: in the former, IC-MSCs were maintained in differentiation medium (DM) to control the osteogenic potential of the cells after the thawing (DM100%); in the latter, IC-MSC were cultured with 50% of the culture medium suitable for the OB cultures, which was stored for 72 hours at 37°C in a 5% CO2 humidified atmosphere and then at -20° C until use, to mimic the effect of the partially exhausted CM derived from OB cultures (DM50%). The ability to generate bone-forming cells was tested by measuring cell proliferation, alkaline phosphatase activity and mineral nodule formation as previously described [2]. Gene expression analysis was performed at T_0 and T_1, by quantifying the transcripts of genes useful to monitor the MSC differentiation into osteoblasts (Runx2, ALPL, BGLAP COL1A1, SPARC, IBSP). Real time PCR was performed by using the Universal ProbeLibrary system. Probes and primers were selected using a web-based assay design software (ProbeFinder https://www.roche-applied-science.com).

Results

Our results can be summarized as follows:

- *IC-MSC obtained from CPT patients are more osteogenic than P-MSC, but less than IC-MSC of the control group*
  The source of the cells was the variable conditioning the osteogenic properties of MSC (ANOVA p values: Alamar Blue test 0.0001; CFU 0.008; ALP positive CFU 0.05; mineral nodules 0.01). In both CPT groups, the metabolic activity and the mineralization ability of MSC cultured with FBS were significantly superior in IC-MSC than in P-MSC. A higher number of CFU and of ALP-positive CFU was observed in IC-MSC cultures, but significant differences were found only in NF1- patients. The number of mineral nodules observed in P-MSC cultures was higher in NF1- rather than in NF1+ group. The most relevant variable influencing the osteogenic properties of IC-MSC was the status of the children (p values: CFU 0.002; ALP positive CFU 0.06; mineral nodules <0.0001). The number of CFU and mineral nodules was significantly lower in both groups of CPT patients, while a significant difference in the number of ALP positive CFUs was found only in the CPT_NF1- group. The expression of bone-related genes in IC-MSC cultured with FBS was influenced by the status of the children. Statistically significant ANOVA p values were found only for ALPL (T_0=0.0009; T_1= 0.001) and COL1A1 (T_0= 0.01), but the post hoc analysis showed that also Runx2 was significantly more expressed in control than in CPT_NF1+. No effect was attributable to the underlying disease, since the gene expression in IC-MSC was similar in NF1+ and NF1- patients. At T_0 bone-related genes tended to be more expressed in IC-MSC than in P-MSC, but the differences were not so pronounced as found with morphological, biochemical and functional analyses; at T_1 this trend disappeared, with the exception of ALPL in NF1+ group.

- *The AUT serum partially changes the osteogenic properties of IC-MSC and P-MSC*
  The AUT serum affected the CFU formation and the ALP activity in control group and the mineral nodules deposition in the CPT_NF1- group, while no significant differences

were observed in CPT_NF1+ patients. In some cases the functional performance of P-MSC cultures was better when AUT serum was used, even though no significant differences were found in comparison to FBS. A down-regulation of bone-related genes was observed in IC-MSC cultured with AUT serum. The overall effect was evident at T_0 (ANOVA p values: Runx2 0.01; ALPL 0.04; COL1A1 0.004; SPARC 0.03; IBSP 0.03) and at T_1 (ANOVA p values: Runx2 0.04; COL1A1 0.04; SPARC 0.01; IBSP 0.05). The post hoc analyses showed that the down-regulation of Runx2 ALPL, COL1A1 and IBSP due to the use of AUT serum was significant in the control group at T_0. At T_1, COL1A1, IBSP and SPARC were decreased in the IC-MSC of CPT_NF1- patients, while no significant differences were observed in NF1+ group. Similar levels of gene expression were observed in P-MSC cultured in FBS and AUT serum.

- *The OB-CM does not affect the differentiation of IC-MSC*
  The experiments with DM100% confirmed that IC-MSC proliferation and viability, CFU and ALP positive CFU number, and mineral nodule number were affected in CPT patients in comparison to the control group. Both DM 50% and OB-CM did not modify the osteogenic properties of IC-MSC, and the differences between CPT and 'No CPT' patients were maintained. The analysis of cells cultured with DM100% confirmed that gene expression levels changed according to the status of the children (ANOVA p values: Runx2 not significant; ALPL < 0.0001; BGLAP 0.05; COL1A1 0.05; SPARC 0.003; IBSP 0.05). In comparison to the control group, ALPL and SPARC were less expressed in all CPT patients, BGLAP and COL1A1 in CPT_NF1+, and IBSP in CPT_NF1- group. The expression of bone-related genes was favored by OB-CM in both control group and NF1- patients, and significant differences were found for Runx2, COL1A1 and BGLAP.

Discussion

Taken together, biochemical, functional and molecular results show that MSC of individuals affected by CPT have a lower osteogenic potential in comparison to the control group, and the defect is more pronounced in NF1+ patients, probably due to the defective neurofibromin function. Neurofibromin is a Ras GTPase activating protein which plays multiple essential roles in skeletal development by regulating the Ras transduced growth signals [4]. Nevertheless, when cells are cultured ex-vivo with FBS, the osteogenic ability is higher in IC-MSC than in P-MSC. These findings imply that the culture conditions are sufficient to promote the IC-MSC differentiation but are not effective for P-MSC, suggesting a functional diversity of the two populations. As a further confirmation of their difference, IC-MSC and P-MSC do not react in a similar way to external stimuli, because only the former shows significant changes when FBS is replaced with AUT serum. In addition, in NF1- patients AUT serum is less effective than FBS in promoting the IC-MSC differentiation, while the IC-MSC of NF1+ patients showed a slight increase in some of the tested parameters, leading to hypothesize that the release of circulating growth factors could be a way to compensate the constitutional defect of the NF1 cells. The bone tissue surrounding the lesion site may favor the differentiation of transplanted MSC, but that occurs only whether bone cells do not have intrinsic defects as in type 1 neurofibromatosis. In conclusion, the use of autologous MSC could be a useful tool for the treatment of recurrent CPT, because it increases the opportunity to obtain an effective bone tissue regeneration. This procedure could be recommended in all patients affected by this rare orthopedic disease, but clinical studies are needed to confirm its real efficacy in comparison to standard therapy.

NEW CELL THERAPY APPROACHES
FOR INFANTILE MALIGNANT OSTEOPETROSIS

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Osteopetrosis is a genetic disease characterized by defective osteoclasts. The Autosomal Recessive Osteopetrosis (ARO) is fatal within the first years of life. Hematopoietic Stem Cell Transplantation (HSCT) cures less than 50% of cases but often leaves severe neurological damages and other dysfunctions. Osteoclast appearance after HSCT is a slow process, during which disease progression continues. We hypothesize that a support osteoclast precursor therapy may contribute to improve the osteopetrotic phenotype.

Peripheral blood or bone marrow osteoclast yield in standard culture conditions is insufficient for therapeutic applications. Therefore, in order to improve the yield and the bone resorbing performance of osteoclasts, we assessed the osteoclastogenic potential of mononuclear progenitors in various culture settings using human cells, which, we believe, has a high translational relevance for future applications in patients. Then we transferred the best working protocol to mouse cells for experimentally testing their therapeutic potential in a mouse model of ARO. To this aim, we treated human peripheral blood mononuclear cells for two weeks with standard human recombinant osteoclastogenic cytokines, M-CSF and RANKL and compared the osteoclast yield with the very same treatment preceded by a single pre-treatment for one week with GM-CSF or a double pre-treatment, for one week with SCF, IL-3 and IL-6 followed by one week with GM-CSF, before standard osteoclastogenic induction with M-CSF and RANKL.

Moreover we tested the effect of StemPro34, a culture medium alternative to DMEM one, developed to support the growth of human HSC. In our condition, the best working osteoclastogenic protocol was the double pre-treatment in StemPro34 medium, which resulted in the appearance of TRAcP-positive osteoclast formation already after 3 days of exposure to M-CSF/RANKL, remarkably earlier than in the other cultures. Using the double-pretreatment protocol after a week of exposure to M-CSF and RANKL, the rate of osteoclastogesis was higher than for other cultures, further improving after two weeks. The double pre-treatment, along with the use of StemPro34, not only improved the yield of multinucleated cells versus standard conditions, but also increased the number of nuclei per osteoclast and the resorption ability, with a pit index about 4 fold greater than that of cells cultured in DMEM with the standard treatment. Similar results were obtained using mouse bone marrow primary cell cultures. For translational purposes, the source of osteoclasts must be readily available and cryopreservation procedures are used to maintain viable cells until use. We therefore tested osteoclast commitment ability after various methods of osteoclast precursor cryopreservation. The best working protocol was preservation at -80° C, in FBS:dimethylsulfoxide (9:1). This condition guaranteed about 65% survival after thawing, assessed by the trypan blue exclusion test and by cytofluorimetric analysis of propidium iodine-positive cells. Osteoclastogenesis and bone resorption potentials were indistinguishable from those of freshly isolated cells.

Next we assessed the *in vivo* impact of our cells in animal models, firstly testing engraftment of human cells in 4 weeks old immunocompromised Balb-c nu/nu mice, which could have a significant translational relevance. Mice were injected once in the left ventricle with 3x105 human peripheral blood mononuclear cells, pre-cultured in StemPro34 with the double pre-treatment with cytokines plus M-CSF and RANKL for 1 day to trigger osteoclastogenic pathways. Animals were sacrificed at various times after cell injection, and assessed for human cell engraftment by PCR for human ALU sequence on extracted DNA from whole limb long bones. We noted ALU PCR amplification from the first to the fourth week from cell inoculation, with no further amplification afterward, indicating transient engraftment of human cells. In situ hybridization in the tibias showed TRAcP-positive multinucleated cells containing ALU-positive nuclei, which were estimated to be 0.82+0.052% of total osteoclast count. This percent is likely to be underestimated as ALU-positive osteoclasts were identified in 60% of ALU-positive mice as assessed by PCR, suggesting that in our hands this latter method is more sensitive than the in situ hybridization. Notably, ALU-negative polymorphic nuclei were also apparent inside the osteoclasts carrying ALU-positive nuclei. They were morphologically distinguishable from the latter and similar to ALU-negative nuclei of surrounding cells presumably belonging to the host. This observation might suggest fusion of injected ALU-positive human precursors with resident mouse ALU-negative osteoclastic cells. Groups of mice treated with 80 µg/kg hrPTH(1-34) showed no further improvement of osteoclast engraftment.

To evaluate any beneficial effect on the disease, we injected newborn osteopetrotic oc/oc mice (intrahepatically for the first 8 days of life and then intraperitoneally) with mouse bone marrow mononuclear cells, isolated from syngeneic wild-type littermates and subjected to the very same treatment as human cells, testing different cell injection protocols, also in association with untreated, immunomagnetically isolated, CD117+ stem cells. Treatments were started at the 2nd day of life and repeated every three days for the lifespan of the animals. We then monitored disease progression and observed improvement of survival, increase of weight and longitudinal growth, and tooth eruption. Histological analysis revealed an improved bone phenotype in cell-injected mice. Tibias of cell-treated oc/oc mice had bigger size and more medullar cellularity than PBS-treated oc/oc mice. They also showed reduced fibrosis and increased hematopoiesis assessed by detection of the Ter119 antigen of red cells. Quantification of erythropoiesis showed an increased number of T119-positive cells in oc/oc mice injected with the osteoclast precursors (red cells/field, 29.78+4.84) compared to oc/oc mice treated with PBS (red cells/field, 16.78+3.34; mean+s.d.; n. 3; p<0.01). Notably, some polarized multinucleated cells delimiting resorption lacunae were observed only in cell-injected oc/oc mice, indicating that functional osteoclasts presumably arise from donor cells. This phenotypic improvement was also confirmed by bone histomorphometry, which showed a reduction of bone volume/total volume in cell-injected compared to PBS-injected oc/oc mice. These results provide first hand information on the feasibility of a support osteoclast precursor therapy in osteopetrosis.

ARO is a paradigm for genetic diseases that would benefit from effective prenatal treatment. Using the oc/oc mutant mouse, we report that *in utero* (IUT) injection of allogeneic fetal liver cells produces a high level of engraftment and rescues the phenotype. Fetal liver cells were obtained from day 12.5 p.c. fetuses heterozygous for the CMV-EGFP transgene in CD-1 background. Cells were injected in day 13.5 p.c. fetuses originated from oc/+ mating. Rescued oc/oc mice showed the expected mutated 7.0-kb band in their tail DNA. Two sets of trials were performed in which alternative methods for disaggregating liver tissue (mechanical or enzymatic) were used. In the first trial (mechanical disaggregation), 19 injected females delivered 60 pups. Among these, only one of the eight oc/oc mutant mice survived more than 4 weeks. This female mouse (F1) was phenotypically normal. At 10 days of age, incisor teeth

showed regular eruption, making the animal able to feed. Nevertheless, a malocclusion of the incisors appeared, requiring their periodic trimming. At sacrifice (30 weeks of ages) the mice weighted 22 grams. In the second trial (enzymatic disaggregation), 20 females were injected and 45 pups were born. Five out of 9 oc/oc mutant mice survived more than 4 weeks: three females (F2, F4, F5) and two males (M3, M6). F5 and M3 showed tooth eruption and were normal except for malocclusion. At sacrifice (24 weeks) F5 weighed about 20 grams. M3 died of unknown causes at 24 weeks (weight around 25 grams). F2 and F4 did not show tooth eruption and were fed with ground pellets and corn pudding. Despite the absence of teeth, they reached the weight of 12 grams and 17 grams, respectively. M6 died at the age of 5 weeks and showed partial tooth eruption. F2, M3, F4, and F5 showed normal capacity of exploring the surrounding environment and were able to climb, search food and eat. No abnormal or turning movements, typical of untreated oc/oc mice, were noted. M3 and F5 were mated with wild-type partners. M3 produced 2 litters of 18 mice total and F5 delivered 4 mice, all of which were oc/+, confirming the homozygous status of the founder. The difference in rescue between the two methods, 5/8 oc/oc mice for mechanical disaggregation versus 5/9 for enzymatic disaggregation, has a χ^2 3.44 [1 degree of freedom, (P=0.06)]. Relative to survival of 5/14 using adult marrow cells previously published, the liver cells did not show significantly higher results, but the rescued phenotype was qualitatively better, although some cured animals suffered from splenomegaly (F1, F2, and F4).

By X-ray we observed that F2, which was the smallest rescued mice, had signs of residual osteopetrosis, F4, F5 and M3 were normal and showed bone marrow cavities in the long bones. Bone densities of F1 and F5, assessed by dual energy X-ray absorptiometry (DEXA), was indistinguishable from that of normal mice. Sections of the spine, tibia, and skull showed microscopic areas of osteopetrosis with retained mineralized cartilage and sclerotic bone, even in the animals apparently normal. Sections of F4 showed no abnormalities, but focal lesions were apparent especially in the skull. The most obviously affected animal was the small male M6, with clear skull lesions observed by DEXA and significant osteopetrosis near the ends of long bones and vertebral plates. M3 had focal sclerotic lesions in the spine and long bones. Tooth growth in several animals was abnormal, but in sections the correlation of tooth abnormalities with regional osteopetrosis was not clear.

In the M6 mouse, bone density analyzed by microCT was highest and the amount of apparent osteopetrotic bone was relatively high, while in animals with intermediate outcomes, focal sclerotic bone was common but the skeleton was, overall, near to normal. In the focal sclerosis areas of F2, increased trabecular thickness, decreased trabecular number and unchanged trabecular spacing were observed. MicroCT was consistent with small-scale focal sclerotic disease inducing tooth eruption abnormalities. It is suggested though that in mice with healthy donor and recipient osteoclast precursors, colonies of abnormal recipient cells cause small foci of osteopetrotic bone to occur in a stochastic pattern, even though the overall skeleton appears normal.

We then tested permanent engrafted of GFP-expressing cells by clonogenic assays in Methocult M03434 containing a cocktail of cytokines to support growth and differentiation of different types of hematopoietic progenitors. The number of bone marrow cells was similar between treated and wild-type mice, with normal frequencies of erythroid and myelomonocytic progenitors. In F1, F4, and F5 mice, a high percentage of the different types of colonies were fluorescent, with values similar to those detected in control cultures raised from the bone marrow of donor CMV-EGFP mice. F2 only gave rise to a smaller fraction of fluorescent colonies. F4 and F5 displayed normal hemoglobin content and numbers of white cells and platelets, and in both mice about half of the cells were GFP+, with 0.26% (F4) and 0.08% (F5) of bone marrow cells Lin-, c-kit+, Sca-1+, similar to those of normal mice. Among them, 58%

(F4) and 49% (F5) were GFP+, in agreement with the data obtained with the colony formation assays. These results suggest that in the analyzed animals the pancytopenia usually associated with the oc/oc phenotype was corrected. However, we cannot exclude that in other mice, especially those with persistent splenomegaly, partial extramedullary hematopoiesis coexisted with bone marrow hematopoiesis. From these results we conclude that the oc/oc pathology appears to be particularly sensitive to early treatment by IUT.

Papers published within the project

Cappariello A, Berardi AC, Peruzzi B, Del Fattore A, Ugazio A, Bottazzo GF, Teti A. Committed osteoclast precursors colonize the bone and improve the phenotype of a mouse model of autosomal recessive osteopetrosis. *J Bone Miner Res* 2009 (in press).

Tondelli B, Blair HC, Guerrini M, Patrene KD, Cassani B, Vezzoni P, Lucchini F. Fetal liver cells transplanted in utero rescue the osteopetrotic phenotype in the oc/oc mouse. *Am J Pathol* 2009;174:727-35.

CHARACTERIZATION OF NEW GENETIC DEFECTS IN MAD

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Mandibuloacral dysplasia type A as a prelamin A-accumulating disease

MAndibuloacral Dysplasia type A (MADA) is a rare progeroid syndrome characterized by dysmorphic craniofacial and skeletal features, lipodystrophy, and metabolic complications. Most patients carry the same homozygous missense mutation (p.R527H) in the LMNA gene C-terminal domain, which encodes lamin A/C, a nuclear envelope component. Prelamin A, the precursor protein of lamin A, undergoes posttranslational modifications at its C-terminal CaaX residue. The finding that accumulation of non-processable prelamin A is the molecular basis of the most severe laminopathies, including MADA, opened new perspectives in the study of their pathogenic mechanisms. In this project, we studied the involvement of defects of prelamin A processing in MADA pathogenesis. In a first study, we reported a patient carrying new compound heterozygous p.R527H and p.V440M mutations and showing an atypical MADA-like phenotype with normal metabolic profiles and absence of clavicle dysplasia. The patient's cells showed accumulation of prelamin A, nuclear shape abnormalities and irregular nuclear lamina thickness. Heterochromatin defects were also detected by electron microscopy, but the major heterochromatin proteins were normally distributed. Subsequently, we investigated the effects on chromatin organization of two drugs inhibiting prelamin A processing.

The farnesyltransferase inhibitor FTI-277 and the drug N-acetyl-S-farnesyl-L-cysteine methylester (AFCMe) were administered to control human fibroblasts. FTI-277 caused accumulation of non-farnesylated prelamin A, while AFCMe caused farnesylated prelamin A accumulation. FTI-277 induced redistribution of heterochromatin domains at the nuclear interior, while AFCMe caused loss of heterochromatin domains, nuclear size increase and nuclear lamina thickening. Heterochromatin-associated proteins and LAP2α were clustered at the nuclear interior following FTI-277 treatment, while they were unevenly distributed or absent in AFCMe treated nuclei. We could conclude that chromatin organization depends on the accumulation of prelamin A forms, and that FTIs or other prelamin A inhibitors are potentially useful for the therapy of laminopathies.

Since MADA features bone resorption, FTI-277 or AFCMe were also applied to peripheral blood monocytes induced to differentiate towards the osteoclastic lineage. We found that monocytes subjected to both drugs, differentiate towards the osteoclastic lineage more

efficiently than untreated monocytes, in terms of number of multinucleated giant cells, mRNA expression of osteoclast-related genes and TRACP 5b activity.

This finding may help to understand the osteolytic processes that characterize MADA and other progeroid laminopathies. Since we demonstrated that drugs acting on prelamin A processing can improve MADA cellular phenotype, it is important to be able to define which type of prelamin A form accumulates in various laminopathies. We studied two new antibodies specific to full-length prelamin A or carboxymethylated farnesylated prelamin A, respectively. Moreover, we were able to show that different prelamin A forms and emerin can influence each other's localization: while emerin absence at the inner nuclear membrane leads to unprocessed prelamin A aberrant localization only, accumulation of prelamin A (non-farnesylated or farnesylated) in human fibroblasts modifies emerin localization. Finally, we generated the first MADA mouse model carrying the p.R527H LMNA missense mutation. The mice show bone defects (resorption/absence of clavicles, round skull shape) and alopecia. Serum MMP9 activity is also changed. We will check the possible molecular targets for developing a therapeutic approach for MADA.

In vitro and *in vivo* treatment with statins and aminobisphosphonates

In MADA cells, prelamin A accumulates at the nuclear rim being responsible for cellular alterations. The post-translational modification of prelamin A involves the isoprenylation of the cysteine residue at the C-terminal domain. Disruption of the mevalonate pathway, with block of the isoprenyl groups biosynthesis, is obtained by means of bisphosphonates and statins. We tested the effects of these drugs at different levels: MADA fibroblasts were treated with statins for 24 hrs and then bisphosphonates for 12hrs in a single dose. A reduction of the number of misshapen nuclei and a partial rescue of the heterochromatin organization was obtained. Then, we developed a therapeutic protocol based on the capacity to reduce, prevent or delay the symptoms of MADA disease.

We evaluated the efficacy and tolerance of pravastatin (statin) and zoledronic acid (bisphosphonate) in association in a non-randomized and open label study. Given the small number of patients, a double-blind placebo controlled protocol could not be envisaged and we decided for a protocol with a Single Group Assignment.

We established the MADA inclusion/exclusion criteria, the dosage and modality of administration for both drugs, and a programmed follow-up. Moreover, we envisaged different primary and secondary outcomes in relation to main disease signs. At the moment, two patients are enrolled. Side effects are not reported to date for both drugs, except for a reaction of acute phase with feverish rise, regressed after a treatment with paracetamol for two days. The patients reported relieve of pain in the distal phalanges of the fingers.

Papers published within the project

Araújo-Vilar D, Lado-Abeal J, Palos-Paz F, Lattanzi G, Bandín MA, Bellido D, Domínguez-Gerpe L, Calvo C, Pérez O, Ramazanova A, Martínez-Sánchez N, Victoria B, Costa-Freitas AT. A novel phenotypic expression associated with a new mutation in LMNA gene, characterized by partial lipodystrophy, insulin-resistance, aortic stenosis and hypertrophic cardiomyopathy. *Clin Endocrinol* 2008;69(1):61-8.

Araújo-Vilar D, Lattanzi G, González-Méndez B, Costa-Freitas AT, Prieto D, Columbaro M, Mattioli E, Victoria B, Martínez-Sánchez N, Ramazanova A, Fraga M, Beiras A, Forteza J, Domínguez-Gerpe L, Calvo C, Lado-Abeal J. Site-dependent differences in both prelamin A and adipogenic genes in subcutaneous adipose tissue of patients with type 2 familial partial lipodystrophy. *J Med Genet* 2009;46(1):40-8.

Camozzi D, Pignatelli S, Valvo C, Lattanzi G, Capanni C, Dal Monte P, Landini MP. Remodelling of the nuclear lamina during human cytomegalovirus infection: role of the viral proteins pUL50 and pUL53. *J Gen Virol.* 2008;89:731-40.

Capanni C, Del Coco R, Mattioli E, Camozzi D, Columbaro M, Schena E, Merlini L, Squarzoni S, Maraldi NM, Lattanzi G. Emerin-prelamin A interplay in human fibroblasts. *Biol Cell.* 2009;101(9):541-54.

Capanni C, Squarzoni S, Columbaro M, Mattioli E, Del Coco R, Camozzi D, Rocchi A, Scotlandi K, Maraldi NM, Foisner R, Lattanzi G. Prelamin A is involved in early steps of muscle differentiation. *Experimental Cell Research* 2008 314:3628-37.

Cenni V, Bertacchini J, Beretti F, Lattanzi G, Bavelloni A, Riccio M, Ruzzene M, Marin O, Arrigoni G, Parnaik V, Wehnert M, Maraldi NM, de Pol A, Cocco L, Marmiroli S. Lamin A Ser404 is a nuclear target of akt phosphorylation in C2C12 cells. *J Proteome Res.* 2008 Sep 23.

Di Masi A, D'Apice MR, Ricordy R, Tanzarella C, Novelli G. The R527H mutation in LMNA gene causes an increased sensitivity to ionising radiation. *Cell cycle* 2008;7(13):2030-7.

Dominici S, Fiori V, Magnani M, Schena E, Capanni C, Camozzi D, D'Apice MR, Le Dour C, Auclair M, Caron M, Novelli G, Vigouroux C, Maraldi NM, Lattanzi G. Different prelamin A forms accumulate in human fibroblasts: a study in experimental models and progeria. *Eur J Histochem.* 2009;53(1):43-52.

Garavelli L, D'Apice MR, Rivieri F, Bertoli M, Wischmeijer A, Gelmini C, De Nigris V, Albertini E, Rosato S, Virdis R, Bacchini E, Dal Zotto R, Banchini G, Iughetti L, Bernasconi S, Superti-Furga A, Novelli G. Mandibuloacral dysplasia type A in childhood. *Am. J. Med. Genet.* 2009, in press

Garg A, Subramanyam L, Agarwal AK, Simha V, Levine B, D'Apice MR, Novelli G, Crow Y. Atypical Progeroid syndrome due to heterozygous missense LMNA mutations. *J Clin Endocr Metab* 2009, submitted.

Lattanzi G, Columbaro M, Mattioli E, Cenni V, Camozzi D, Wehnert M, Santi S, Riccio M, Del Coco R, Maraldi NM, Squarzoni S, Foisner R, Capanni C. Pre-Lamin A processing is linked to heterochromatin organization. *J Cell Biochem* 2007;102(5):1149-59.

Lombardi F, Fasciglione GF, D'Apice MR, Vielle A, D'Adamo M, Sbraccia P, Marini S, Coletta M, Novelli G. Increased release and activity of MMP-9 in patients with Mandibuloacral Dysplasia, a rare premature aging syndrome. *Clin Genet* 2008;74(4):374-83.

Lombardi F, Gullotta F, Columbaro M, Filareto A, D'Adamo M, Vielle A, Guglielmi V, Nardone AM, Azzolini V, Grosso E, Lattanzi G, D'Apice MR, Masala S, Maraldi NM, Sbraccia P, Novelli G. Compound heterozygosity for mutations in LMNA in a patient with a myopathic and lipodystrophic mandibuloacral dysplasia type A phenotype. *J Clin Endocrinol Metab.* 2007;92:4467-71.

Maraldi NM, Capanni C, Mattioli E, Columbaro M, Squarzoni S, Parnaik WK, Wehnert M, Lattanzi G. A pathogenic mechanism leading to partial lipodistrophy and prospects for pharmacological treatment of insulin resistance syndrome. *Acta Biomed* 2007;78:207-15.

Maraldi NM, Lattanzi G. Involvement of prelamin A in laminopathies. *Crit Rev Eukaryot Gene Expr* 2007;17(4):317-34.

Marmiroli S, Bertacchini J, Beretti F, Cenni V, Guida M, De Pol A, Maraldi NM, Lattanzi G. A-type lamins and signaling: The PI 3-kinase/Akt pathway moves forward. *J Cell Physiol* 2009;220(3):553-61

Mattioli E, Columbaro M, Capanni C, Santi S, Maraldi NM., D'Apice M.R, Novelli G, Riccio M, Squarzoni S, Foisner R, Lattanzi G. Drugs affecting prelamin A processing: effects on heterochromatin organization. *Exp Cell Res* 2008;314(3):453-62.

Meaburn KJ, Cabuy E, Bonne G, Levy N, Morris GE, Novelli G, Kill IR, Bridger JM. Genome mis-organisation, genomic instability and apoptosis in primary fibroblasts containing A-type lamin or emerin mutations. *Aging Cell* 2007;6:139-53.

Novelli G. Terapia farmacologica delle sindromi progeroidi: inizio di un trial clinico. *Not Ist Super Sanità* 2008;21(11 Suppl.1): 9-13.

Zini N, Avnet S, Ghisu S, Maraldi NM, Squarzoni S, Baldini N, Lattanzi G. Effects of prelamin A processing inhibitors on the differentiation and activity of human osteoclasts. *J Cell Biochem* 2008;105(1):34-40.

DOES A RELATIONSHIP EXIST BETWEEN GH TREATMENT AND BMI IN PATIENTS AFFECTED BY SCOLIOSIS IN PRADER-WILLI SYNDROME?

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Introduction

The purpose of this study is to develop a protocol defining a clinical diagnostic procedure for the patients to be admitted to the authors' Institute to receive treatment for either suspected or confirmed diagnosis of spine deformity in Prader-Willi syndrome. The aim is to evaluate every subject from the diagnostic point of view, assessing variability of clinical expression and evolution of spinal deformity in the light of the related genetic aspects, thus providing a univocal protocol.

Material and methods

The present case series includes 18 patients; 7 (5 males, 2 females) were surgically treated and aged 12.8 years (range, 10 to 14.6 yrs) on average, whether the remaining 11 (mean age, 4.2 yrs; range 0.5 to 16.1 yrs) were males who received orthopaedic treatment at the outpatient department of Rare Diseases. As regards origin, 87% of the patients came from outside of the Region Emilia-Romagna and 61% from the central-southern regions, mainly from Latium (44%). Both standard BMI (weight/stature) and BMI Z-score (only for children aged over 2 years) were calculated.

The values of the BMI Z-score are included in 8 charts, each one related to a specific Z-score (-2, -1.5,-1,-0.5,0, 0.5,1,1.5,2), corresponding to the 8 various different growth curves of the children. The scores are the standard deviations from the mean values calculated on the reference population. The corresponding percentiles for standard normal distribution are calculated from the Z-scores. The various different weight categories derive from the range of the percentile values and are the following: underweight, normoweight, overweight and obesity. The percentiles are plotted in graphs, one per sex, depicting growth by age (Figure 1). This procedure allows researchers to observe, even on the graphs, each child's BMI compared to the other children of the same age and sex. Moreover, the GH treatment was recorded for each group of patients as follows: present, suspended or absent. The Chisquare and Fisher Exact statistical tests were used when the BMI Z-score could be calculated; when only standard BMI could be assessed, the non-parametric ANOVA test (Analysis of Variance) was used to calculate the difference between average BMI values.
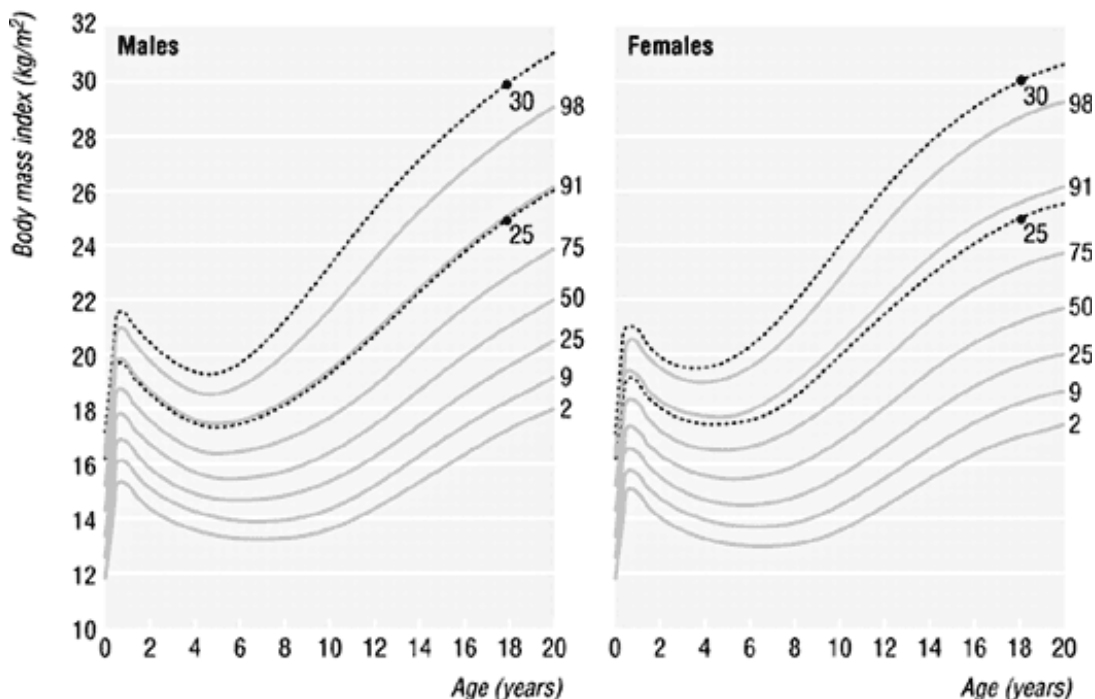
Figure 1. BMI values per age percentiles, divided by sex

Statistical analysis

The BMI and BMI Z-score of the Prader-Willi children receiving ambulatory care were equal to 17.54 (range 11.49 to 29.48) and 0.7 (range -0.7 to 1.7), respectively. Clinical records provided information about GH treatment: 8 patients had been treated with GH, in 2 cases treatment had been suspended and no data were available about the remaining 2 subjects.

As often reported in the literature (references, 1 to 10), there is a relationship between these 2 parameters; in the present case series, a statistically significant difference (p < 0.05) in terms of average BMI was observed and it was less significant in the little patients under GH treatment (16.8, SD=1.1) than in those who had suspended it (24.7, SD=6.8).

The BMI and BMI Z-score of the 7 Prader-Willi subjects, who underwent surgery for scoliosis, were equal to 29.41 (range, 21.03-41.72) and 1.8 (range, 0.51-2.77), respectively. This value was notably higher than that observed for the outpatients, whose average age was even much lower. The BMI Z-score could be calculated for all of the surgical patients, thus making it possible to identify the weight category through the growth graphs by age percentiles: only 2 subjects could be classified as normoweight.

Regarding GH treatment, 4 of the surgical patients had received it; however, the correlation with standard BMI and BMI Z-score did not prove to be statistically significant.

To increase the number of subjects in the case series, and since surgery cannot be considered as an effect modifier of the correlation between GH and standard BMI, the test was repeated including surgical and non-surgical patients: even in this case, no statistically significant difference was found, although the BMI of patients who had either suspended or never received

GH was equal to 28 (SD=8.7), whereas it was 21 (SD=6.1) in the remaining subjects under treatment.

In this latter group of patients, genetics was compared with BMI: 8 cases presented with a microdeletion of chromosome 15 and an average BMI of 15 (SD=3.4), in 3 uniparental disomy and an average BMI of 17 (SD=1.5) were observed, whereas no information on genetics were available in the remaining case. The difference was not statistically significant.

Discussion

The increased number of patients to enter the study will either confirm or deny the current findings which, so far, seem to highlight a significant relationship between GH treatment and standard BMI only in patients with less severe scoliosis (outpatients receiving orthopaedic treatment) and not in those who underwent surgery for more severe spinal deformities. These different results, however, might depend on the lower number of patients belonging to the surgical group. Finally, the present study revealed no correlation between BMI and genotype.

CYTOKINE-BASED IMMUNOTHERAPY AND TUMOR MICROENVIRONMENT IN MODELS OF CUTANEOUS AND OCULAR MELANOMA

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Uveal melanoma is a rare disease, distinct from the more frequent cutaneous melanoma, since metastatic ocular melanoma patients develop predominantly liver metastases. The general aims of this project were: 1) the development of of cytokine and antibody-based immunotherapy strategies in cutaneous and ocular (uveal) melanoma pre-clinical models; 2) the study of the redox potential and of chronic inflammation as tumor-promoting factors; and 3) the identification of new potential targets for immunotherapy in the extracellular matrix proteins of uveal melanoma.

The antitumor effects of the pro-inflammatory cytokine TNFalpha are mainly due to the preferential toxicity for the tumor-associated vasculature and to the ability to potentiate the immune response against tumors. For these reasons, TNFa may be used as an adjuvant in the formulation of antitumor vaccines.

L19mTNFa is a fusion protein composed by the scFv L19, specific for the highly conserved ED-B domain of fibronectin, a tumor-associated antigen identical in humans and mice, and m(ouse)TNFalpha that, in different mouse tumor models, induces a therapeutic T cell-mediated immune response that protects the host against syngeneic tumors of different histological origin. In the purpose of studying the efficacy of L19mTNFa as an adjuvant in vaccination protocols with melanoma homogenates, two melanoma models (B16F1 and B16BL6B17) were established in the syngeneic C57 black mice. These melanomas are low immunogenic tumors, that grow fast and do not respond to the systemic therapy with L19mTNFa and melphalan that, on the contrary, cures 80% of WEHI-164 fibrosarcoma and 20-30% of C51 colon carcinoma-bearing mice. In the attempt to induce an efficient immunological response against melanoma, a mix of the two melanoma homogenates, supplemented or not with L19mTNFa, was used. The vaccination protocol was chosen after a number of different attempts and consisted in 4 s.c. injections of melanoma homogenate supplemented or nor with L19mTNFa. After the fourth vaccination all the mice were s.c. challenged with a tumorigenic dose of B16F1 melanoma cells. The therapy with L19mTNFa and melphalan was given at day 10 and 11 after tumor challenge.

At day 15 after s.c. tumor cell injection, B16F1 tumor reached a volume of about 0.4 cm3 in all untreated control mice. In mice vaccinated with melanoma homogenate the tumor was reduced to 0.16 cm3, in average. The addition of L19mTNFa to the vaccine determined per se tumor rejection in 40% of the mice and, where present, a very reduced volume (0.02 cm3). Moreover, the treatment of mice with L19mTNFa and melphalan further improved the results in the above described groups of mice. In fact, with the treatment, 60% of the mice vaccinated with homogenate supplemented with L19mTNFa totally rejected tumor that, when present (40%), reached an almost negligible volume. Also in the group of mice vaccinated with homogenate alone the tumor, while palpable in all mice, had a volume in average 0.03 cm3. These results indicate that, in the melanoma models tested, the vaccination with L19mTNFa counteracts the tumor growth better than vaccination per se and that in both cases the therapy with L19mTNFa and melphalan further ameliorates the results. Noteworthy, after the third injection, all the mice vaccinated with homogenate supplemented with L19mTNFa presented

patches of alopecia areata and hair depigmentation usually associated to autoimmunity and described in mice actively immunized in the presence of adjuvants.

At different times during and after vaccination, we evaluated the variations of different immunological parameters in the spleen and lymphnodes. In the spleen of vaccinated mice, we observed that the percent and absolute number of CD4+ and CD8+ T cells significantly increased, with respect to naive control mice, from the second to the fourth vaccination. A CD8+ T cell-mediated specific lysis against B16F1 cells and a mixed Th1/Th2 type of response were also found in all vaccinated mice. On the contrary, no modulation of the CD11b+/Gr1+ myeloid-derived suppressor cells and of the CD4+/CD25+/FoxP3+ T regulatory cells was observed.

Compared to tumor-bearing control mice, a considerable down-modulation of the CD11b+/Gr1+ myeloid-derived suppressor cells (at the spleen level) and of the CD4+/CD25+/FoxP3+ T regulatory cells (in the tumor-draining LNDs) was observed after B16F1 tumor challenge in all vaccinated mice both treated and untreated with L19mTNFa and melphalan. While immune cell infiltrates (IIC) are very rare in B16F1 induced in naive mice, a significantly increased number of CD4+ and CD8+ T cells, B cells, dendrytic cells, granulocytes and macrophages was found in the tumors of all vaccinated mice, while no major variations respect to controls were observed in the number of CD11b+/Gr1+ cells.

Since no reliable syngeneic models of uveal melanoma exist, we used the B16LS9 melanoma subline, which mimics some characteristics of uveal melanoma. B16LS9 was previously derived from B16 cutaneous melanoma by repeated splenic injection and collection of liver metastases and retain metastatic tropism for the liver when injected i.v. or in the posterior eye chamber. By i.v. injection of B16LS9 100% of mice developed lung and liver metastases within 25 days (mean survival time 20.7+2.3), indicating that B16LS9 is a highly aggressive tumor. We engineered GM-CSF with an RGD peptide, which binds to the alpha-V/beta-3 integrins, with the aim to enhance local immune-stimulating effects of GM-CSF and reduce unwanteted systemic effects. GM-CSF/RGD-transfected B16F10 melanoma cells released biologically active GM-CSF/RGD (10ng/mL/24h/106 cells) and displayed it on the cell membrane. These cells were then admixed with B16L69, lethally irradiated and used as vaccine to treat B16LS9 melanoma metastases. Immunotherapy by the irradiated cellular vaccine beginning two days after metastasis induction produced only a slight increase in mean survival time (22.9+4), whereas the vaccine was active in an immunoprophylactic setting in naïve mice. To verify whether pre-existing or tumor-induced CD4+CD25- immunosuppressive Treg cells may limit the effects of immunotherapy we combined this treatment with an anti-CD25 mAb (PC61, 0.5mg per mouse), which targets Treg cells. Although the administration of anti-CD25 antibody alone had no effect on tumor-free survival, when it was combined with the cellular vaccine a significant increase in tumor-free survival was observed (41.1+9, P<0.001). Also an immune-enhancing anti-OX40 agonistic mAb, which has been reported to inhibit Treg cell functions and co-stimulate effector T cell functions, enhanced the effect of the vaccine leading to an increased survival time (28.1+13). More importantly, the effect of a "triplex" combination of anti-CD25+ anti-OX40 mAbs + B16GM-CSF vaccine showed a superior effect on disease-free survival of mice bearing B16LS/9 i.v. metastases and cured 31.2% of them at long term (>80 days) (Survival time 58.5+38; P<0.0001), while a combination of anti-CD25 and anti-OX40 mAb without vaccine allowed only to enhance the mean survival time, but no mice were cured. Re-challenge of cured mice with wild-type B16LS/9 showed immunity to melanoma in 40% of cured mice. Spleen cells of these mice showed enhanced IFN-gamma secretion by ELISPOT when challenged *in vitro* with irradiated B16LS/9 cells. The effects of the triplex immunotherapy were dependent on both CD4 and CD8 T cell responses as shown by *in vivo* antibody-mediated CD4+ or CD8+ cell depletion experiments, which showed a clear-cut

reduction of triplex therapy effects in tumor-bearing CD4- or CD8-depleted mice. These data indicate that a Treg cell depleting anti-CD25 antibody can be effectively combined with a co-stimulatory anti-OX40 mAb and a GM-CSF-based cellular vaccine in an aggressive model of metastases, which mimics uveal melanoma metastatic behavior.

We also investigated the redox of the tumor microenvironment and the role of redox changes in the inflammatory processes and in tumor progression. The results obtained indicate that redox modulation is strongly implicated in both the innate and adaptive immune response: in particular, the secretion of IL-1ß, a pro-inflammatory cytokine involved in tumor progression, is strongly increased by a reducing extracellular milieu. Moreover, in advanced neoplasia the oxidative stress is overwhelmed by an antioxidant response, with strong production and secretion of non protein thiols (GSH and/or reduced cysteine) and overexpression of the oxido-reductase thioredoxin. The reduced microenvironment may thus support the production of IL-1ß, worsening the chronic inflammatory state. Analyses of tumor cell lines at different levels of aggressiveness revealed a correlation between redox and malignancy: cells with a more reduced phenotype displayed a more aggressive behaviour both *in vitro* and *in vivo*. Moreover, the redox phenotype of tumor cells dictates the response to pro-oxidant drugs such as Arsenic trioxide suggesting that the redox phenotype may represent a new valuable marker of prognosis and response to therapy.

The composition and the role of the extracellular matrix (ECM) in melanoma and the possible use of tumor matrix proteins as targets for immunotherapy, were also examined. Cancer cells can alter their adjacent stroma to form a permissive and supportive environment for tumor progression and produce a range of growth factors and proteases that modify their stromal environment and induce angiogenesis and inflammation. The induction of inflammation in the tumor stroma also results in production of a range of factors as ECM components that promote tumour progression. Periostin, a secretory protein of the ECM that has been involved in cell adhesion and tumor formation, is produced in the stromal environment. In conflicting reports the origin of the periostin is attributed to cancer cells or to stromal cells surrounding the cancer cells. It has been reported that Periostin is not expressed in human normal melanocytes. We studied periostin isoforms as new potential tumoral markers.

We found that both fibroblasts and melanoma cells isolated from melanomas specimen produced mRNA of periostin but each cell type has its own typical pattern of alternative splicing mRNA expression. We have also studied the different periostin isoforms expression in five uveal melanoma tissues. Preliminary results indicate that both types of tumor-derived cells produce periostin mRNA, but only melanoma cells express exon 17 in 50% of cases (3/6). On the contrary, all (4/4) the uveal melanoma tissues analyzed so far express isoforms of periostin containing the exon 17, thus suggesting that exon 17 of periostin is a potential marker of this tumor.

We have produced the full-length cDNA and fragments of human periostin and expressed them in E. coli. Recombinant proteins were used for mice immunization to produce murine monoclonal antibodies and to select a phage display library to produce human recombinant antibodies.

Finally we have also expressed human periostin in mammalian cells (human renal epithelial cell line HEK-293) to obtain a protein with complete post-translational modifications. The periostin, purified using an affinity column, will be used in studies of biological activities.

Papers published within the project

Balza E, Sassi F, Ventura E, Parodi A, Fossati S, Blalock W, Carnemolla B, Castellani P, Zardi L, Borsi L. A novel human fibronectin cryptic sequence unmasked by the insertion of the angiogenesis-associated extra type III domain B. *Int J Cancer* 2009;125(4):751-8.

Carta S, Castellani P, Delfino L, Tassi S, Venè R, Rubartelli A. DAMPs and inflammatory processes: the role of redox in the different outcomes. *J Leuk Biol* 2009;86(3):549-55.

Castellani P, Angelini G, Delfino L, Matucci A, Rubartelli A. The thiol redox state of lymphoid organs is modified by immunization: role of different immune cell populations. *Eur J Immunol* 2008;38(9):2419-25.

Ceccarelli J, Delfino L, Zappia E, Castellani P, Borghi M, Ferrini S, Tosetti F, Rubartelli A. The redox state of the lung cancer microenvironment depends on the levels of thioredoxin expressed by tumor cells and affects tumor progression and response to prooxidants. *Int J Cancer* 2008;123(8):1770-8.

Piccini A, Carta S, Tassi S, Lasiglié D, Fossati G, Rubartelli A. ATP is released by monocytes stimulated with pathogen-sensing receptor ligands and induces IL-1beta and IL-18 secretion in an autocrine way. *Proc Natl Acad Sci USA* 2008;105(23):8067-72.

Rubartelli A, Lotze MT. Inside, outside, upside down: damage-associated molecular-pattern molecules (DAMPs) and redox. *Trends Immunol.* 2007;28(10):429-36.

Rubartelli A, Sitia R. Stress as an intercellular signal: the emergence of stress associated molecular patterns (SAMP). *Antioxid Redox Signal* 2009;11(10):2621-9.

Tassi S, Carta S, Venè R, Delfino L, Ciriolo MR, Rubartelli A. Pathogen-induced Interleukin-b processing and secretion is regulated by a biphasic redox response. *J Immunol* 2009;183(2):1456-62.

MULTIDRUG RESISTANCE DEPENDANCE ON CYTOSKELETON INTEGRITY IN SOLID TUMOURS

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Introduction

Osteosarcoma is an orphan disease with approximately 1,200 new cases diagnosed in the United States each year. A similar incidence of the disease exists in Europe. According to the Children's Oncology Group (COG), the survival of children with osteosarcoma has remained at 60-65 percent since the mid-1980s. Chemotherapy is the mainstay of treatment for disseminated solid tumors, including ostesarcoma. Multidrug resistance to antitumor agents is a major cause of treatment failure in patients with cancer. Overexpression of the mdr1 gene-encoded P-glycoprotein (Pgp, p170) exerts a major role in reducing effectiveness of cytotoxic therapy.

Mounting evidence highlighted the involvement of Pgp/actin cytoskeleton interactions in tumor multidrug resistance. Ezrin exerts a key role in linking actin filaments and several plasma membrane proteins and we previously depicted its crucial role in conferring Pgp mediated resistant phenotype in lymphoid cells. Herein we address ezrin's role in osteosarcoma chemoresistance, employing a multidrug resistant Pgp over-expressing osteosarcoma cell line (Saos-2 DX580) and its stably transfected counterpart that expresses a deletion mutant of ezrin(Δ146) devoid of the C-terminal actin-binding domain (Saos-2 DX580Δ146).

Our results highlight that Pgp functionality in multidrug resistant cells depends on its plasma membrane expression and colocalization with both ezrin and the plasma membrane ganglioside Gm1 which selectively partitions into lipid rafts. Cytotoxic assays and drug retention studies (data not shown) revealed that in Saos-2 DX580Δ146 the expression of ezrin deletion mutant restored drug susceptibility consistently with Pgp-1 dislocation to cytoplasmic compartments. These data depict ezrin's key role in the MDR phenotype of human osteosarcoma cells and indicate the possibility of ezrin mutants as MDR reverting agents.

The prognosis of patients with osteosarcoma remains guarded, although in recent years treatment strategies have relied on advances in both surgery and available chemotherapeutic agents. Tumor multidrug resistance develops after repeated exposure to antineoplastic agents. A key signature of multidrug resistance is that selection of drug-resistant cell lines with a single agent can confer resistance to several structurally unrelated compounds, including vinca alkaloids, epipodophyllotoxins, anthracyclines and actinomycin D.

Multidrug resistance frequently correlates with overexpression of the ABC superfamily efflux pumps-related genes among which the first discovered and best characterized member in humans (mdr-1), encodes a surface membrane glycoprotein of approximately 170,000 daltons: P-glycoprotein [also referred to as: ABCB1, ATP-binding cassette sub-family B member 1, MDR-1, PGY1, CD243 and Pgp]. A direct correlation between Pgp expression levels and the degree of drug resistance has been established both *in vitro* and *in vivo* in cell lines of different origins, selected for multidrug resistance, allowing cancer cells to circumvent otherwise lethal effects of a wide variety of antineoplastic drugs. In osteosarcoma, Pgp expression and involvement in drug resistance, has also been proposed at diagnosis, as a prognostic factor.

Herein, we addressed ezrin's role in Pgp functional membrane localization and subsequent pharmacological response in human osteosarcoma. To this end, we employed an ezrin deletion

mutant (Δ146) which lacks the C-terminal actin-binding domain but is still capable of interacting with plasma membrane transmembrane proteins.

The MDR Pgp over expressing human osteosarcoma cell line (Saos-2 DX580) was used in parallel with its stably transfected counterpart (Saos-2 DX580 Δ146) in order to analyze: 1) cellular expression, distribution and molecular association of Pgp with ezrin in human osteosarcoma and 2) the effects of the latter on drug mediated cytotoxicity, drug efflux and Pgp subcellular localization as well as its interaction with actin.

The results depict a critical role for Pgp/ezrin linkage in Pgp plasma membrane localization, which parallels the establishment of a MDR phenotype in human osteosarcoma. Interestingly, the MDR phenotype is abrogated following the overexpression of an ezrin deletion mutant devoid of actin binding properties (data not shown); concomitantly with loss of plasma membrane Pgp localization.

Discussion

P-glycoprotein expression correlates with treatment failure in patients with osteosarcoma. However, the mechanism underlying cellular accumulation and distribution of cytotoxic drugs and their correlation with Pgp functionality are still poorly defined. In this study we evidenced that the ezrin-to-Pgp connection is key in the establishment of Pgp mediated multidrug resistance in an *in vitro* human osteosarcoma model comprising both the MDR and the chemo sensitive counterparts.

In conclusion, we further confirm that the activity of a transporter such as Pgp, may be altered not only by deranged expression of the former but also by translocation and/or deregulated cellular localization. Extracellular acidosis in solid growing tumors have been recently shown to cause a chemoresistant phenotype due to increased Pgp activity; providing evidence that that the surrounding micro environment reflects on the protein activity and therefore the protein cellular localization is crucial for its performance. Preliminary data lets us envisage that the ezrin deletion mutant we employed may abrogate MDR also in other solid tumors.

To this end, we have previously demonstrated that tumor aggressiveness parallels capacity of survivin in harsh acidic environment and the development of a series of characteristics such as cannibalic activity and that over expression of the truncated ezrin Δ146 abrogated cannibalic activity in human melanoma cells. Over all, we provide evidence that deranged cytoskeletal plasma membrane interactions target more than one aspect defining a tumor's phenotype and aggressiveness, despite tumor hystology.

IMMUNOBIOLOGIC AND CLINICAL ACTIVITY OF DNA HYPOMETHYLATING AGENTS IN HUMAN SARCOMAS

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Sarcomas account for approximately 1% of human malignancies and are frequently locally aggressive and/or metastasize. Adjuvant therapy reduces local disease recurrence, although no convincing effects on overall survival have been demonstrated yet; thus, newer treatments are urgently needed for human sarcomas. In this respect, an appealing option is represented by immunotherapeutic treatments, such as those targeting the Cancer Testis Antigens, that are currently being utilized with promising results in other solid tumors. Moreover, the DNA hypomethylating agent (DHA) 5-aza-2'-deoxycytidine (5-AZA-CdR) was recently demonstrated to possess important immunomodulatory properties, among which: i) inducing/up-regulating the expression of CTA in neoplastic cells; ii) functionally reverting the intratumor clonal heterogeneity of CTA expression; iii) up-regulating a set of "immune molecules" (e.g., HLA class I, costimulatory molecules), which positively modulate the recognition of tumor cells by immune effectors. Based on these notions, we focused at defining the expression of CTA and the immunomodulatory potential of DHA in human sarcomas to eventually design novel therapeutic strategies for this malignancy. The study was mainly conducted on the controversial entity of the Malignant Fibrous Histiocytoma (MFH) both to evaluate new molecular profiles that could extend its morphological classification criteria, and to provide alternative therapeutic approaches to patients affected by this heterogeneous malignancy. RT-PCR analyses on 35 unrelated MFH tissues demonstrated that CTA are expressed in this malignancy, albeit at different frequencies: 11% for MAGE-A4, 17% for MAGE-A10, 20% for NY-ESO-1, 23% for MAGE-A3, 37% for MAGE-A2, 43% for MAGE-A1, 51% for SSX 1-5 and 60% for GAGE 1-6. The expression of at least one CTA was observed in 71% of lesions, and their expression appeared to be co-regulated, resulting in their clustered expression in investigated tissues. Chi-squared statistical analysis showed a significant ($p<0.05$) association between the expression of MAGE-A1 and that of MAGE-A2, -A3, GAGE 1-6 and SSX 1-5, between MAGE-A2 and MAGE-A3 and GAGE 1-6, between MAGE-A3 and MAGE-A10 and SSX 1-5, between MAGE-A4 and NY-ESO-1, between MAGE-A10 and NY-ESO-1, and between GAGE 1-6 and SSX 1-5. Noteworthy, RT-PCR analysis on metachronous lesions demonstrated that the pattern of CTA expression was highly conserved through disease progression in 5 out of 7 MFH patients, confirming previous observations in other malignancies. MFHs can be morphologically divided into the "spindle cell" and the "pleomorphic" subtypes. Since these two principal categories associate with different clinical outcomes, we asked the question whether CTA expression is differentially distributed among these subtypes. Although the limited number of patients analyzed prevents to draw general conclusions, it is intriguing the observation that the "pleomorphic" type, which represents the more aggressive form of the tumor, was associated with a much higher frequency of CTA expression as compared to the "spindle cell" variant. In order to evaluate presence of their protein and their intratumoral distribution, the characterization of the constitutive expression of CTA in MFH tissues was

finally completed by immunohistochemical (IHC) detection of MAGE-A family antigens. Concordant results were observed between RT-PCR and IHC data, nevertheless intratumor distribution of CTA appeared to be heterogeneous, with concomitant presence of CTA-positive and -negative neoplastic foci.

Based on the epigenetic regulation of CTA expression and on the immunomodulatory activity of 5-AZA-CdR already demonstrated in cutaneous melanoma, dose-finding experiments were performed to characterize the potential immunological effects of DHA in human sarcomas. Ten sarcoma cell lines, either established in culture from metastatic lesions surgically removed at our institution or available from trade, were treated with scalar doses of 5-AZA-CdR (0.1μM, 0.2μM, 0.5μM, or 1μM). Both quantitative RT-PCR and indirect immunofluorescence analyses demonstrated a dose-dependent induction/up-regulation of CTA in sarcoma cell lines, though even the lowest concentrations of the drug were able to generate high levels of CTA expression. Furthermore, the single cycle of 5-AZA-CdR employed was demonstrated to be sufficient to induce the ex-novo expression of either NY-ESO-1 or MAGE-A in 20 to 60% of the neoplastic cells of the treated populations. Concomitantly, 5-AZA-CdR treatment up-regulated ($p<0.05$) the cell surface expression of HLA class I antigens and induced/up-regulated ($p<0.05$) the expression of ICAM-1 mRNA and cell surface protein in the panel of sarcoma cell lines under investigation, showing a dose-dependent response. This modulation was paralleled by an increase in the mRNA levels of the antigen processing molecules TAP1 and TAP2 following 5-AZA-CdR treatment in 7 out of 10 investigated cell lines, while no effect was seen on the expression of tapasin.

Overall, these data, though preliminary, suggest that CTA-based immunotherapies are worth consideration as alternative, feasible, therapeutic options for MFH patients, and strongly suggest that DHA may represent useful therapeutic agents to comprehensively increase immunogenicity and immune recognition of sarcoma cells, providing the rationale for their use in new combined chemo-immunotherapeutic approaches in the sarcoma clinic. Laying on these data, a phase I/II clinical study will be designed and finalized for the second line treatment of patients with metastatic sarcoma.

Papers published within the project

Nicolay HJ, Sigalotti L, Fonsatti E, Covre A, Fratta E, Coral S, Maio M. Epigenetically-regulated Tumor-Associated Antigens in melanoma. *Expert Rev Dermatol* 2009;4(2):145-54.

INNOVATIVE BURKITT'S LYMPHOMA THERAPY

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Introduction

Burkitt Lymphoma (BL), one of the most aggressive human cancers, is a very rare form of adult cancer in the western world (sporadic form 3 cases per 10.000.000) usually HIV-associated, but it's quite frequent (endemic form)1 among children from Central Africa, New Guinea and South America (6-7 cases per 100 000) with a peak incidence at 6 or 7 years of age and often malaria associated.

BL is a monoclonal proliferation of B lymphocytes, often carrying EBV(Epstein-Barr virus), characterized by small non cleaved cells that are uniform in appearance and produce a diffuse pattern of tissue involvement. Histological and morphologic characteristics are: medium sized cells, round nuclei, multiple nucleoli, relatively abundant basophilic cytoplasm all with high rate of proliferation, cell death (apoptosis). A "starry sky" pattern, imparted by numerous benign macrophages that have ingested apoptotic tumor cells is shown in the histological preparations

Most Burkitt's lymphomas are characterized by chromosomal translocations and in particular by the t(8;14) that juxtaposes the c-myc oncogene to one of the Ig loci. Since c-myc determines some of the phenotypic and functional characteristics like: CD10 and CD38 expression, cell proliferation, propensity to apoptosis, inhibition of differentiation, because of the new altered topography this gene becomes up regulated and promotes continuous cell proliferation.

Now a days treatment of adults patients with an intense dose of CODOX-M-IVAC (cyclophosphamide; doxorubicin, vincristine; high methotrexate-isofosfamide, etoposide and high cytarabine) a regimen validated also for HIV-positive patients is promising. The situation is far bleaker in BL patient with high proliferative cells rate since this form of the disease is refractory to therapy and relapses after treatment even with high-dose chemotherapy coupled by stem cell rescue. Among these patients refractory or not eligible for aggressive treatments, are several HIV/BL patients.

For all the above reasons alternative experimental protocols should be considered. In this perspective we propose a new gene therapeutic approach based on the selective inhibition of c-myc oncogene.

In our studies we devised new strategies for BL therapy based on anti-gene PNAs. Specifically we used a PNA that binds to a unique c-myc sequence (PNAmyc); a second PNA that is complementary to the core of the Eμ enhancer (PNAEμwt)5. PNAEμwt has few advantages since not only inhibits c-myc over expression and consequent hyper proliferation of BL cell (like the BRG cell line where the translocated c-myc is under the control of Eμ) but also it selectively down regulates the translocated oncogene therefore specificity inhibiting the "pathological" c-myc expression.

Therefore, based upon the already reported *in vitro* results, BL cells treatment with PNAEμ should result in the loss of a number if not of all the malignant properties also *in vivo*, therefore acting as antineoplastic agent.

As a model system, we initially used SCID mice injected s.c. with BL cells which progressed to reproducibly form subcutaneous tumor masses. A number of pharmacokinetic parameters for

PNAEµ were also evaluated. In particular, we determined that the therapeutically active portion of PNA persisted in tumor tissues for a prolonged time after administration and that the compound did not exhibit acute toxicity even after chronic injection at the highest possible dose and this molecule did not present any immunogenicity in immunocompetent mice.

Both pre-treatment of BL cells with PNAEµwt before inoculum and chronic intravenous administration of PNAEµwt to mice already inoculated with BL cells selectively caused increased latency of tumor appearance and decreased final tumor size. Tumors from PNAEµwt-treated animals showed substantial areas of cell necrosis and of c-myc down regulation. Inhibition of tumor growth was specific and was not observed with PNAEµmut treatment, with at least two bases sequence mutations, and in the BL cell lines where the translocated c-myc is not under the control of the Eµ enhancer.

PNAEµ can also significantly reduce Burkitt's lymphoma tumor burden in a SCID mice model with cells dissemination similar to the human disease. In a recent study the therapeutic potential of PNAEµ was evaluated in a systemic mouse model. BRG-BL cells transfected with the luciferase gene were inoculated intravenously into SCID mice resulting in a preferential expansion, similar to the one of human adult patients, in the abdominal cavity, central nervous system and bone marrow.

Bioluminescent imaging was carried out with a highly sensitive CCD camera mounted in light-tight specimen box (IVIS, Xenogen 100 imaging system). Mice were injected intraperitoneally (150 mg/kg^{-1} in phosphate buffered saline) with D Luciferin (Biosynth International Inc., Naperville, IL), and anesthetized with 1-3% isofluorane and then placed in the supine position on the stage inside the camera box with continuous exposure to isofluorane. Imaging gives a linear response in photons/second for exposure times ranging from 1 to 5 min. A colorimetric coded reference bar (from purple: minimum to red: maximum) allows a 'first glance' intensity quantization of the luminescence. Bioluminescent signals from BRG-BL-Luc cells, as detected by the camera system, were recorded, integrated, digitalized, displayed and quantified (in photons/second) using the Living Image (Xenogen) software15. Tumor cells growth in the PNAEµwt or control PNAEµ-treated groups was calculated (photons/seconds ± s.d.) by measuring the luminescence emitted in each mouse by the BRG-Luc cells, during tumorogenesis at increasing times and number of treatments, with 2.5 min of exposure and with a homogeneous sensitivity setting.

To define the optimal animal model system, groups of female SCID mice were injected intravenously with BRG-BL-Luc increasing cells ($1x10^5$, $2x10^5$ and $4x10^5$ per mouse/experiment). Luminescent images of mice inoculated with different BRG-BL-Luc doses at increasing exposure times (1.0, 2.5, 5.0 min) were recorded at various time points after inoculation. Average total luminescence per time point per mice ± s.d. was determined using the appropriate software.

From these experiments, $2x10^5$ cells per mouse was determined to be the optimal dose capable of inducing tumors, as they were already detectable at day 7 and were consistent for site and time of appearance in different experiments.

The mice were chronically injected intraperitoneally either with PNAEµwt or with control PNA. The treatment was stopped when the control animals developed severe neurological symptoms. Overall tumor growth in PNAEµ-treated mice decreased by 80% as detected both by imaging and inspection at necropsy.

Histological and immunohistochemical studies showed, only in PNAEµ-treated mice, a substantially reduced BL cell growth at the major sites of invasion and vast areas of necrosis in the lymphomatous tissues with concomitant c-myc expression down regulation. Cells at boundaries of necrotic areas displayed nuclear changes including shrinkage, increased

basophilia and karyorrhexis. Furthermore, abnormal nuclei intermingled with coagulated non-nucleated eosinophilic cells were predominant near to the center of necrotic areas.

Collectively, the above findings indicate that two mechanisms possibly operated in the PNAEµ-treated animals to prevent tumor spreading. One was represented by the block of cell proliferation caused by c-myc down regulation and the other by cell lysis (which probably occurred after the c-myc down regulation) and resulted in growth arrest.

Indeed, earlier *in vitro* observations showed that the inhibition of c-myc expression by PNAEµwt is followed by growth arrest of the proliferating BL cells and concomitant apoptosis inhibition. In these conditions, some of the cells undergo terminal plasma-cell differentiation.

Altogether, the data support the therapeutic potential of PNAEµ in human adult BL.

Another aim of this project was to synthesize specific anti gene PNAs for the genes encoding the critical latent antigens of EBV. This strategy deserves further in depth investigation in the attempt to "cure" EBV- driven B cell lymphoproliferative disorders typical of immuno suppressed patients (AIDS later stages or organ transplanted).

Therefore, in this additional series of studies, PNAs specific for the genes encoding the latent antigens of EBV EBNA1 and LMP1 were designed and synthesized together with their control matching scrambled sequence:

- PNAEBNA1-$_{wt}$ 3' VKRKKKP-tggttagagagtc-5'(NH2); PNAEBNA-1-$_{scr}$ 3' VKRKKKP-ctgtgtgtggaaa-5'(NH2)
- PNALMP1-$_{wt}$ 3' VKRKKKP-atggaacacgacctt-5'(NH2); PNALMP1-$_{scr}$ 3' VKRKKKP-cttatggacagacac-5'(NH2)

The EBNA1 and LMP1 antigens are utilized by the latent virus to promote infected B cells growth.

Anti gene PNAs for EBNA-1 and LMP1 were tested *in vitro* in B lymphoblastoid (LCL) or Burkitt Lymphoma (BL) cell lines expressing both EBV antigens (LATIII). They were found -to effectively block the expression of both viral proteins furthermore -*in vitro*/in culture, in the presence of either anti gene PNA, B cells latently infected with EBV stopped growing within 24-48 hrs. Experiments are now in progress to test these reagents *in vivo* in the SCID mice model.

Therefore this type of approach too may be useful in the treatment of EBV-positive BL. In fact these cells have a proven expression of the EBNA-1 antigen that appears to facilitate their survival/ expansion *in vivo*.

Papers published within the project

Boffa LC, Cutrona G, Cilli M, Matis S, Damonte G, Mariani MR, Millo E, Moroni M., Roncella S, Fedeli F, Ferrarini M. Inhibition of Burkitt's lymphoma cells growth in SCID mice by a PNA specific for a regulatory sequence of the translocated *c-myc*. *Cancer Gene Therapy* 2007;14(2):220-26

Boffa LC, Menichini P, Bolognesi C, Cutrona G, Roncella S, Damonte GL, Millo E, Mariani MR, Matis S, Russo D, Ciliutti P, Ferrarini M. Lack of mutagenicity and clastogenicity of a PNA targeted to a regulatory sequence of the translocated *c-myc* oncogene in Burkitt's Lymphoma. *Mut Res Genet Toxicol Environ Mutagen* 2007;628(2):129-37.

Cutrona G, Boffa LC, Mariani MR, Matis S, Damonte G, Millo E, Roncella S, Ferrarini M. The PNA targeted to a regulatory sequence of the translocated *c-myc* oncogene in Burkitt's Lymphoma lacks immunogenicity. Follow up characterization of PNAEµ-NLS. *Oligonucleotides* 2007,171(1):146-50.

Matis S, Mariani MR, *et al.* PNAEµ can significantly reduce Burkitt's lymphoma tumor burden in a SCID mice model: cells dissemination similar to the human disease *Cancer Gene Ther* advance online publication, 10 April 2009; doi:10.1038/cgt.2009.26

Recorded and under review patents

PCT/EP2008/008052 (published March 25, 2009) Lidia Boffa, Giovanna Cutrona, Manlio Ferrarini; PNA conjugates targeted to the Eμ enhancer of the Ig locus as therapeutic agent for BCL2 translocation-driven Follicular Cell Lymphoma clonal expansion.
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TARGETING THE PROGNOSTIC AND METASTASIS-PREDICTING SURFACE PROTEOGLYCAN NG2 FOR IMMUNOTHERAPEUTIC TREATMENT OF SELECTED SARCOMAS

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Transcriptional levels of NG2 cell surface ProteoGlycan (PG) are highly upregulated in a variety of soft-tissue sarcoma histologic subtypes represented within a cohort of >100 patients and the PG appears de novo expressed in primary lesions when compared to the adjacent connective tissue from which the tumour may have arose. This implies that NG2 is part of a transcriptional program that is activated upon neoplastic transformation of mesenchymal cells. Metastatic lesions of lung occurring in a subset of these patients showed a >50-fold increase of NG2 mRNA levels when compared to those detected in the primitive tumour masses and this enhanced expression was corresponded by a more abundant distribution of both the PG core protein and its principal molecular ligand contained by the tumour microenvironment, collagen type VI (Col VI). Interestingly, surface expression of NG2 in both primitive and metastatic lesions was heterogenous and restricted to certain subsets of neoplastic cells, the identity of which remains, however, to be established. Given its notorious malignancy-associated role, these findings suggest that in soft-tissue sarcoma NG2 expression could be restricted to the more malignant subpopulation of neoplastic cells. Parallel experiments involving NG2+ and NG2- cells immunosorted from a variety of sarcoma cell lines and primary sarcoma cells isolated in the laboratory from metastatic lesions and transplanted into nude mice show corroborates a more aggressive behaviour of NG2-expressing subsets. Comparative whole-genome DNA microarray gene profiling identifies a number of gene expression differences in NG2+ and NG2- cells which are currently under characterization. To complement this post-genomic analyses we have designed global comparisons of the protein expression profiles of the two cell phenotypes. These experiments involve the DIGE technology and MALDI-TOF identification of differentially expressed protein spots appearing aftrer bi-dimensional SDS-PAGE.

In a more accurate retrospective analysis in which we compared the transcriptional profile of NG2 in primitive lesions of patients who developed post-surgery metastases within a 7-years follow-up period and patients who remained free from secondary lesions metastatic lesions revealed an unprecedented role for the PG in predicting with >55% probability post-operative metastatic disease within 12 months following removal of the primitive lesions. These observations finding, taken together with the lower survival rates of patients with enhanced NG2 expression, assigns to the PG a significant value as an independent prognostic factor and a multicenter extension of our investigations is currently ongoing to more firmly establish the clinical importance NG2 expression as a molecular marker in soft-tissue sarcomas. A corollary

interest is to define whether high expression of NG2 in primitive pre-surgery lesions may correlate with tumour relapse or be indicative of therapeutic responses.

By employing more than 30 sarcoma lines established from surgical specimens, *in vitro* growth, adhesion and cell migration assays and transplantation into wild type and Col VI knockout mice we have addressed some of the cellular and molecular mechanisms underlying the modes through NG2 may promote tumour growth and metastasis formation. Through this combination of cellular assays *in vitro* and tumorigenic assays *in vivo* a crucial role was assigned to the interaction of NG2 with Col VI and phosho-proteomic profiling was employed to delineate the gene and signalling networks triggered by NG2-Col VI interplay. Finally, our previous investigations have documented a central role for NG2 in governing the behaviour of pro-angiogenic pericytes, possibly by contributing to the growth factor responses of these cells. We have therefore hypothesised that NG2 may be implicated in the control of FGF signalling. Indeed, in mice lacking the NG2, FGF-2-induced corneal angiogenesis is strongly impaired due to a failure of vascular pericytes to undergo normal proliferation and extension. Detailed analyses of isolated NG2-expressing and NG2-deficient cells indicates that NG2 governs both paracrine and autocrine FGF mitogenic responses of the cells and does so in elective cooperation with FGFR1 and FGFR3. The high-affinity co-receptor activity of the PG seems to operate at three distinct levels: through glycosaminoglycan-independent sequestering and activation of latent FGFs; through compensation for reduced surface levels of FGFR available for ligand interaction; and through modulation of the receptor-induced levels of signal transduction. Upon FGF stimulation NG2 becomes phosphorylated but it not capable of autonomously transducing pro-mitogenic stimuli when engaging in FGF binding. The multifunctional cell surface action exerted by NG2 during growth factor-induced angiogenesis appears to define an alternative GAG-unrelated cell autonomous strategy for the perception of FGF gradients and for regulation of the magnitude of cellular responses to these gradients. The findings expand our knowledge on the fine equilibrium between the FGF/FGFR/co-receptor interplay in controlling signalling events that occur during neovascularization and other growth factor-dependent processes.

The combined importance of NG2 in the regulation of the sarcoma cell interaction with the host microenvironment and growth factor-iunduced propagation of tumour and vascular cells suggested to us that the PG could also mediate some of the events involved in the processes of intra- and extravasation. Accordingly, we have investigated the effect of the PG in the transendothelial movement of sarcoma cells *in vitro* and *in vivo*. Intravasation *in vitro* was simulated in transmigration assays in which we used various types of hematic and lymphatic endothelial cells in a monolayered configuration. The extravasation phenomenon was simulated by using a parallel-flow chamber system in which tagged sarcoma cells are perfused at defined shear rates over the same endothelial monolayers and the contacts established between the two cell types is assessed through high-speed video cameras supported by dedicated image analysis softwares. The ability of NG2 to promote lymphatic intravsation is currently under investigation using a syngenic setting in rat. Tagged rat sarcoma cells transduced to express NG2 or in which the constitutive levels of NG2 have been knockdown by RNAi are implanted into the diaphragmatic region of rats and entrance of the sarcoma cells into the lymphatic capillaries is monitored by intravital microscopy. Preliminary experiments suing this *in vivo* paradigm have provided promising results showing the feasibility of the system and the first hints about a putative function of NG2 in mediating the interaction of spreading sarcoma cells with the lymphatic vessels. These findings provide novel insights into a multifunctional role NG2 suggesting that the PG may act at both cell-cell and cell-ECM interfaces for the promotion of the tumour cells' dissemination capabilities.

The acquisition of information regarding the role NG2 as a prognostic and metastasis formation-predicting factor and as multivalent controller of tumorigenesis strongly points to NG2 as a suitable therapeutic target. Thus, we have undertaken two different approaches to exploit the potential of the PG in immunotherapeutic approaches. On one hand, we have generated a series of 63 monoclonal antibodies against the extracellular portion of NG2 and have started a systematic screening of the putative effects of these antibodies on tumour cells *in vitro* and *in vivo*. Particular emphasis is given to the potential of the antibodies to affect cell movement, while *in vivo* tests focus on local and metastatic growth of our sarcoma cells in xenogenic settings. Through these assays we have thus far identified two antibodies, among the close to 20 examined, that interfere with sarcoma cell migration *in vitro* and local (subcutaneous) growth in nude mice and are currently addressing the cellular and molecular mechanisms through which these agents affect the process of cell motility. In another series of experiments we are investigating if binding of the antibodies to cell surface NG2 causes phosphorylation of the cytoplasmic tail of PG, which is known to be associated with Erk and PI-3K signalling cascades. Subsequent analyses of the signalling pathways potentially elicited by binding of the antibodies to NG2 will be examined by global phospho-proteomic screenings based upon antibody arrays as described above.

Parallel studies have been devoted to the understanding of the immunomodulatory effects exerted by an anti-idiotypic antibody – MK2.23 - mimicking a segment of the central portion of the ectodomain of NG2 and previously shown to have a potential as anti-melanoma vaccine. In fact, a recent trial on advanced melanoma patients vaccinated with this antibody showed remarkable curing effects and suggested that the antibody may be similarly effective in immunotherapeutic tretament of sarcoma patients. To gain further knowledge about how the antibody act as an anti-neoplastic immunomodulator, we have specifcally examined the anti-NG2 immune responses evoked in melanoma patients extensively vaccinated with the antibody. Indeed, high titers of circulating anti-NG2 antibodies could be detected in these cells using recombinant NG2 and these results asserted that the vaccination had re-activated immune-recognition of the tumour cells NG2 antigen. In parallel studies we also found that T cells from the immunized patients could be stimulated *in vitro* by recombinant NG2, suggesting a certain degree of cross-reactivity between the anti-idiotypic antibody and NG2-derived epitopes and recalls observations made with a similar NG2 anti-idiotypic antibody, which displays a partial amino acid sequence homology with 10-17 residue stretches of NG2 and encompasses at least one sequence inducing HLA-A2-restricted T cell responses cross-reactive with a corresponding native NG2 peptide. Since a similar partial sequence homology can be observed between the variable heavy chain of the former anti-idiotypic antibody and NG2, the possibility remains that antibody MK2-23 may behave as a "heteroclitic vaccine", capable of exploiting the low-affinity T cell repertoire that usually does not undergo thymic deletion or tolerance.

In light of this fact, we also have approached the design of alternative NG2 peptide vaccines by selecting NG2 sequences that per se are not immunogenic due to their low affinity for HLA molecules, but which still would allow the targeting of NG2-expressing tumour cells by immune effectors. To model NG2 peptides optimized for their binding properties to different HLA class I supertypes and for proteasomal processing, a "reverse immunology" strategy aimed at identifying NG2 sites cleavable by the proteasome was employed. Stretches of 15-25 residues comprised between two NG2 cleavage sites predicted to be low binders of most HLA class I supertypes were searched by using the NetMHC2.1 software. Then, to further improve the immunogenicity of the selected sequences, amino acid substitutions were introduced at the C-terminus and at position -1 with respect to the internal cleavage sites that would favour the anchoring of the peptides to specific HLA supertypes. The sequences have been reproduced as

synthetic peptide and are under testing *in vitro* against isolated cytotoxic T cells and *in vivo* in sarcoma tumorigenic xeno-models.

Papers published within the project

Benassi MS, Pazzaglia L, Chiechi A, Alberghini M, Conti A, Cattaruzza S, Wasserman B, Picci P, Perris R. NG2 expression predicts the metastasis formation in soft-tissue sarcoma patients. *J Orthop Res* 2009;27,135-40.

Cattaruzza S, Nicolosi PA, Perris R. Proteoglycans in the control of tumor growth and metastasis formation. *Connect Tissue Res* 2008;49,225-9.

Moskovszky L, Szuhai K, Krenács T, Hogendoorn PC, Szendroi M, Benassi MS, Kopper L, Füle T, Sápi Z. Genomic instability in giant cell tumor of bone. A study of 52 cases using DNA ploidy, relocalization FISH, and array-CGH analysis. *Genes Chromosomes Cancer* 2009;48,468-79.

Palmerini E, Staals EL, Alberghini M, Zanella L, Ferrari C, Benassi MS, Picci P, Mercuri M, Bacci G, Ferrari S. Synovial sarcoma: retrospective analysis of 250 patients treated at a single institution. *Cancer* 2009;115,2988-98.

Pazzaglia L, Chiechi A, Conti A, Gamberi G, Magagnoli G, Novello C, Morandi L, Picci P, Mercuri M, Benassi MS. Genetic and molecular alterations in rhabdomyosarcoma: mRNA overexpression of MCL1 and MAP2K4 genes. *Histol Histopathol* 2009;24,61-7.

Perris R, Cattaruzza S, Braghetta P, Nicolosi PA, Lacrima K, Rossi S, Stallcup WB, Bonaldo P, Pazzaglia L, Benassi MS, Picci P, Girolamo F, Virgintino D, Roncali L, Ozerdem U, Sigalotti L, Bertani N, Wasserman B, Spessotto P, Bruni L, Maccarana M, Maio M. Proteoglycans in metastasis formation and anti-neoplastic therapeutic approaches. Connect. *Tissue Res* 2007;48,338-63.

Tahara H, Sato M, Thurin M, Wang E, Butterfield LH, Disis ML, Fox BA, Lee PP, Khleif SN, Wigginton JM, Ambs S, Akutsu Y, Chaussabel D, Doki Y, Eremin O, Fridman WH, Hirohashi Y, Imai K, Jacobson J, Jinushi M, Kanamoto A, Kashani-Sabet M, Kato K, Kawakami Y, Kirkwood JM, Kleen TO, Lehmann PV, Liotta L, Lotze MT, Maio M, Malyguine A, Masucci G, Matsubara H, Mayrand-Chung S, Nakamura K, Nishikawa H, Palucka AK, Petricoin EF, Pos Z, Ribas A, Rivoltini L, Sato N, Shiku H, Slingluff CA, Streicher H, Stroncek DF, Takeuchi H, Toyota M, Wada H, Wu X, Wulfkuhle J, Yaguchi T, Zeskind B, Zhao Y, Zocca MB, Marincola FM. Emerging concepts in biomarker discovery; The US-Japan workshop on immunological molecular markers in oncology. *J Transl Med* 2009;7,45.

INNOVATIVE MANAGEMENT OF PATIENTS WITH DIFFUSE MALIGNANT PERITONEAL MESOTHELIOMA

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Introduction

Diffuse Malignant Peritoneal Mesothelioma (DMPM) is a rare tumour, accounting for 10% to 20% of the 2200 cases of all malignant mesotheliomas registered each year in the United States. The prognosis for patients with DMPM is poor, with a median overall survival of 12.5 months in the best series treated with Systemic Chemotherapy (sCT) alone. The biology of DMPM is largely unknown and the cellular and molecular bases, responsible for the proliferative potential and the relative resistance to current therapies of DMPM, cells have not been elucidated yet. Clinical and research effort in this field are important because there is an increasing of incidence in Europe with the expected pick between 2015 and 2020.

The end point of this project where:

– *Clinical study*
  To evaluate the impact of a new combination treatment named Cytoreductive Surgery (CRS) and Heated Intra Peritoneal Chemotherapy (HIPEC) associated with sCT on survival of patients affected by DMPM.

– *Experimental biology*
  Evaluating the prognostic relevance of telomere maintenance mechanisms, ii) validating the anti-apoptotic factor survivin as a new therapeutic target, and iii) assessing the activity of new anticancer agents, in peritoneal mesothelioma.

– *Experimental pathology*
  To verify the relevance of the expression of tyrosine kinase receptor EGFR on clinical outcome of patients. We planned to ascertain if the activation of EGFR, PDGFRA and PDGFRB could offer alternative therapeutic targets in MPM.

Methods and results

Clinical study

During the study period 33 patients were enrolled and treated. Prospectively collected clinical data on 83 consecutive patients with DMPM undergoing CRS and HIPEC with cisplatin

and doxorubicin were reviewed.. When technically possible, fresh specimens where sent at the time of surgical treatment to the laboratory of pathology and experimental oncology. For the overall series, median follow-up was 52 months (range 1-126) and 5-year overall survival (OS) 49.5%. At multivariate analysis, pathologically negative (*vs* positive/not assessed) nodes (relative risk (RR)=2.81; 95% confidence interval (CI)=1.12-7.05; P=.027), epithelial subtype (RR=2.93; CI=1.24-6.95; P=.015), mitotic count ≤5/50 HPF (RR=5.34; CI=1.96-14.54; P=.001), completeness of cytoreduction (RR=2.06; CI=1.19-3.56; P=.001) correlated to increased OS.

Experimental biology

Telomere maintenance mechanisms (telomerase activity [TA] and alternative lengthening of telomeres [ALT]), were detectable in 86.4% of 44 diffuse malignant peritoneal mesothelioma (DMPM) specimens tested: ALT or TA alone was found in 18.2% or 63.6% of lesions, respectively, whereas 2 cases (4.6%) were ALT+/TA+. TA and ALT proved to be inversely associated (P=0.002). In the overall series, TA was prognostic for 4-year relapse (TA+ *vs* TA-, hazard ratio, 3.30; 95% Cl, 1.23-8.86; P = 0.018) and cancer-related death (TA+ *vs* TA-, hazard ratio, 3.56; 95% Cl, 1.03-12.51; P = 0.045), whereas ALT failed to significantly affect clinical outcome. These results held true also in the subset of patients submitted to uniform treatment with cytoreductive surgery and hyperthermic intraperitoneal chemotherapy. The results indicate that both known telomere maintenance mechanisms, TA and ALT, are present in DMPM and differentially affect patient prognosis.

The expression of survivin and other members of the inhibitors of apoptosis proteins (IAP) family (IAP-1, IAP-2 and X-IAP) was assessed in 32 DMPM specimens. Survivin expression was observed in 29 (91%) surgical DMPM specimens, whereas the positivity rate for the other IAPs ranged from 69% to 100%. Transfection of a human DMPM cell line with a survivin siRNA induced a marked inhibition of survivin protein expression, a time-dependent decline in cell growth and an enhanced rate of spontaneous and drug-induced apoptosis, with a concomitant increase in the catalytic activity of caspase-9. These results show for the first time that survivin, as well as other IAPs, is largely expressed in clinical DMPMs and suggest that strategies aimed at down-regulating survivin may provide a novel approach for the treatment of the malignancy. As far as new anticancer agents are concerned, we assessed the cellular effects of a new series a nortopsentin heteroanalogues, 3-[2-(1H-Indol-3-yl)-1,3-thiazol-4-yl)-1H-4-azaindole in a DMPM cell line. Selected compounds, which were able to inhibit the activity of CDK1, consistently reduced cell growth and induced a concentration-dependent cell cycle arrest at the G2/M phase and an increase in the apoptotic rate, with a concomitant down-regulation of the anti-apoptotic protein survivin. Notably, the addition of these compounds to paclitaxel-treated cells resulted in a marked increase of the cytotoxic effect, as a consequence of increased activation of caspases. Finally, considering that EGFR-, PDGFR-, and mTOR-mediated signalling are activated in DMPM, we evaluated the effect of the EGFR inhibitor Gefinitib, the mTOR inhibitor RAD001 and the multiple tyrosine kinase inhibitor Sorafenib on the cellular proliferation of a DMPM cell line. As single agents, Gefitinib and RAD001 were somewhat ineffective against mesothelioma cells, exhibiting an IC50 value of 40.08±1.35 μM and 15.23±0.82 μM, respectively, whereas a higher cytotoxic activity was observed after exposure to Sorafenib, with an IC50 value of 0.55±0.07 μM. Interestingly, the treatment with RAD001 followed by Sorafenib induced marked synergistic effects in these cells.

Experimental pathology

Receptor Tyrosine Kinase (RTK) biochemical analysis was performed on 20 fresh untreated MPM samples obtained immediately after surgical resection and snap frozen. RTK immunohistochemical, mutational and cytogenetic analyses, as well as the detection of ligand expression were performed on the corresponding fixed material. Additionally, we explored the status of RTK downstream pathways through biochemical and mutational analysis and fluorescent in situ hybridization (FISH) of PI3K/PTEN/AKT and RAS/BRAF/ERK pathways, as well as mTOR and its effector S6. RTK analysis. Immunoprecipation/Western blot experiments showed a significant EGFR (90% cases) and PDGFRB (75% cases) activation, while PDGFRA resulted to be phosphorylated in 40% of MPMs, despite its expression in all cases. Coactivation of these receptors, along with an autocrine loop activation were suggested by the presence of EGFR/PDGFRB heterodimers and the expression of the cognate ligands (TGF-α, PDGFA and PDGFB), respectively. All cases showed absence of both EGFR, PDGFRA and PDGFRB activating mutation and gene amplification. An increased gene copy number of these receptors through low polysomy was found in only 3 (15%) cases. RTK downstream pathway analysis. All MPMs showed, by biochemical experiments, expression and activation of AKT, ERK1/2 and mTOR, as well as of S6. Increased PI3KCA gene copy number, represented by low polysomy of chromosome 3, was found in 25% of the cases, while all MPMs showed absence of loss of PTEN gene and expression PTEN protein. Half of the cases were investigated for PI3KCA, PTEN, KRAS and BRAF mutations and the results showed absence of mutations. The ligand- and heterodimerization-dependent activation/expression of EGFR and PDGFRB seems to make these receptors promising molecular targets for tailored treatments in MPM. If we will confirm the absence of PI3KCA, PTEN, KRAS and BRAF alterations by extending mutational analysis to all cases, the data indicate that the observed activation of the RTK downstream effectors AKT, ERK1/2, mTOR and S6 is mediated by the upstream activated receptors, providing a rationale to apply combined RTK and mTOR inhibitor treatments.

Conclusion

Clinical study. A variety of treatments options have been proposed to treat DMPM, alone or in combination, but most of them have failed to demonstrate a significant impact in palliation or disease free/overall survival. The chemotherapy resistance of the tumour is well known. Thus, it seems wise to try and combine systemic therapies, though still under development, with radical surgery and locoregional therapies. This study confirms that the recent advent of a combined approach of CRS and HIPEC has dramatically changed the natural evolution of the disease representing a effective salvage therapy for this clinical entity.

Experimental biology and pathology. The biological results indicate that both known telomere maintenance mechanisms, TA and ALT, are present in DMPM and differentially affect patient prognosis. These results show that survivin is largely expressed in clinical DMPMs and suggest that strategies aimed at down-regulating survivin may provide a novel approach for the treatment of the malignancy. The activation of the RTK downstream effectors AKT, ERK1/2, mTOR and S6 is mediated by the upstream activated receptors, providing a rationale to apply combined RTK and mTOR inhibitor treatments. Interestingly, the treatment with RAD001 followed by Sorafenib induced a marked synergistic effect in these cells.

Papers published within the project

Baratti D, Kusamura S, Cabras AD, Dileo P, Laterza B, Deraco M. Diffuse malignant peritoneal mesothelioma: Failure analysis following cytoreduction and hyperthermic intraperitoneal chemotherapy (HIPEC). *Ann Surg Oncol* 2009;16(2):463-72. [Epub 2008 Dec 12].

Baratti D, Kusamura S, Cabras AD, Laterza B, Balestra MR, Deraco M. Lymph-node metastases in diffuse malignant peritoneal mesothelioma. *Annals of Surgical Oncology* Oct 24. [(Epub ahead of print].

Baratti D, Kusamura S, Deraco M. Circulating CA125 and diffuse malignant peritoneal mesothelioma. *Eur J Surg Oncol* 2009;35(11):1198-9.

Deraco M, Baratti D, Zaffaroni N, Cabras AD, Kusamura S. Advances in clinical research and management of diffuse peritoneal mesothelioma. *Recent Results Cancer Res* 2007;169:137-55.

Deraco M, Bartlett D, Kusamura S, Baratti DJ. Consensus statement on peritoneal mesothelioma. *Surg Oncol* 2008;98(4):268-72.

Laterza B, Kusamura S, Baratti D, Oliva GD, Deraco M. Role of explorative laparoscopy to evaluate optimal candidates for cytoreductive surgery and hyperthermic intraperitoneal chemotherapy (HIPEC) in patients with peritoneal mesothelioma. *In Vivo* 2009;23(1):187-90.

Perrone F, Jocollè G, Brich S, Cabras A, Deraco M, Baratti D, Pilotti S. Analysis of EGFR, PDGFRA and PDGFRB and related pathways in malignant peritoneal mesothelioma. Poster presented at the 9th International Mesothelioma Interest Group (IMig) Conference in Amsterdam, 25-27th of September 2008

Villa R, Daidone MG, Motta R, Venturini L, De Marco C, Vannelli A, Kusamura S, Baratti D, Deraco M, Costa A, Reddel RR, Zaffaroni N. Multiple mechanisms of telomere maintenance exist and differentially affect clinical outcome in diffuse malignant peritoneal mesothelioma. *Clin Cancer Res*. 2008;14(13):4134-40.

Yan TD, Deraco M, Elias D, Faheez M, Gilly FN, Glehen O, Levine EA, Moran BJ, Morris DL, Piso P, Shen P, Baratti D, Kusamura S, Sugarbaker PH. Cytoreductive surgery combined with hyperthermic intraperitoneal chemotherapy for peritoneal mesothelioma – a multi-institutional registry study. *Journal of Clinical Oncology* 27(36):6237-42.

Zaffaroni N, Costa A, Pennati M, De Marco C, Affini E, Madeo M, Erdas R, Cabras A, Kusamura S, Baratti D, Deraco M, Daidone MG. Survivin is highly expressed and promotes cell survival in malignant peritoneal mesothelioma. *Cell Oncol* 2007;29(6):453-66.

PHENOTYPE CORRECTION OF ADAMTS13 DEFICIENCY AND PROTECTION FROM THE DEVELOPMENT OF THROMBOTIC THROMBOCYTOPENIC PURPURA THROUGH INTRAVASCULAR AND SKELETAL MUSCLE ADAMTS13 GENE DELIVERY IN MICE

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ADAMTS13 is a plasma metalloprotease which regulates the size of von Willebrand factor (VWF) multimers. Genetic or acquired deficiency of ADAMTS13 causes Thrombotic Thrombocytopenic Purpura (TTP) in humans, a life-threatening illness characterized by fever, hemolytic anemia, thrombocytopenia, neurological symptoms and renal dysfunction. Plasma infusion is the treatment of choice for patients with congenital ADAMTS13 deficiency. However, this practice exposes patients to the risk of infections, allergies and fluid volume overload. The search for alternative treatments is needed.

In the present project we tested the ability of systemically administered adenovirus encoding human ADAMTS13 (Ad-ADAMTS13) to restore the deficient protein in circulation of Adamts13-/- mice3. Adamts13-/- mice were injected with 1×10^9 pfu Ad-ADAMTS13 or Ad-$\beta-$Gal and one week later the transgene expression was evaluated in different organs by RT-PCR and X-gal staining, respectively. Both techniques showed that adenovirus efficiently transduced the liver, kidney, lung, heart, and spleen, but not the brain. Ad-ADAMTS13 injection induced a remarkable increase in the protease activity as detected by Collagen Binding Assay (CBA) ($293 \pm 68\%$ in respect to the 100% activity of normal human plasma) not seen upon treatment with Ad-βGal. A reduced area of thrombi ($49 \pm 18.5\%$, $P<0.05$) was observed when blood from Ad-ADAMTS13 treated mice was perfused over a collagen-coated surface in a parallel plate flow chamber compared with blood of Ad-βGal treated controls. The secreted ADAMTS13 protein was functionally active even after 4, 8 and 12 weeks from injection ($16.7 \pm 2.9\%$, $11.6 \pm 0.6\%$ and $13.2 \pm 0.7\%$, respectively), although at much lower levels than those measured 1 week after injection. This reduction of activity correlates with the formation of antibodies against both the adenovirus and ADAMTS13. Despite the presence of antibodies, whole blood from Ad-ADAMTS13 treated mice sacrificed at 12 weeks still showed a lower platelet deposition to collagen-coated surface in respect to Ad-βGal controls, resulting in a $10.2 \pm 2.9\%$ reduction of the area covered by thrombi.

These data show that intravenous administration of Ad-ADAMTS13 induces the release into the circulation of a functionally active protease for at least 12 weeks at levels consistently above the 5-10% sufficient to protect patients from disease recurrences. The vector used in the present study, however, bears some limitations. The main disadvantage of adenovirus resides in its inherent immunogenicity that could be circumvented by genetic or chemical modifications of the adenoviral genome and/or capsid. PEGylated adenovirus can indeed protect the vectors from

pre-existing and adaptive immune responses allowing new protein production following a second administration4. An alternative approach is the use of a non-pathogenic adeno-associated virus (AAV) whose main limit is actually represented by its cargo capacity that is of 4,3kb, similar to the size of ADAMTS13 cDNA, which results in an AAV vector with low infectivity. We are now testing AAV hybrid serotypes with extended packaging capacity (up to 8.9 kb).

Collectively, these findings represent the proof of principle that gene therapy can restore ADAMTS13 plasma levels and activity in ADAMTS13 knockout mice. More studies are mandatory to better identify the ideal gene delivery system. Once established the most suitable one, novel therapeutic perspectives could open for patients affected by the congenital form of TTP.

Papers published within the project

Trionfini P, Tomasoni S, Galbusera M, Motto D, Longaretti L, Corna D, Remuzzi G, Benigni A. *In vivo* adenoviral-mediated gene transfer restores plasma ADAMTS13 level and function in ADAMTS13 knockout mice. *JASN* 2008;19:112A.

Trionfini P, Tomasoni S, Galbusera M, Motto D, Longaretti L, Corna D, Remuzzi G, Benigni A. Adenoviral-mediated gene transfer restores plasma ADAMTS13 levels and function in ADAMTS13 knockout mice. World congress of Nephrology, WCN 2009, Milan Abstract Book M053.

Trionfini P, Tomasoni S, Galbusera M, Motto D, Longaretti L, Corna D, Remuzzi G, Benigni A. Adenoviral-mediated gene transfer restores plasma ADAMTS13 antigen and activity in ADAMTS13 knockout mice. *Gene Therapy* 2009;16(11):1373-9.

NEW THERAPEUTICAL APPROACHES
IN THE HUMAN BETA-THALASSEMIA TREATMENT:
IN VITRO AND *IN VIVO* STUDIES

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The human β-thalassemias are characterized by insufficient or absent production of β-globin chains, due to mutations affecting the β-globin gene complex. The disease is caused by the imbalanced α and non α-globin chain synthesis, which leads to accumulation and precipitation of unpaired α-globin chains and, consequently, to ineffective erythropoiesis and hemolysis. Although the relationship between phenotype and genotype in β-thalassemia is complex, the α/non α ratio correlates with the severity of disease. In β-thalassemia intermedia, one or two β-globin genes are defective, but concurrent genetic factors (HPFH, α and δβ-thal determinants) can reduce or enhance the globin chain imbalance, thus ameliorating or exacerbating the clinical conditions. In homozygous β0-thalassemia major a very high α/non α ratio is associated with severe ineffective erythropoiesis and dependence on red blood cell transfusions for survival. These observations suggest that the severity of the disease can be reduced by γ-globin reactivation. In spite of extensive studies on pharmacological induction of HbF synthesis, clinical trials based on HbF reactivation in human β-thalassemia produced inconsistent results.

In the first part of this project we demonstrated that Stem Cell Factor (SCF) markedly stimulates cell proliferation reactivating fetal hemoglobin (HbF) synthesis in human thalassemic erythroid precursors and pharmacological doses of Dexamethasone (Dex) potentiate these stimulatory effects, thus paving the way for a future clinical application of SCF in β-thalassemia treatment. Particularly, in unilineage erythroid cultures of 20 patients with either intermedia or major β-thalassemia, addition of SCF, alone or in combination with Dex, remarkably stimulated cell proliferation (3-4 logs over control cultures), while decreasing the percentage of apoptotic and dyserythropoietic cells (<5%). More important, in both thalassemic groups the addition of SCF+Dex induced a strong increase of γ-globin chains reaching values of HbF content three fold higher than those observed in erythroid controls (81% *vs* 27%, mean values, in the β-thalassemia major). A series of experiments was focused on the characterization of molecular mechanisms underlying the proliferative and antiapoptotic effects of SCF ± Dex in normal and thalassemic CD34+ hematopoietic progenitor cells grown in unilineage erythroid culture.

Our results obtained by RT-PCR showed that in normal erythroid precursors SCF activates a set of genes including Notch2, HES-1, IMPDH2, S100A and HMG (High Mobility Group) family members (HMGI-Y) that play an essential role in the regulation of cell survival and proliferation. Preliminary investigations performed on erythroblasts derived from two β-major thalassemic patients showed a stronger SCF mediated induction of Notch 2 as compared with normal control. Concerning HbF reactivation, the SCF effect may be mediated by Id2 and/or Tal1 transcription factors 4, as well as the MEK signalling pathway 5 which in turn down-regulates the expression of transcription factor COUP-TFII, a repressor of γ-globin genes 6. These factors may mediate the SCF stimulus on HbF synthesis, independently of or in relationship to the miR-221/222 mechanism as recently observed by our group 7. In the second

part of this project, in order to design a protocol for pre-clinical studies, we analyzed the SCF effects in C57/BL6 normal mice treated with high doses of cisplatin, a chemotherapic agent which induces a state of myelosuppression and consequent anemia. This represents an optimal model for evaluating the SCF ability to increase haematological parameters such as red blood cell (RBC) number and total hemoglobin content. In particular, we observed that, by administering subcutaneous SCF doses of 50 μg/kg 4h before and after cisplatin injection and every 8h for one week, RBCs increased from 7 to 10 x106/μl and Hb raised from 11 to 14 g/dL in SCF-treated mice, thus reaching normal values; more important, no collateral effect were observed in the animals. Moreover, since the most important complication in β-thalassemic patients is the progressive iron overload into the organs, in preliminary experiments we evaluated the expression of genes involved in iron metabolism. It was previously demonstrated that hepcidin protein, which regulates the iron release bounding ferroportin, is in turn under control of growth differentiation factor 15 (GDF15), a TGFβ superfamily member secreted from erythroblasts during human erythropoiesis.[8] Hence, we analyzed the ferroportin and GDF15 expression both at mRNA and protein level during *in vitro* erythroid differentiation of β-thalassemic progenitor cells treated or not with SCF.

Preliminary results clearly demonstrate a key role of SCF in the regulation of both these genes and, as well as the HbF reactivation, they should be confirmed *in vivo* on thalassemic mice, particularly transgenic mice expressing human γ-globin chain. In conclusion, our studies provide a significant experimental basis for the development of preclinical models of SCF-based therapy in human β-thalassemia, aiming to possibly decrease or eliminate the need for transfusions and improve the quality of life in these patients.

Papers published within the project

Gabbianelli M, Morsilli O, Massa A, Pasquini L, Cianciulli P., Testa U, Peschle C. Effective erythropoiesis and HbF reactivation induced by kit ligand in β-thalassemia. *Blood* 2008;111:421-429,.

Gabbianelli M, Morsilli O, Massa A, Pasquini L, Cianciulli P, Testa U, Peschle C. Stimulation of erythropoiesis and fetal hemoglobin reactivation induced by stem cell factor in human β-thalassemia. In: Taruscio D, Salvatore M (Ed.). *Workshop. Rare Diseases and Orphan Drugs. Rome, November 7-8, 2007. Abstract book.* Roma: Istituto Superiore di Sanità; 2007. (ISTISAN Congressi 07/C8). p. 51

Zeuner A, Bartucci M, Morsilli O., Sposi N M, Baiocchi M, Cianciulli P, De Maria R, Gabbianelli M. Therapeutic potential of stem cell factor in the human beta-thalassemia treatment: *in vitro* and *in vivo* studies. In: Taruscio D, Salvatore M (Ed.). *Workshop. Projects on rare diseases funded within the bilateral agreement Italy (Istituto Superiore di Sanità) and USA (NIH, Office for Rare Diseases) on joint research and development of public health actions. Istituto Superiore di Sanità. Rome, October 29-31, 2008. Abstract book.* Roma: Istituto Superiore di Sanità; 2008. (ISTISAN Congressi 08/C10). p. 145

EFFECTS OF THE INHIBITION OF PDGFR PHOSPHORYLATION ON THE ACTIVATION OF CIRCULATING PLATELETS AND NEUTROPHILS IN PATIENTS WITH SYSTEMIC SCLEROSIS: RESULTS FROM A DOUBLE-BLIND PLACEBO-CONTROLLED CLINICAL TRIAL

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Disease modifying therapy for Systemic sclerosis (scleroderma, SSc) is an unmet medical need. Excessive oxidative stress has been implicated in the natural history of the disease, since Reactive Oxygen Species (ROS) generation is a putative bridge between the Ha-Ras and growth-factor activated extracellular signal-regulated kinases 1/2 (Ha-Ras, ERK1/2): this circuit is amplified in scleroderma fibroblasts. The recently identified stimulatory autoantibodies against PDGF (Platelet-Derived Growth Factor) receptor possibly provide a link between autoimmunity and fibrosis in SSc: they induce ROS production via Ha-Ras and ERK1/2 recruitment and are ultimately responsible for SSc fibroblast activation via the intracellular kinases system, with collagen overproduction. Inhibition of this pathway is therefore a candidate strategy for molecular intervention in SSc patients. Imatinib mesylate, the standard therapy for chronic myeloid leukemia, is a specific inhibitor of ABL kinases, which normally phosphorylate the PDGF receptor. Kinase activation is also a critical event sustaining the reciprocal activation of circulating platelets and leukocytes, which contributes to the vascular inflammation characteristic of the diseases.

We are carrying out a multicentic, randomized double-blind, placebo-controlled trial to address the action of Imatinib in SSc patients. SSc patients with refractory disease (worsening of skin involvement or visceral damage despite adequate immunosuppressive and vasoactive therapy at standard doses for at least 3 months) have been randomly assigned to receive or not Imatinib 200 mg die orally for 6 months (+6 months of follow-up) in addition to the conventional treatment. We are actively evaluating the action of the treatment on circulating leukocytes and platelets of SSc patients, including in particular the modulation of beta2integrin

expression and function, the presence of platelet-neutrophil and –monocyte heterotypic aggregates, the clearance of activated P-selectin platelets by circulating leukocytes and correlating the results with clinical endpoints, including skin thickness evaluated by the modified Rodnan skin score, and amelioration of the quality of life and the patient physical and emotional well being, evaluated by the Italian version of the Health Assessment Questionnaire (HAQ) and SF-36 score.

Preliminary results reveal the existence of an activatory cross-talk among circulating cells in scleroderma patients and suggest a potential action of kinase activation in sustaining this loop.

AUTOIMMUNE PEMPHIGUS: DYNAMICS OF AUTOREACTIVE B CELLS, AN INTEGRATED DATABASE AND DIAGNOSTIC/THERAPEUTIC RECOMMENDATIONS

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Pemphigus is a life-threatening autoimmune blistering disorder of skin and mucosae associated with pathogenetic autoantibodies directed to keratinocyte cell surface antigens, specifically desmoglein 1 and 3 (Dsg1 and Dsg3). The binding of IgG antibodies to Dsg on epidermal keratinocytes leads to intraepithelial blister formation. At present, the involvement of anti-Dsg antibodies in pemphigus pathogenesis is well-established, while the mechanism of blister formation is only partly defined.

The first aim of the present project has been to analyse the repertoire of autoreactive memory B cells in pemphigus patients. To this purpose, we have used a previously developed method for the efficient immortalization of IgG+ memory B cells with Epstein Barr virus and a Toll-like receptor agonist. After cloning of selected B cells the human monoclonal antibodies (hMAbs) obtained have been characterized by: (i) ELISAs based on the ectodomain of Dsg1 and Dsg3; (ii) staining of different epithelial tissues and cell substrates; (iii) immunoblotting and immunoprecipitation analysis on keratinocyte extracts. Several hMAbs reactive for various epithelial antigens were cloned: 19 anti-Dsg3 and/or Dsg1 hMAbs as well as 20 hMAbs reactive with intracellular epithelial antigens and 2 hMAbs recognizing membrane antigens other than DSG. Four of 19 anti-Dgs hMAbs recognized a calcium-dependent conformational epitope o Dsg3 and showed pathogenic activity as demonstrated by using a keratinocyte dissociation assay. Furthermore, six of 19 anti-Dsg hMAbs have been epitope mapped by ELISA based on Dsg3 extracellular sub-domain constructs (EC1-EC5): two hMAbs reacted with EC1, one with EC3 and three against EC5. Cross-competition experiments indicated that the epitopes recognized by 2 pathogenic hMAbs are located in the NH2-terminal region of the Dsg3 ectodomain. Seven out of 19 anti-Dsg hMAbs were IgG4 and 13 of 16 used κlight chains. In addition, the sequencing of the variable portions of the heavy and light chain immunoglobulin genes of 10 Dsg-specific hMAbs isolated from a patient affected with pemphigus vulgaris (PV) showed restricted patterns of heavy and light chain gene usage. Immunoblotting and immunoprecipitation studies indicated periplakin and a 100 kDa protein as possible targets of 2 hMAbs that did not react with Dsgs. In conclusion, we isolated pathogenetic and non pathogenetic hMAbs recognising Dsg1 and Dsg3, as well as other epithelial antigens that could represent valuable tools for disease diagnosis and for investigating mechanisms of blister formation in pemphigus.

In parallel, in order to study the efficacy of the anti-CD20 specific immunotherapy, 10 PV patients corticosteroid-refractory disease have been recruited and treated with rituximab administered at 750 mg/m^2 in two infusions at 2 week interval. The severity of the disease was assessed according to the revised severity index for pemphigus described by Ikeda. The primary endpoint was the rate of complete response and secondary endpoints included the time to disease control and adverse effects of treatment. Complete response (CR) was defined as

absence of lesions for at least one month and either no corticosteroid/adjuvant treatment or treatment with prednisone ≤ 5 mg/day; partial response (PR) was defined as the presence of 1 to 5 new oral or cutaneous blisters per week with treatment with prednisone ≤ 10 mg and no adjuvant. Disease control (DC) was defined as the lack of new blister formation and healing of established lesions. Before starting rituximab infusions, all patients had a severity score of ≥ 6 according to Ikeda index and received a prednisone dosage of 40 mg/day or more. During rituximab infusions the prednisone dose was reduced to 25 mg/day; then a 12.5 mg/day dose was continued with a subsequent gradual tapering according to clinical conditions. Concomitant immunosuppressive therapies were stopped at the time of the first rituximab administration. In all the cases there was a positive response after treatment. All but 3 patients experienced DC one month after the end of the rituximab infusions. Three months after therapy 8 patients were in CR and 2 in PR. These 2 patients received an additional 500 mg rituximab infusion and three months later were in CR. Relapses were recorded in nine patients during a follow-up period of two years, and 4 of them experienced two relapses. Disease severity of the relapse was always mild, not exceeding the Ikeda score of 3. An additional administration of 500 mg rituximab cleared the disease in all these cases, without the need for steroids. All the patients were in CR at the end of the two-year follow-up period. No serious side effects, in particular no infections, occurred following rituximab treatment. The changes in anti-Dsg1 and Dsg3 autoantibody levels were consistent with the clinical response. The antibody titre showed a slow but progressive decline over time after rituximab infusions, while it always increased before disease relapse. A slight decrease in the serum immunoglobulin level, not exceeding 20% of the basal value, was observed in all patients. As expected, the B-cell count in peripheral blood dropped to 0 after the first infusion and remained undetectable for at least six months in all the treated patients. No disease relapse was observed while the B-cell count remained to 0. Our findings confirm that rituximab can be considered an important treatment option in patients with widespread recalcitrant or life-threatening PV. This drug has a good safety and tolerability profile. Although its use is currently limited to selected cases of PV, controlled clinical trials on greater number of patients are urgently needed.

Another aim of the project was to investigate the impact of pemphigus on quality of life (QoL) and psychological status. A data-base has been created: 156 pemphigus patients were registered and are in follow-up. The data-base is integrated and interactive: rates about health status and quality of life (QoL) are related with the clinical and laboratory data. For QoL evaluation, a set of instruments has been routinely used for all pemphigus cases. Specifically, the Medical Outcome Study 36-item short form health survey questionnaire (SF-36) was employed to assess the health status, the Skindex-29 to evaluate the impact of dermatology-specific aspects, and the 12-item General Health Questionnaire (GHQ-12) to detect patients with psychological problems. In parallel, clinical severity of the disease was assessed by the Physician Global Assessment index (PGA) and the Ikeda index.

The SF-36 scores in pemphigus patients showed a strong impact of the disease on all scales compared to the normative score for the general Italian population and an association between greater disease severity and lower SF-36 values was observed. Female patients exhibited significantly lower physical and mental SF-36 scores values than men. Patients aged 50 years or older and patients with mucocutaneous lesions also had significantly lower SF-36 values. The Skindex-29 showed a marked impairment of the overall QoL in pemphigus patients compared with healthy controls on all scales (symptoms mean score 37 *vs* 8, in patients and control subjects, respectively; emotions 37 *vs* 14; functioning 33 *vs* 4; p<0.001). Disease severity correlated with all three Skindex-29 scale scores, for both PGA and Ikeda values (p<0.05). Women had poor QoL scores on Skindex-29 symptoms and emotions items (p=0.01; p=0.02), while no significant differences were seen for age or disease duration. Minor psychological

distress was detected in 39.7% of patients. Patients with anxiety had severe disease as measured by the PGA. Overall our results show a strong impact of pemphigus on patients QoL both for the dermatology-specific and the general health aspects. The introduction of a minimal set of QoL evaluation tools (SF-36, Skindex-29, GHQ-12, PGA and Ikeda) in the clinical routine could provide additional useful information to guide clinicians in the treatment of these patients. A new study is now in progress to compare the efficacy, safety and impact on quality of life of rituximab and of three different adjuvant treatment regimens commonly used as steroid-sparing agents in PV (azathioprine, mycophenolate mofetil, cyclophosphamide).

The last aim of the project was the development and validation by a National Dermatology Society of a consensus statement for definitions of disease clinical parameters and therapeutic response. An expert committee of the National Society of Hospital Dermatologists (Associazione Dermatologi Ospedalieri Italiani, ADOI), composed of 40 dermatologists with a specific expertise in the management of pemphigus, including a member of the present project, has been established (3). The aim of the Italian committee has been to evaluate and translate at the national level the consensus statements elaborated by the International Pemphigus Committee (which also included a member of the present project as Italian representative) regarding the definitions of disease, end points, and therapeutic response for pemphigus (4). Following several rounds of review and revisions, there was a unanimous agreement regarding the definitions proposed by the International Pemphigus Committee for end points (control of disease activity, end of consolidation phase, complete and partial remission), relapse/flare, while the Italian experts decided to slightly modify the reported definition on therapy failure, in order to better comply with criteria of common availability of diagnostic tools as well as current use of specific immunosuppressive drugs in national dermatological centres. In particular, it was agreed not to specify the minimum dose of adjuvant drug (azathioprine, mycophenolate mofetil, cyclophosphamide or methotrexate) required to establish the failure of therapy. The Italian expert committee is at present working on the establishment of a consensus for measurement for the extent of disease and disease activity in patients with pemphigus.

Papers published within the project

Cianchini G, Veller Fornasa C, Lupi F, Amato S, Bellosta M, Bergamo F, Bettoli V, Bruni PL, Campisi G, Cardinali C, Carducci M, Colonna L, Congedo M, Corona R, Deledda S, De Giorgi V, De Pità O, Di Carlo A, Di Giuseppe D, Filosa G, Galluccio AG, Griseta V, Kokelj F, Lo Scocco G, Mazzanti C, Milioto M, Mulas P, Mussi A, Pallotta S, Pellicano R, Pigatto P, Provenzano E, Puddu P, Puglisi Guerra A, Ricciuti F, Sedona P, Simonacci M, Valenti G, Valenzano L, Valenzano MC. Definizioni di malattia, end points e risposta terapeutica nel pemfigo: international consensus statement e raccomandazioni GIDI e GIPMO. *Esperienze Dermatologiche* 2009;11:3-6.

Murrell DF, Dick S, Ahmed AR, Amagai M, Barnadas MA, Borradori L, Bystryn JC, Cianchini G, Diaz L, Fivenson D, Hall R, Harman KE, Hashimoto T, Hertl M, Hunzelmann N, Iranzo P, Joly P, Jonkman MF, Kitajima Y, Korman NJ, Martin LK, Mimouni D, Pandya AG, Payne AS, Rubenstein D, Shimizu H, Sinha AA, Sirois D, Zillikens D, Werth VP. Consensus statement on definitions of disease, end points, and therapeutic response for pemphigus. *J Am Acad Dermatol* 2008;58:1043-6.

Paradisi A, Sampogna F, Di Pietro C, Cianchini D, Didona B, Ferri R, Abeni D, Tabolli S. Quality of life assessment in patients with pemphigus using a minimum set of evaluation tools. *J Am Acad Dermatol* 2009;60:261-9.

Tabolli S, Mozzetta A, Antinone V, Alfani S, Cianchini G, Abeni D. The health impact of pemphigus vulgaris and pemphigus foliaceus using Medical Outcomes Study 36-item short form health survey questionnaire. *Br J Dermatol* 2008;158:1029-34.