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"Molecular bases of Down syndrome: differential gene expression and pathway dysregulation associated with trisomy 21"

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Chapter 1

General Introduction

Down syndrome

Down syndrome (DS) (MIM 190685), described by Down in 1866, is by far the most common and best known chromosomal disorder in humans with a frequency in the general population ranging from 1:650 to 1:1,000 live births and 1:150 conceptions (Hook 1982). Mental retardation, dysmorphic facial features and other distinctive phenotypic traits characterize the syndrome but the DS phenotypes are often variable among subjects.

DS, caused by trisomy of either the entire or a critical portion of chromosome 21 (21q22.1-21q22.3), occurs in 95% of cases as a result of meiotic nondisjunction (NDJ) mainly of maternal origin and in 2 to 4% of cases as a mosaicism of trisomic and normal cells (mitotic non-disjunction) (Antonarakis et al., 1991; Antonarakis et al., 1992; Antonarakis et al., 1993; Sherman et al., 1991).

In about 5% of patients, chromosome 21 is translocated to another acrocentric chromosome, most often chr 14 or 21.

1. Etiopathogenesis: The origin of the extra copy of chromosome 21

Trisomy 21 is due in 95% of cases to full trisomy 21 caused by meiotic non-disjunction or abnormal segregation of chromosomes occurring mainly during maternal oogenesis (95%) (Antonarakis, 1991; Freeman et al., 2007), particularly at meiosis I (MI) (80%) (Antonarakis, 1992; Sherman et al., 2007).

The age of the mother at the time of the conception is, by far, the most significant risk factor for having a fetus with DS. In fact, the birth prevalence increases with maternal age from 0.6 in 1,000 live births at maternal age of 20 up to 11 in 1,000 live births at maternal age of 40 (Morris et al., 2003).

Aside from maternal age, an association between maternal non-disjunction errors and reduced recombination along chromosome 21 was suggested (Warren et al., 1987). Furthermore, some studies have shown that not only the

absence but also the site of the exchanges is an important susceptibility factor for non-disjunction (Lamb et al., 1997). A single telomeric exchange leads to an increased risk for MI error, whereas pericentromeric exchanges increase the risk for MII error (Hawley et al., 1994).

More recently, the relationship between maternal age and recombination has been explored in order to further elucidate the causes of chromosomal NDJ (Lamb et al., 2005; Oliver et al., 2008).

The process of oogenesis is lengthy and involves meiotic arrest, which makes it more vulnerable to malsegregation of chromosomes than spermatogenesis (Oliver et al., 2008). Moreover, with increasing age, there is rapid degradation of cellular proteins involved in spindle formation (Hawley et al., 1994), sister chromatid cohesion (Wolstenholme and Angell, 2000) or anaphase separation of sister chromatids in oocytes, which imposes the risk of NDJ both at MI and MII (Yoon et al., 1996).

Recombination, initiated in the fetal ovary, stabilizes the tetrad and ensures proper segregation of chromatids to opposite poles. But the process is random and may be absent even in euploid samples (Cheung et al., 2007). These achiasmate meioses are at risk for NDJ, and this risk increases with age due to rapid deterioration of ovarian proteins that control the separation of these non-exchange chromosomes (Cheslock et al., 2005).

It has been shown that nondisjoined chromosomes often show altered patterns of recombination (MacDonald et al., 1994; Hassold et al., 1995; Koehler et al., 1996b) and for trisomy 21, achiasmate meioses contribute about 45% of maternal MI cases (Sherman et al., 2007). Therefore, for MI errors, a greater proportion of single telomeric exchanges, within the distal region was showed by younger mothers while for MII cases, older age group mothers revealed a preferential occurrence of single chiasma within the proximal 5.2 Mb of 21q (Oliver et al., 2008).

In conclusion, chromosomal NDJ is a complex and multi-factorial event for which the underlying mechanisms are related to two different sets of factors; one age dependent and another age independent.

1.1 Polymorphisms of genes involved in homocysteine/folate metabolism and risk of Down syndrome offspring

Despite huge efforts, the mechanisms underlying meiotic nondisjunction and the maternal age effect on trisomy 21 are not completely understood. Recently, genetic and metabolic risk factors have been studied in human chromosome non-disjunctions and in particular a relationship between trisomy 21 and maternal polymorphisms in genes of folate and homocysteine metabolism have generated considerable interest. James et al., (1999) were the first to propose the hypothesis that altered DNA methylation patterns resulting from abnormal folate metabolism may increase the risk of chromosome nondisjunction and in particular, suggetsed maternal polymorphisms in enzymes involved in this metabolic pathway as a risk factor for DS.

Methylenetetrahydrofolate reductase (MTHFR) is one of the most important enzymes in folate/homocysteine metabolism. It is responsible for the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate and regulates the intracellular flow of folate through the conversion of homocysteine to methionine for nucleotide synthesis. This reaction is important for the synthesis of S-adenosylmethionine (SAM), the major intracellular methyl donor for DNA, protein and lipid methylation.

Two polymorphisms (C677T and A1298C) in the MTHFR gene affect this pathway by reducing enzyme activity (Frosst et al., 1995; Van der Put et al., 1998), leading to an increased need of folic acid to allow the remethylation of Hcy to Met. An increase in Hcy levels associated to a decrease in methionine results in a decreased SAM to S-adenosylhomocysteine (SAH) ratio whose optimal value, known as methylation index (10:1) is required for a correct functioning of the methylation machinery (Round et al., 1998). Recently, a third common mutation of the MTHFR gene (G1793A) has been described but its effect on the folic acid metabolism is still unknown (Rady et al., 2002).

The remethylation of Hcy to Met is catalysed by the methionine synthase (MTR) enzyme, maintained in its functional state by the methionine synthase reductase (MTRR) reducing system. Two polymorphisms have been

described in the MTR and the MTRR genes, A2756G and A66G respectively, and the former has been associated with aberrant methylation in the homozygous state (Castro et al., 2004).

Furthermore, the mutation 844ins68 in the cystathionine beta-synthase (CBS) gene, reported by our group as a common polymorphism in the general population and carried by nearly 10% of the general population (Sebastio et al., 1995; Sperandeo et al., 1996; De Franchis et al., 2002), may affect Hcy levels and cause lower tHcy levels compared to non-carriers, in particular after methionine loading (Tsai et al., 1997).

Other mutations of genes involved in Hcy and folate metabolism include: A80G in the reduced-folate-carrier gene (RFC1), associated with higher plama folate levels and moderate hyperhomocysteinemia if in double homozygous with MTHFR 677TT genotype, and G1958A in the methylenetetrahydrofolate dehydrogenase gene (MTHFD), which encodes for a trifunctional enzyme that catalyses the conversion of tetrahydrofolate to 10-formyl, 5,10-methenyl, and 5,10-methylenetetrahydrofolate, donor cofactors for nucleotide and DNA biosynthesis (Chango et al., 2000).

James and coworkers were the first to report that the variant 677T allele of the MTHFR gene might be a maternal risk factor for having a child with DS in a North American population, and observed increased plasma Hcy levels in mothers of DS individuals (MDS), compared to mothers of healthy children (James et al., 1999).

After these studies, a number of research groups focused on case–control studies to further investigate the effect of folate pathway polymorphisms as possible risk factors for DS.

Some of these studies reported an association between maternal polymorphisms and trisomy 21 (James et al., 1999; Hobbs et al., 2000; O'Leary et al., 2002; Da Silva et al., 2005; Scala et al., 2006), whereas others could not find any association (Chadefaux-Vekemans et al., 2002; Stuppia et al., 2002; Bosco et al., 2003).

Another complexity to our understanding of the role of folate metabolism in DS risk is the fact that several metabolic genes, including those encoding for the reduced folate carrier (SLC19A1 or RFC1) and for cystathionine beta

synthase (CBS), are located on chromosome 21. Trisomy for these genes might increase folate demand or availability in developing DS fetuses. Therefore, the current opinion is that maternal and embryonic combinations for variants in folate metabolizing genes, coupled with the maternal nutritional and life style status during pregnancy, may strongly influence the probability that some embryos with trisomy 21 survive to the birth (Martínez-Frías et al., 2006).

Furthermore, the contribution of maternal age at conception adds complexity to the investigation of the role of folate metabolism in DS risk and emphasize the fact that human non-disjunction is a multifactorial trait that must be dissected into its component parts to identify specific associated risk factors (Lamb et al.,2005; Oliver et al., 2008; Hultén et al., 2008)

The discordant data among subjects of distinct geographical regions may be explained by differences in the nutritional habits, genetic background of the populations, ethnicity. Furthermore, the conflicting results suggested that the presence of a single polymorphism of a gene participating in the folate/Hcy metabolic pathway might be insufficient to increase the risk of having a child with DS, whereas the combined presence of two or more of them in the genome could increase DS risk.

2. The phenotypic variability of Down syndrome

Although caused in the majority of cases by full trisomy of chromosome 21, the DS phenotype is often variable among subjects. Almost all individuals show specific morphologic features, mental retardation and hypotonia that, taken together, constitute the hallmark of the syndrome. However, the degree of these clinical manifestation is largely variable and a wealth of other clinical manifestations occurs only in a proportion of subjects. Congenital cardiac anomalies, including ventricular septal defects, A/V communis, arterial septal defects, and patent ductus arteriosus, are present in about 40% of Down syndrome infants. Other congenital malformations include duodenal

stenosis or atresia (in about 10% of cases), congenital cataract, Hirschsprung disease. Down syndrome subjects also display immune-hematologic anomalies such as lymphopenia, impairment of cell mediated immunity and of serum immunoglobin patterns and increased susceptibility to dermal, mucosal, gastrointestinal and respiratory infections. Children with Down syndrome are also at greater risk of developing leukemia, usually the acute lymphocytic type, with an increase of 10-15 fold confined to the pediatric age group. Auto-immune and endocrine disorders such as hypothyroidism, gluten intolerance, diabetes, alopecia are also observed at increased frequency in Down syndrome compared to the general population. Finally, premature aging and early onset of Alzheimer's disease are included among DS features.

3. Metabolic imbalance in Down syndrome: homocysteine/folate metabolism, the oxidative stress and the mitochondrial dysfunction

Although the perception of DS as a metabolic disease is not prevalent, overexpression of genes encoding specific enzymes directly leads to biochemical aberrations that affect multiple interacting metabolic pathways, culminate in cellular dysfunction and contribute to the pathogenesis of DS.

A metabolic derangement of the Hcy/folate pathway and abnormal DNA methylation have been observed in children with DS, in whom a lack of active methyl groups and altered protein activity have been proposed as possible pathogenetic mechanisms of various manifestations, including mental retardation (Gueant JL et al., 2005; Butler C et al., 2006). In fact, at least six genes involved in the one-carbon/transulfuration pathway are mapped on chr 21, including: the cystathionine beta-synthase (CBS) gene; two folate transporter genes (RFC1 and FTCD respectively); the glycyneamide ribotide formyltransferase (GART) gene, involved in purines biosynthesis; a DNA methyltransferase 3-like gene (DNMT3L); and an arginine methyltransferase 1-like protein (HRMT1L1), involved in protein

post-translational methylation.

A major point of interest relies in the study of metabolic impairment of the Hcy/folate pathway due to the 3 copies of the CBS and RFC1 genes and of the influence of polymorphisms on biochemical data. However, a wealth of studies points to a higher grade of complexity of the pathogenesis of DS including the gene-dosage effect of selected genes localized on chr. 21, signal transduction pathway impairment and mithocondrial dysfunction (Butler et al., 2006, Chang et al., 2005; Arron et al., 2006).

In children with DS in whom the CBS gene resulted overexpressed with an activity increase of 157%, reduced Hcy levels were documented (Chadefaux et al., 1988, Chadefaux et al., 1985). Pogribna and coworkers (Pogribna et al., 2001) evaluated that the three copies of CBS resulted in a significant decrease of plasma Hcy, Met, SAM, SAH while the lymphocyte DNA was surprisingly hypermethylated (Pogribna et al., 2001).

Until now, there are no available data on the metabolic effects of the three copies of the CBS gene combined to polymorphisms of genes of the sulfur amino acid and folate pathway in patients with DS.

An imbalance of the cell redox activity was suggested as another key player in DS pathogenesis. In fact, protein modifications by reactive oxygen species (ROS) may have a crucial role in the pathogenesis of a number of DS features, such as neurodegeneration, premature aging, autoimmune disease and cataract. However, there is no definitive consensus on whether OS is mediated by increased oxidation, decreased antioxidant levels, or insufficient clearance of oxidized proteins.

In DS, markers of OS such as lipid peroxidation, protein modifications and DNA damage are increased as well as the gene expression of superoxide dismutase 1 (SOD1) and amyloid precursor protein (APP), both mapping on chr 21 (Zana et al., 2007, Galletti et al., 2007). Published data from our group showed a dramatic increase of L-isoaspartyl (L-isoAsp) residues in erythrocytes membrane proteins of DS subjects under basal condition and after oxidative treatment (Galletti et al., 2007). In proteins, L-isoAsp residues arise from deamidation of intrinsically labile L-asparagines, and might be related to cell ageing, impairment of cognitive function associated with

abnormal processing of amyloid beta precursor APP (Lott et al., 2006).

As to the total antioxidant capacity, it was shown to be decreased in one study and preserved in another (Carratelli et al., 2001; Zitnanova et al., 2006). In particular, impairment of the glutathione redox state has been observed in DS subjects (Pastore et al., 2001; Michova et al., 2007).

Both disruption of the sulfur amino acid pathway and oxidative damage can be related to mitochondrial dysfunction and may independently lead to impairment of the methylation machinery. Damage of mtDNA induced by enhanced oxidative stress in DS brains and fibroblasts (Busciglio J et al., 1995, Busciglio J et al., 2002), can result in ATP depletion and interfere with DNA methylation, since the synthesis of SAM, the major intracellular methyl donor for DNA, is a high-energy form of methionine and requires ATP. Also the alteration of the one-carbon metabolism modifies the levels of SAM, leading to different methylation reactions also in mithocondria, in which the status is already defective in DS pathogenesis.(Arbuzova et al., 2002; Infantino et al., 2011)

The mechanism and the cellular site (cytosol and/or mitochondria) responsible for the overproduction of ROS have not been fully elucidated. The up-regulation of the SOD1 gene, located on human chr 21 (Torsdottir et al., 2001; Sinha, 2005), has been proposed as a potential culprit. However, an animal model of DS, the TS1Cje mouse, carrying a subset of triplicated human chr 21 orthologs that lacks SOD1, shows OS and mitochondrial dysfunction (Shukkur et al., 2006).

In fact, ROS are generated inside mitochondria by respiratory chain complexes I and III. Dysfunctions of these complexes, as well as of ATP synthase, can result either in reduced ATP levels or in increased ROS production (Raha et al., 2000) and were associated to neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntington's diseases and in the normal aging process (Schon et al., 2003; Ames, 2004; Kidd, 2005; Gandhi et al., 2005).

In human DS cells, gene expression variations of oxidative phosphorylation units Krebs cycle enzymes (Kim et al., 2000; Kim et al., 2001; Lee et al., 2003; Bajo et al., 2002), reduced mithocondrial redox activity and membrane

potential (Busciglio et al., 1995), ATP depletion (Valenti et al., 2010), impaired mtDNA status and repair systems (Infantino et al., 2010; Druzhyna et al-, 1998) were described.

4. Gene expression variation

The simplest model for gene expression in DS would predict that each gene on chromosome 21 would be expressed at 150% of the levels seen in euploid individuals. A number of investigators have examined this question both in tissues or cell lines from human DS and in mice trisomic for regions of Mmu16 homologous to HSA21. In general, the outcomes of these studies seem to support the hypothesis that many HSA21 genes are expressed at the expected 150% of normal, but there are numerous exceptions, including genes on HSA21 that are not overexpressed at all or overexpressed by more than 150%. In some studies, only overall global chromosome 21 gene expression could be concluded to be overexpressed. That is, the set of HSA21 genes taken together is overexpressed by 150%, but overexpression of individual genes could not be assessed in a statistically significant way (Mao et al., 2003). In some cases, expression levels of individual HSA21 genes appeared to differ in different tissues and cells (Li et al., 2006). In some tissues, HSA21 genes are overexpressed while in others these same genes are not overexpressed. A similar situation exists in the Ts65Dn mouse model of DS. In this case, expression of the trisomic gene GABPA protein is elevated only in brain and skeletal muscle (O'Leary et al., 2004). Clearly, an answer to whether or not each HSA21 gene is overexpressed, to what extent, in which tissues, and during which developmental stage is an exceedingly important information to have when attempting genotype –phenotype correlations.

An additional level of complexity comes from the recent observations of extensive gene-expression variation among unaffected individuals (Storey et al., 2007; Cheung et al., 2003; Monks et al., 2004) and that some HSA21 genes varying up to 40-fold among individuals (Prandini et al., 2007; Deutsch

et al., 2005; Antonarakis et al., 2004; Stranger et al., 2005).

Therefore, some authors suggested that overexpressed genes in DS can be considered good candidates for the pathogenesis only if tightly regulated in euploid cells, and that may play a role in the constant features of the syndrome if tightly regulated also in DS, while they could be responsible of the variable phenotypic features if expressed with an intermediate value (Sultan et al., 2007; Prandini et al., 2007; Ait-Yahya Graison et al., 2007).

5. Mechanisms underlying gene expression variation

The inference of data from gene expression studies is complicated by the great number of factors that may influence gene expression besides trisomy 21, such as copy number variations, transcription factors variation, conserved non-coding regions, post-transcriptional regulation, DNA methylation and gene-gene interactions (Patterson, 2007).

5.1 Copy number variations.

Copy number variations (CNVs) can be defined as regions of the genome, often several kb or larger, that vary in copy number from individual to individual (Iafrate et al., 2004; Sebat et al., 2004; Hegele, 2007). Often, these CNVs contain genes. A recent study presents evidence that duplication of a region of HSA21 including the APP gene can lead to familial Alzheimer's disease (AD) in five separate families. In these families, the duplication ranges from 0.58 to over 6 Mb (Rovelet- Lecrux et al., 2006). Importantly, a detailed phenotypic analysis of the five families in which this duplication was observed demonstrated that there was no clinical evidence of DS (other than the appearance of AD) (Cabrejo et al., 2006). This observation is consistent with the idea that trisomy of APP may explain, at least in part, the observation that persons with DS virtually always develop the neuropathology associated with AD, but that trisomy of APP is not sufficient

to cause DS. However, there are four other genes in the smallest duplicated region reported in these families, and the level of gene expression was not reported. Thus, the conclusion that APP is the responsible gene is attractive, but not definitively proven yet. A recent report indicates that duplication of a 4.3 Mb region of HSA21 containing slightly more than 30 genes causes a DS phenotype in three family members (Ronan et al., 2007). These family members had the facial gestalt of DS but had mild cognitive disability. Again, however, no measurement of the level of overexpression of the genes in the duplicated region was reported. Moreover, two genes hypothesized to be involved in the phenotype of DS, DSCR1 and DSCAM, were not included in the duplicated region. Thus, it appears that duplication of this region, while contributing to the phenotype of DS, is probably not sufficient to cause all the features of DS. Although these data implicate CNVs as a reason for variation in gene expression, a recent study estimates that single nucleotide polymorphisms detect about 84% of gene expression variability while CNVs capture about 17% (Stranger et al., 2007).

5.2 Transcription Factors and Variability of Expression.

There are at least 25 genes on HSA21 that directly or indirectly regulate gene transcription (Gardiner, 2006). Many if not most of these function as parts of multiprotein complexes. Alterations in the relative abundance of members of a protein complex may affect its activity in ways that are difficult to predict. Moreover, many genes are regulated by sets of transcription factors acting in concert.

5.3 Conserved Noncoding Regions.

As the essentially complete genomic sequences of mammals in addition to humans have become available, genomic sequence comparisons have revealed the existence of a large number of conserved non-coding regions (Dermitzakis et al., 2005). Conserved noncoding regions (CNCs) are not repetitive sequences and the vast majority are not transcribed (Dermitzakis et

al., 2005). HSA21 was the first chromosome completely analyzed for CNCs, and 2,262 were identified. Possible functions for CNCs include roles as cis- or trans-regulatory regions or elements required for chromatin or chromosome structure (Dermitzakis et al., 2004).

5.4 Post-transcriptional regulation.

As the discussion above demonstrates, assessments of transcript levels are crucial for understanding DS and other conditions. However, post-transcriptional regulatory mechanisms clearly play important roles, since transcript levels do not always accurately reflect the levels of the proteins they encode. Some unexpected mechanisms of posttranscriptional regulation have been revealed by functional genomics studies.

5.5 MicroRNAs.

MicroRNAs (MiRNAs) are endogenous small RNA molecules of about 22 nt derived from larger transcripts (Bartel, 2004; Kosik and Krichevsky, 2005). Hundreds of miRNAs have been identified (Nam et al., 2005). They bind to the 30 UTRs of mRNA molecules and can interfere with mRNA translation or subject mRNA to degradation (Bartel, 2004; Bartel and Chen, 2004; Fahr et al., 2005; Maas et al., 2006). An intriguing hypothesis is that miRNAs serve to dampen translation of mRNAs rather than have an all or none effect. In this way, they may be able to exert more rapid and subtle modifications of protein levels than transcriptional control mechanisms (Bartel and Chen, 2004; Kosik and Krichevsky, 2005). At least five putative miRNAs are encoded by genes on HSA21. Recent work shows that miRNAs constitute up to 5% of human genes (Niwa and Slack, 2007). Each miRNA may contribute to the regulation of expression of hundreds of mRNAs. It appears that miRNAs may be particularly relevant to regulation of genes in the nervous system, genes that control expression of other genes, e.g., transcription factor genes, or genes that control signaling. There is evidence suggesting that this mechanism of gene regulation is relatively rare for genes that are widely

expressed in many cell types, and that may be essential for cellular survival, for example, metabolic genes (Cui et al., 2007; Gaidatzis et al., 2007).

5.6 DNA methylation.

DNA methylation is strictly speaking an epigenetic change, not a genetic change. However, it clearly plays a role in gene expression, and genes on HSA21 play a role in the phenomenon. Since DNA methylation is critical for meiosis, and recent experiments suggest that it may play a major role in memory formation, a brief discussion is included here. Methylation of the 50 position of cytosines located in cytosine-guanosine dinucleotides resulting in m5C is the primary, if not the only, modification of mammalian DNA (Turek-Plewa and Jagodzinksi, 2005). Generally, DNA methylation is thought to play a role in long-term silencing of gene expression, and to be critical for mammalian development (Turek-Plewa and Jagodzinksi 2005; Shames et al., 2007). In some cases, tissue-specific DNA methylation may be important for tissue specificity of gene expression in mice (Song et al., 2005). In cancer, global genome hypomethylation is often observed as coupled with hypermethylation of specific genes, for example, tumor suppressor genes (Shames et al., 2007). It has been hypothesized that altered dietary folates can affect DNA methylation, and correlations have been drawn between folate deficiency and increased risk for many types of cancer (Kim, 2005). The gene encoding the reduced folate carrier, considered the primary protein responsible for cellular internalization of folates, is located on HSA21 (Moscow et al., 1995). Thus, one could hypothesize that elevated levels of the reduced folate carrier protein might enhance cellular uptake of folates mitigating the effects of low folate, including the effects of low folate on DNA methylation. This might be related to the observed low incidente of solid tumors in individuals with DS (Patja et al., 2006). Recently, demethylation of methylated miRNA resulting in its activation has been observed in cancer cells (Lujambio et al., 2007). This finding implies a link between regulation of gene expression by DNA methylation and miRNA. DNA methylation is carried out by a family of DNA methyltransferase

(DNMT) enzymes. A member of this gene family, DNMT3L, is located on HSA21 (Aapola et al., 2000). The DNMT3L protein has significant homology to the DNMT3 DNA methyltransferases, but its DNA methyltransferase activity has not yet been demonstred. However, it interacts directly with DNMT3A. DNMT3L knockout mice are viable, but sterile. Study of these mice has shown that DNMT3L is required for establishment of maternal imprints during oogenesis. Moreover, it is necessary for normal spermatogenesis (Hata et al., 2006). It is difficult to extrapolate from knockout mice to the possible effects of trisomy of the DNMT3L gene in persons with DS. However, one could speculate that overexpression of this gene might alter meiotic DNA methylation and be related to aberrant chromosomal segregation and also to the sterility seen in men with DS.

5.7 Interactions between genes.

Overexpression of individual genes cannot be considered indipendently when one considers phenotype–genotype correlations. Recent evidence demonstrates that trisomy of two or more genes acts synergistically to affect phenotype. A well-developed example of this type of synergy is the accumulating evidence that elevated expressions of the DYRK1A and DSCR1 genes on HSA21 act synergistically to prevent the nuclear localization of the NFATc transcription factor (Arron et al., 2006). Thus, trisomy of either of these genes individually may not be sufficient to cause this dysregulation, but trisomy of both is sufficient to cause dysregulation.

6. Chromosome 21 genes and Down syndrome phenotype

An essential step in understanding how the genes on HSA21 lead to DS has been the publication of the virtually complete DNA sequence of the long arm of HSA21 (21q) and the annotation of this sequence (Hattori et al., 2000). Annotation of HSA21 is an ongoing process and, currently, about 430 genes

have been identified (http://chr21db.cudenver.edu/). Along with the increasing knwoledge of the HSA21 structure, an unexpected complexity in genome structure and in gene regulation emerged with profound implications for understanding genotype/phenotype relationships.

A specific region approximately 5.4 Mb onchromosome 21 (21q22.3), named Down syndrome Critical Region (DSCR) (Delabar et al., 1993) has been found to be associated with several of the major DS phenotypes including hypotonia, short stature, facial dysmorphisms, and mental retardation (Olson et al., 2007; Roper and Reeves 2006), even if it was observed that trisomy of this region is necessary but not sufficient to cause the brain phenotype in the trisomic mouse model (Olson et al., 2007).

Currently, two different hypotheses have been proposed to explain the DS phenotype: the "gene-dosage effect" and the "developmental instability" hypothesis. The first hypothesis postulates that the overexpression of specific chromosome 21 genes directly contributes to different aspects of the disease phenotype (Korenberg et al., 1990), while the alternative "developmental instability" hypothesis states that the expression of extra genes, regardless of their identity or function, globally disturbs the correct balance of gene expression in DS cells during development (Saran et al., 2003; Shapiro et al., 2001).

To determine which hypothesis applies to the etiology of DS, a number of investigators have examined this question both in tissues or cell lines from individuals with DS and in mice trisomic for regions of Mmu16 homologous to HSA21. Several methods have been used, including DNA microarray analysis, serial analysis of gene expression (SAGE), and, less commonly, qRTPCR or proteomic approaches.

In mouse models of DS most of these studies reported a generalized overexpression of triplicated genes at the mRNA level (Amano et al., 2004; Lyle et al., 2004; Kahlem et al., 2004; Dauphinot et al., 2005), while studies performed on human trisomic tissues indicated apparently discrepant results with some studies essentially showing the selective overexpression of a limited subset of chr21 genes (Altug-Teber et al., 2007; ; Mao et al., 2003; Mao et al., 2005), whereas other studies describe a more generalized

transcriptional dysregulation (Conti et al., 2007; FitzPatrick et al., 2002; Gross et al., 2002; ; Lockstone et al., 2007; Tang et al., 2004; Rozovski et al., 2007).

In particular, among studies underscoring essentially the dysregulation of chr 21 genes in DS, Mao et al., (2003 and 2005) reported that, in fetal cerebral cortex, heart, and primary cultures of cortical astrocytes, the most consistently dysregulated chr 21 genes, were those involved in mitochondrial function (ATP5O, ATP5J and MRPL39) metabolic pathways (oxidoreductase, NADH dehydrogenase), antioxidant defenses (SOD1, superoxide dismutase I), and extracellular matrix (COL6A1, Collagen type VI alpha I). In fibroblast and fetal hearts, Li et al., (2006) proposed hyperactive interferon signaling as candidate pathway for cell senescence and autoimmune disorders in DS, and abnormal purine metabolism for a potential role in cardiac defects (Li et al., 2006). A partial overlap with their overexpression patterns of chr. 21 genes, was reported also in amniocytes and chorionic villus cells by Altug-Teber et al., (2007).

In audult T cells from DS, the most consistent finding was the overexpression of the superoxide dismutase gene (SOD1), MHC DR beta 3 (HLA-DRB3), GABA receptor A gamma 2 (GABRG2), acetyltransferase Coenzyme, A 2 (ACAT2) and ras suppressor protein 1 (RSU1) genes (Giannone et al., 2004).

On the other hand, to support the "developmental instability" hypothesis, studies on amniocytes (FitzPatrick et al., 2002) and fetal hearts (Conti et al., 2007) found a vast majority of differentially-expressed genes not located on chr 21. In particular, the authors found upregulation of extracellular matrix (ECM) genes including metalloproteases in both tissues, and down regulation of genes associated with growth factors and mitochondrial function in fetal heart where oxidative phosphorylation was indicated as the most affected pathway.

In cultured trophoblasts, Rozovski and colleagues (2007) showed an association between trisomy 21 and various biological processes connected to oxidative phosphorylation, ubiquitin cycle, purine nucleotide biosynthesis, rRNA metabolism and Alzheimer disease.

In post-mortem adult cortex (Lockstone et al., 2007) and in whole blood

RNAs (Tang et al., 2004), dysregulated pathways encompassing developmental, lipid transport, cellular proliferation, and transcriptional regulation genes were found.

Finally, one microarray study on cell-free fetal mRNA from uncultured amniotic fluid supernatant samples (Slonim et al., 2009), identified a deregulation of genes mostly belonging to functional classes pertaining to oxidative stress, phospholipids, ion transport molecules, heart muscle, structural proteins, and DNA repair and suggested compounds related to oxidative stress and to potassium/calcium signalling.

In conclusion, these contrasting results, deriving at least partly from differences due to tissue specificity, developmental stages and the applied experimental platforms, suggested that the two hypotheses are not mutually exclusive and that it is possible that the DS phenotype is caused in part by genes that are over-expressed from chromosome 21, and in part by a generalized dysregulation.

Recently, to extract more consistent and uniform informations, Vilardell et al., (2011) performed a statistical meta-analysis from 45 heterogeneous publicly available DS data sets, from human and mouse, at transcriptome and proteome level. The authors identified 324 genes with significant genome-wide dosage effects, including 77 HSA21 genes such as SOD1, APP, RUNX1 and DYRK1A, and 247 genes located on other chromosome, highlighting the genome-wide impact of DS. Pathway analysis, performed on all the 324 significant genes, referred mainly to nervous system development, neurodegeneration (e.g. Huntington's disease, Alzheimer's disease or Parkinson's disease) and defects in synapsis (e.g. Axon guidance, NGF signaling), while Gene Ontology analysis of the 247 non-HSA21 genes revealed their association to human development (organ, tissue and cell development). Additionally, 70 different transcription factors, identified as being, directly or indirectly, affected by dosage imbalance, resulted associated to neurological development, organ development and stress response. Finally, 62 new genes, associated to neurodegenerative disorders including Alzheimer's disease and age-related degeneration, were identify as novel candidates.

However, this comparison have some limitations since the probes for the orthologous mouse and human genes do not correspond well and genes associated to nervous system might be over-represented, being the brain the dominant sample source.

7. EXPERIMENTAL BACKGROUND

Genome-wide expression analysis of lymphoblastoid cell lines from subjects with Down syndrome

In the last years our group investigated the gene expression profiles of DS lymphoblastoid cell lines by DNA microarray analysis. Affymetrix HU133 plus 2.0 oligonucleotide arrays containing 47,000 probe sets allowed us the screening of about 38,500 well characterized human genes.

The expression values of the RNA derived from 6 DS subjects and 6 controls were determined using the Affymetrix GeneChip software v. 5.0 and then normalized to the values of the entire microarray by the GeneSpring software (Silicon Genetics, Redwood City, CA). Age, gender, karyotype and phenotypic features of the analyzed samples are provided in Table 1.

ANOVA was used to identify all genes that differed at level of $p < 0.01$ and $p < 0.05$ and these were further filtered for data quality and for differences in hybridization signals of 1.2-fold above the experiment mean. Corrections for multiple tests comparisons were been also applied. Finally, the Gene Tree function was used to group the differentially expressed genes into over-expressed and under-expressed.

Table 1. Characteristics of the samples analysed in the study

Samples	Karyotype	Age (years)	Phenotype
Trisomy 21	47, XX +21	5	MR, CHD, HT
	47, XY +21	6	MR, cataract
	47, XX +21	4	MR, CHD
	47, XY +21	5	MR, CHD, HT
	47, XX +21	4	MR, R-URTI
	47, XY +21	6	MR, HT
Control	46, XX	5	normal
	46, XY	6	normal
	46, XX	4	normal
	46, XY	3	normal
	46, XY	5	normal
	46, XX	6	normal

MR: mental retardation; CHD: congenital heart defect; HT: hypothyroidism; R-URTI: recurrent upper- respiratory tract infections.

7.1 Deregulation of functional classes and pathway perturbation

For the the genome-wide expression profiles data analysis was performed using a p-value cut-off of 0.01 and a list of 406 differentially expressed transcripts, throughout the genome, was generated, corresponding to 83% down-regulated (n=335) and 17% up-regulated (n=71).

By using DAVIDBionfomatics, all differentially expressed genes were categorized according to GO classification based on their hypothetical biological processes and molecular functions. This analysis, performed by submitting the two lists of down and up-regulated genes separately, revealed that down regulated categories were mostly associated to: ubiquitin metabolism; cell signalling, with a particular enrichment for NF-kappaB cascade; cell cycle; protein localization and regulation of gene expression (Table 2).

Among up-regulated categories the most enriched resulted: developmental

processes and transport and localization with a strong enrichment for calcium ion transport (Table 3)

Pathway analysis, performed by submitting the list of all 406 deregulated genes, identified ubiquitin mediated proteolysis as the pathway mostly influenced by trisomy 21 (p=2.9 E-5; fold enrichment= 4.8).

Table 2. Enriched GO categories of down-regulated gene (FDR 0.01), sorted by p-value

Categories	n. of genes	% of genes in list category	p-value	fold enrichment
Ubiquitin metabolism				
ubiquitin-dependent protein catabolic process	16	5.4	5.2E-6	4.3
modification-dependent protein catabolic process	25	8.5	1.4E-5	2.7
proteolysis involved in cellular protein catabolic process	25	8.5	2.9E-5	2.6
proteolysis	29	9.8	0,0093	1.6
proteasomal ubiquitin-dependent protein catabolic process	6	2.0	0,025	3.6
ligase activity	17	5.8	0,001	2.6
ubiquitin-protein ligase activity	8	2.7	0,013	3.2
Cell signalling				
positive regulation of I-kappaB kinase/NF-kappaB cascade	9	3.1	0,00021	5.5
positive regulation of protein kinase cascade	10	3.4	0,0024	3.5
positive regulation of signal transduction	12	4.1	0,01	2.4

Cell cycle				
mitotic cell cycle	14	4.7	0,0084	2.3
cell cycle phase	15	5.1	0,0086	2.2
M phase of mitotic cell cycle	10	3.4	0,013	2.7
mitosis	9	3.1	0,031	2.4
nuclear division	9	3.1	0,031	2.4
interphase	6	2.0	0,033	3.3
Protein localization				
establishment of protein localization	25	8.5	0,0017	2.0
Regulation of gene expression				
gene expression	62	21.0	0,022	1.3
mRNA processing	12	4.1	0,023	2.2
RNA splicing	11	3.7	0.024	2.3
Vescicle related				
ER to Golgi vesicle-mediated transport	4	1.4	0,033	5.6

Table 3. Enriched GO categories of the up-regulated gene (FDR 0.01), sorted by p-value

Categories	n. of genes	% of genes in list category	p-value	fold enrichment
Development				
cellular developmental process	14	23.3	0,002	2.5
anatomical structure development	17	28.3	0,004	2.0
cell differentiation	13	21.7	0,005	2.4
system development	16	26.7	0,005	2.1
multicellular organismal development	17	28.3	0,015	1.8
organ development	12	20.0	0,021	2.1
Transport and localization				
calcium ion transport	5	8.3	0,0010	10.6
transmembrane transport	7	11.7	0,01	3.7
metal ion transport	6	10.0	0,016	3.9
localization	17	28.3	0,022	1.7
cation transport	6	10.0	0,032	3.3
transport	15	25.0	0,034	1.7
ion transport	7	11.7	0,035	2.8
establishment of localization	15	25.0	0,037	1.7

7.2 Chromosome 21 expression profiles

Of the 168 chromosome 21 transcripts with FC ≥|1.2|, 86 corresponding to 69 known genes resulted differentially expressed in DS vs controls lymphoblasts, with FC ranging from 1.20 to 2.57 and a mean DS/control ratio of 1.36 ± 0.2, suggesting that a substantial number of triplicated genes escapes the gene-dosage rule, and underscoring the presence of complex mechanisms of regulation of gene expression.

To identify functional categories associated to DS, the list of the trisomic deregulated genes was submitted to a GO analysis. Results indicated regulation of ATPase activity coupled to transmembrane movement of substances and oxidative phosphorilation as the major enriched categories (Table 4).

The analysis from three different databases (GenMapp, BioCarta and KEGG), showed Alzheimer's, Parkinson's and Huntington's disease as the most deregulated biological pathways (Table 5).

Table 4. Enriched GO categories of HSA21 up-regulated genes

Term	n. of genes	Genes	%	P-Value	Fold Enrichment
ATPase activity, coupled to trans membrane movement of substances	4	*ATP5O, ATP5J, ABCC13, ABCG1*	6.2	0.005	11.3
Cofactor metabolic process	4	*SOD1, FTCD, PDXK, CRYZL1*	6.2	0.029	5.8
Regulation of cholesterol biosynthetic process	2	*ABCG1, SOD1*	3.1	0.031	62.2
Oxidative phosphorylation	3	*ATP5O, ATP5J, NDUFV3*	4.7	0.04	9.2

Table 5. Enriched pathways of HSA21 up-regulated genes

Term	n. of genes	Genes	%	P-Value	Fold Enrichment
Parkinson's disease	4	*ATP5O, ATP5J, NDUFV3, UBC6/7*	6.2	0.010	8.3
Alzheimer's disease	4	*ATP5O, ATP5J, NDUFV3, BACE*	6.2	0.023	6.1
Huntington's disease	4	*ATP5O, ATP5J, NDUFV3, SOD1*	6.2	0.030	5.5

8. Aims of the thesis

The aims of the present thesis were to expand the knowledge on the molecular bases of DS and to provide new tools that could be useful for the development of innovative therapeutic approaches.

For this purpose, different objectives and different strategies have been applied.

The previous genome-wide analysis, which allowed us to compare DS and euploid lymphoblasts expression profiles, identified ubiquitin mediated proteolysis as the pathway most influenced by trisomy 21, whereas ubiquitin metabolism and regulation of NFkappaB cascade resulted to be two of the most significant down regulated GO categories.

On the basis of these results and of the data present in the literature, the objectives developed in the present thesis were:

- Functional analysis of the ubiquitin-proteasome system (UPS), both to validate microarray data and to investigate its involvement in DS phenotype.

- Functional analysis of the NFkappaB pathway, both to validate microarray results and to study model proteins regulated by ubiquitination in association to DS.
- Analysis of the DYRK1A/DSCR1/NFAT pathway, suggested as a candidate pathway with a role in mice models of trisomy 21 (Arron et al., 2006), but still not well investigated in human DS samples.

Finally, since MCPH1, a causative gene of primary microcephaly in consanguineous Pakistani families (Roberts et al., 2002; Woods et al., 2005), was found significantly down-regulated in our population of subjects with Down syndrome, in whom syndromic microcephaly has been described, we performed a sequencing analysis of the entire gene to identify the presence of possible alterations also in a population of unrelated patients with primary microcephaly from different ethnic groups.

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Chapter 2

Impairement of the Ubiquitin-Proteasome system in Down syndrome

INTRODUCTION

1. Ubiquitin-proteasome system

The ubiquitin proteasome system (UPS) is the major proteolytic pathway used by eukaryotic cells to metabolize proteins (Ciechanover, 2005).

The substrate is first marked for degradation by covalent linkage to ubiquitin, a small 8.5 kDa protein. After the first ubiquitin is attached to the protein to be degraded, further ubiquitin molecules (4 to 7) are bound to form a polyubiquitin chain through the action of three different enzymes termed E1, E2, and E3, in ATP dependent manner. The polyubiquitinated protein is then targeted to a multi-subunit protease termed proteasome 26S which has a 20S catalytic core, that possesses three different proteolitic activities: chimotrypsin-like; trypsin-like and caspase-like (Rechsteiner et al., 2005; Kisselev et al., 2002); and two 19S regulatory caps (Baumeister et al., 1998; Wolf et al., 2004).

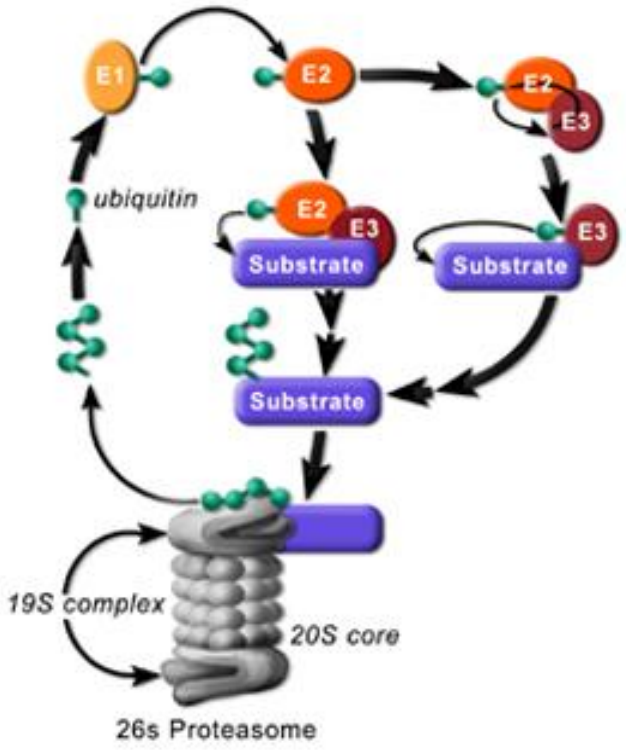
At the proteasome, the polyubiquitinated substrate is degraded to small peptides and amino acids, while the polyubiquitin chain is disassembled to ubiquitin monomers that are released for future use (Glickman et al., 2002; Rajkumar et al., 2005; Nussbaum et al., 1998) (Fig. 1)

UPS is known to metabolize not only misfolded, oxidized and damaged proteins, but also proteins involved in many cellular processes such as cyclins, transcriptional activators and their inhibitors, and components of signal transduction pathways. Ultimately, a link between protein synthesis and degradation by UPS has been proposed (Ding et al., 2006).

Intracellular protein degradation is relevant for most cellular and physiological functions including: apoptosis, cell cycle progression, differentiation, stress response, DNA repair and antigen presentation in the immune response.

The pathological states associated with the ubiquitin system can be classified into two groups: those that result from loss of function, such as mutation in a ubiquitinating enzyme, that result in stabilization of certain proteins; and those that result from gain of function and abnormal or accelerated degradation of the protein target.

Fig. 1 Ubiquitination of proteasomal substrate proteins is performed by a complex system consisting of ubiquitin activating (E1) enzymes, ubiquitin-conjugating (E2) enzymes and substrate recognition proteins (E3 enzymes).



By http://ocw.mit.edu/courses/biology/7-340-ubiquitination-the-proteasome-and-human-disease-fall-2004/

Alterations in ubiquitination and deubiquitination reactions have been directly implicated in the etiology of many malignancies through the stabilization of oncoproteins or destabilization of tumor suppressor genes. Furthermore, accumulation of ubiquitin conjugates and/or inclusion bodies associated with ubiquitin, proteasome, and certain disease-characteristic proteins have been reported in a broad array of chronic neurodegenerative diseases, such as the neurofibrillary tangles of Alzheimer's disease (AD), brain stem Lewy bodies (LBs) in Parkinson's disease (PD) and intracellular bodies in Huntington's disease (reviewed by van Tijn et al., 2008; Glickman MH et al., 2002). Finally, the ubiquitin system is also involved in immune and inflammatory

response: it degrades, in a nondiscriminatory manner, both intracellular “self ” as well as foreign “non-self” proteins such as viral proteins. Peptides from both populations are presented to CTLs, but those that are derived from self proteins do not elicit a T-cell response. It is easy to imagine that aberrations in processing of these proteins may lead to autoimmune diseases.

1.1 UPS activity, oxidative stress (OS) and mitochondrial function

An imbalance of the cell redox activity is known to be a feature of DS (Zana et al., 2007; Galletti et al., 2007; Carratelli et al., 2001; Zitnanova et al., 2006; Pastore et al., 2001; Michova et al., 2007) and was proposed as a key player in the pathogenesis since the accumulation of proteins modified by reactive oxygen species (ROS) may have a crucial role in neurodegeneration, premature aging, autoimmune diseases and cataratta also present in DS.

However, the causes of chronic oxidative stress observed in DS subjects are not yet known and there is no definitive consensus on whether OS is mediated by increased oxidation, decreased antioxidant levels, or insufficient clearance of oxidized proteins.

Several studies reported that oxidized proteins are selectively cleared by the ubiquitin-proteasome system (UPS) upon OS (Poppek et al., 2006; Breusing et al., 2008) and that there is a decline of the proteasomal activity after sub-lethal OS, followed by a functional recovery in the after-stress period (Reinheckel et al., 1998).

Furthermore, it was reported that as the proteasome is made by proteins, it may be itself damaged by oxidative stress, in particular the 19S regulatory particle (Prendmore et. al., 2010; Reinheckel et al., 2000). Other components of the UPS are regulated by OS as well, with a transient decrease of Ub-conjugates during stress, followed by increase in the Ub-conjugates during recovery (Shang et al., 1997).

Since mitochondria are the main source of ROS, UPS activity is tightly connected with mitochondrial function. A close relationship between mitochondrial abnormalities and oxidative damage have been described in DS

brains and fibroblasts (Busciglio et al., 2002 ; Raha et al., 2000) and gene expression variations of oxidative phosphorylation units, increased levels of the Krebs cycle enzymes and impaired mtDNA repair systems (Kim et al., 2000; Kim et al., 2001; Lee et al., 2003; Bajo et al., 2002; Druzhyna et al., 1998) were described in human DS cells.

Furthermore, also some mitochondrial proteins are degraded by UPS that, along with autophagy, is involved also in the clearance of damaged mitochondria from the cell (Carlucci et. al., 2008; Carrard et al., 2002). At least 4 E3 ligases are localized to the mitochondria. One of these, MULAN, is also an activator of NF-kappaB, thus providing a mitochondria-to-nucleus signalling (Li et al., 2008). Deubiquitinating enzymes (USP30) present in the mitochondrial outer membrane regulate mitochondrial morphology as well (Nakamura et al., 2008). Finally, a dinucleotide deletion in Ub-B mRNA leads to formation of polyubiquitin (UbB)+1 and causes mitochondrial stress and neuronal degeneration in primary neurons (Tan et al., 2007).

OBJECTIVE

A preliminary genome-wide expression analysis performed by our group, revealed that ubiquitn mediated proteolysis is the pathway mostly influenced by trisomy 21 in lymphoblastoid cell lines, in particular among the down-regulated GO categories.

UPS is mainly involved in the degradation of oxidized or damaged proteins and chronic oxidative stress is a feature of DS.

Hence, since there has been increasing interest in the ubiquitin-proteasome system (UPS) because of its possible role in neurodegenerative diseases, autoimmune disorders and cancer, functional analyses were performed to investigate its involvement in the DS phenotype.

MATERIALS AND METHODS

Cell culture and treatment

Peripheral blood samples were collected, after informed consent, from control and karyotipically confirmed full trisomy 21 children. All subjects were recruited at the Department of Pediatrics, Federico II University of Naples, the regional referral center for DS.

Lymphocytes were isolated by using Ficoll-Hypaque (Gibco) and then immortalized by Epstein-Bar Virus (EBV) to generate lymphoblastoid cell lines (LCLs).

Cells were grown in RPMI-1640 (Cambrex) supplemented with 20% fetal bovine serum, 5000U/ml penicillin-streptomycin, 2 Mm glutamine (Gibco) and 0.16 mg/ml gentamicin, at 37°C.

Oxidative stress was induced with 0.1 mM H_2O_2 for 30 minutes and the recovery was evaluated 1, 2 and 4 hours after resuspention in fresh RPMI.

The proteasome blocking was induced with 50μM MG-132 (Calbiochem) for 6 hours at 37°C for western blot experiments, and 100 μM MG-132 for 2 hours for the proteasome activity assay.

Protein extraction

Total protein extraction was carried out lysing the cells with the Tropix lysis solution (Applied Biosystems) supplemented with 0.1 M DTT, protease inhibitor cocktail 1X (Sigma), 50 μM MG-132 (Calbiochem) and 5 mM Manganese according to Mimnaugh and Neckers protocol (Mimnaugh and Neckers, 2002)

Western blot analysis

Protein extracts (20-40 μgr) were separated by 10% SDS-PAGE and then transferred on PVDF membrane (Millipore). Membranes were then blocked for 1 hour in milk 5% - PBS 1X-0.1% Tween-20 (T-PBS) and then incubated overnight with diluitions of specific primary antibodie anti-ubiquitina (P4D1) (CellSignalling).

Anti-mouse IgG horseradish peroxidase conjugated (GE-Healthcare) was

used as secondary antibody and the specific bands were visualized by ECL reaction (GE-Healthcare).

Luminescent proteasome assay

To individually measure the chymotrypsin-like, trypsin-like and caspase-like protease activity associated with the proteasome complex in cultured cells we used the Proteasome-Glo™ 3 substrates Cell-Based Assay (Promega). Reagents from this kit contain three specific luminogenic proteasome substrates in a buffer optimized for cell permeabilization, proteasome activity and luciferase activity. For the Chymotrypsin-Like Assay, there is the Suc-LLVY-Glo™ Substrate; for the Trypsin-Like Assay the Z-LRR-Glo™ Substrate; and for the Caspase-Like Assay there is the Z-nLPnLD-Glo™ Substrate.

Each sample was performed in duplicate and in two separate experiments and for each of them were considered :

• Blank: Proteasome-Glo™ Cell-Based Reagent + culture medium (without cells) and vehicle control used (DMSO), to measure the background luminescence

• Test : Proteasome-Glo™ Cell-Based Reagent + culture medium containing cells and vehicle control (DMSO)

• Treatment-Test: Proteasome-Glo™ Cell-Based Reagent + culture medium containing cells with treatment compound (H_2O_2) and vehicle control (DMSO)

• Inhibitor Control: Proteasome-Glo™ Cell-Based Reagent + culture medium containing cells with a specific proteasome inhibitor (MG-132 in DMSO), to identify nonspecific protease activity not related to the proteasome.

Lymphoblastoid cell lines, in concentration of 20,000/well in a 96-well plate, were incubated at 37°C overnight and then treated with 100 μM MG-132 for 2 hours and 0.1 mM H_2O_2 for 30 minutes, before adding the Proteasome-Glo™ Cell-Based Reagents. The luminescence was read after 15 minutes in a plate-reading luminometer (TECAN)

Statistical Analysis

The data were reported as the means ± S.E. and the statistical significance of differences between means was assessed by using the two-tail unpaired Student's t test. The differences between the means were accepted as statistically significant at the 95% level ($p = 0.05$).

RESULTS

The ubiquitn-proteasome system was investigated in DS lymphoblastoid cell lines by Western blot analysis and proteasome activity assay.

Western blot analysis, performed on total proteins extracted before and after proteasome blocking with MG132, showed that in DS there is a significant reduction of ubiquitinated protein levels before treatment and a surprising increase of their accumulation following MG132 treatment (Fig. 2, 3).

The same experiments, performed before and after oxidative stress induction with H_2O_2, showed that in DS there is an increase of the accumulation of the ubiquitinated proteins also under stress conditions and that there is a slower recovery of their basal levels compared to controls. (Fig 4, 5)

The subsequent proteasome activity assay, performed on cells before and after H_2O_2 induction, revealed a significative increase in the trypsin-like and in the chymotripsin-like activitity ($p \leq 0.01$) in DS subjects, both in basal and stress conditions, while no difference was observed for the caspase-like activity (Fig. 6)

Fig. 2 Western blot analysis of total protein levels and their ubiquitination state in basal condition and following proteasome blocking (+ 50 µM MG132 for six hours).

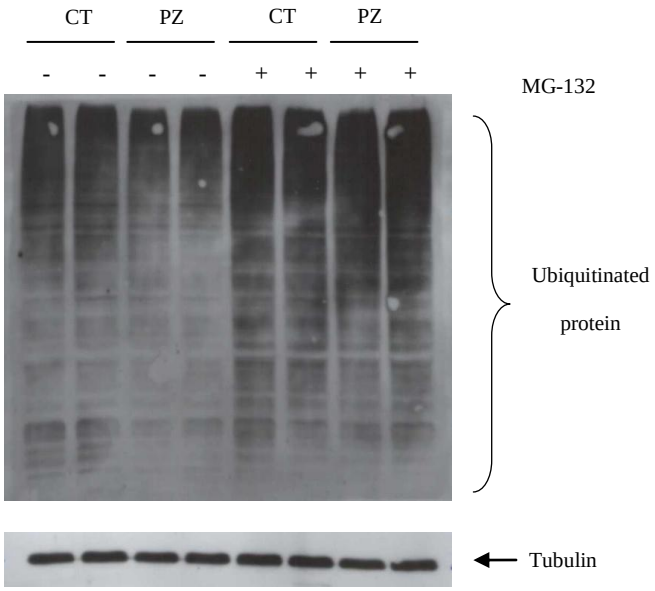


Fig. 3 Densitometry of the ubiquitinated protein levels showed by Western blot analysis in basal condition and following proteasome blocking

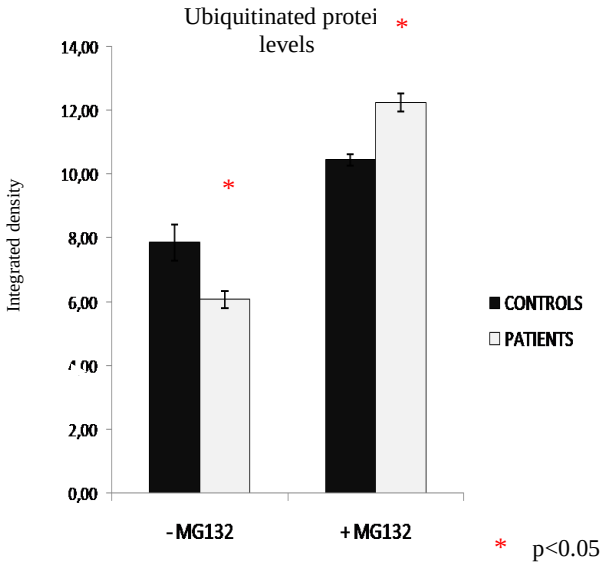


Fig. 4 Western blot analysis of the ubiquitinated protein levels in basal condition and after oxidative stress (0,1 mM H_2O_2 for 30 minutes) and after 1, 2 and 4 hour of recovery

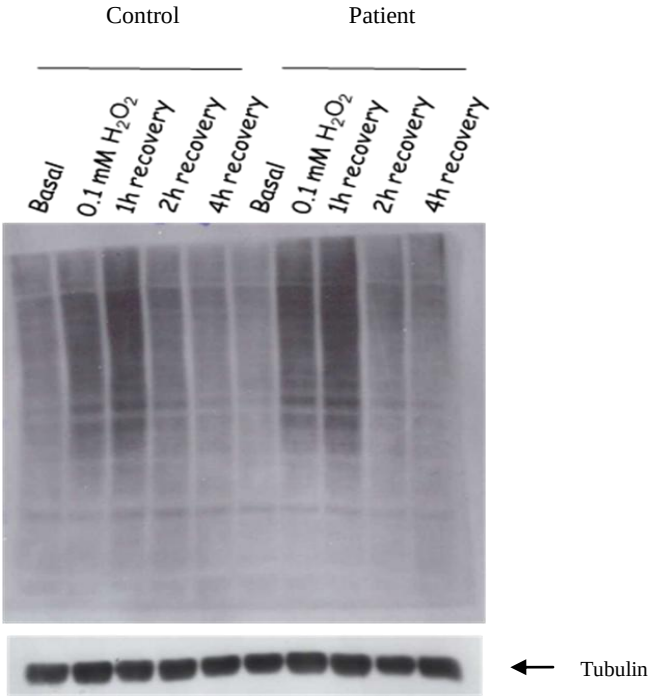


Fig. 5 Densitometry of the ubiquitinated protein levels observed after oxidative stress conditions and recovery.

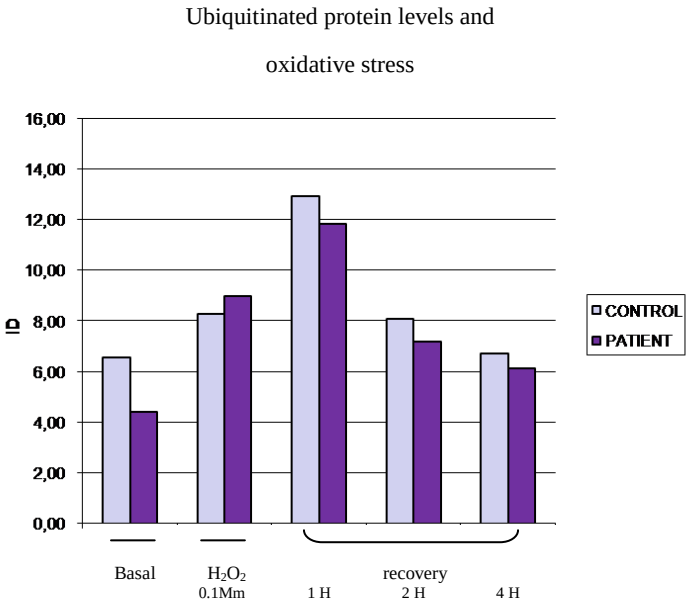
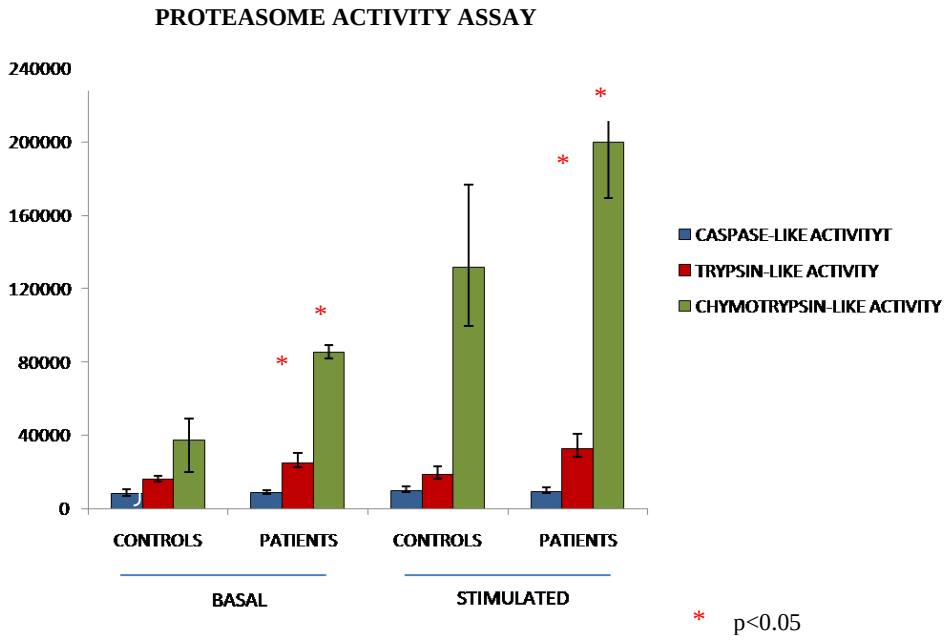


Fig. 6 Analysis of the three proteasome activities in basal conditions and after treatment with H_2O_2 (0,1 mM for 30 minutes). The average of the sample activities of pool of DS subjects versus controls (n=3) was evaluated in duplicate and repeat two times.



DISCUSSION

The 26S proteasome is the enzymatic core engine of the ubiquitin dependent proteolytic system (UPS), the major eukaryotic pathway for regulated protein degradation. The UPS plays a pivotal role in cellular protein turnover, protein quality control, antigen processing, signal transduction, cell cycle regulation, cell differentiation and apoptosis. Hence, aberrations in this system underlie, directly or indirectly, the pathogenesis of several diseases including some observed in DS such as neurodegenerative diseases (Alzheimer's, Parkinson's and Huntington's disease), autoimmune disorders and cancer.

Genome-wide expression analysis and bioinformatic tools revealed that

ubiquitin mediated proteolysis is the pathway mostly influenced by trisomy 21 in lymphoblastoid cell lines and in particular among the down-regulated GO categories.

In the present study, functional analyses of the UPS revealed that there is a reduction of the ubiquitinated protein levels and a consistent increase in two of the three proteasome activities in DS cell lines in basal conditions. Furthermore, it was observed an increase in the accumulation of ubiquitinated proteins in DS compared to controls under oxidative stress conditions, and a significant increase of the same proteasome activities observed in basal conditions.

Taken togheter, this results suggested that in human DS lymphoblastoid cell lines there is a defective protein degradation system that could result from a primary dysfunction of UPS and from an excess of damaged proteins, such as oxidated proteins, that could cause an UPS overload

To date, except to a recent expression study in cultured DS trophoblasts that showed a down-regulation of the Ub-mediated proteolysis (Rozovski et al., 2007), no data are available on the function of the UPS in DS. Only one study on the cerebellum of a DS mouse model reported a reduction of the proteasome chymotrypsin-like activity and an increase in ubiquitinated proteins (Necchi et al., 2011) in contrast with the present findings.

Furthermore, it was reported that a decline of the proteasomal activity occurs after sub-lethal OS, followed by a functional recovery in the after-stress period (Reinheckel et al., 1998). Increased accumulation of highly oxidized proteins has been attributed to decreased proteasome function during aging (Das et al., 2007; Sitte et al., 2000; Stadtman et al., 2001), but UPS modifications induced by stress response in DS are still unexplored. Only Engidawork and co-workers (2001) showed a significant overexpression of the proteasome zeta chain and of the *T isopeptidase* deubiquitinating enzyme in DS fetal brain compared to controls, probably due to ROS accumulation (Busciglio and Yanker, 1995).

However, since most studies were performed on DS brains, our findings, assessing an impairments of the UPS in human DS lymphoblastoid cell lines, offer new perspective to study this system in association to DS phenotipic

features not only of neurodegenerative origin.

Further studies are needed to investigate if also the activities of some of the enzymes involved in the ubiquitin system degradation (E1, E2 and E3 and deubiquitinating enzymes) are affected.

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Chapter 3

Down-regulation of the NF-KappaB pathway in Down syndrome

INTRODUCTION

NF-κB is a dimeric transcription factor formed by members of a highly conserved family of proteins that share an ~300-amino-acid region termed the Rel Homology Domain (RHD) (Karin and Lin 2002) through which they can form homodimers and heterodimers that bind to 9-10 base pair DNA sites, known as kB sites, in the promoters and enhancer regions of genes, thereby modulating gene expression.

In mammals, the NFkB family is composed of five related transcription factors: p50, p52, RelA (p65), c-Rel and RelB (Moynagh 2005; Hoffmann et al., 2006).

RelA, c-Rel and RelB contain C-terminal transcriptional activation domains (TADs), which enable them to activate target gene expression. In contrast, p50 and p52 do not contain C-terminal TADs; therefore, they are bound to a protein RelA, c-Rel or RelB acting as transcriptional co-activators. In particular, the p50/p65 heterodimer is the most abundant form in eukaryotic cells.

NF-Kappa B is activated by two-step proteolytic mechanism: a primary processing of the precursor protein (p100 and p105 to yield the active subunit p52 and p50 rispectively) and signal induced phosphorylation and subsequent degradation of its inhibitor IkB .

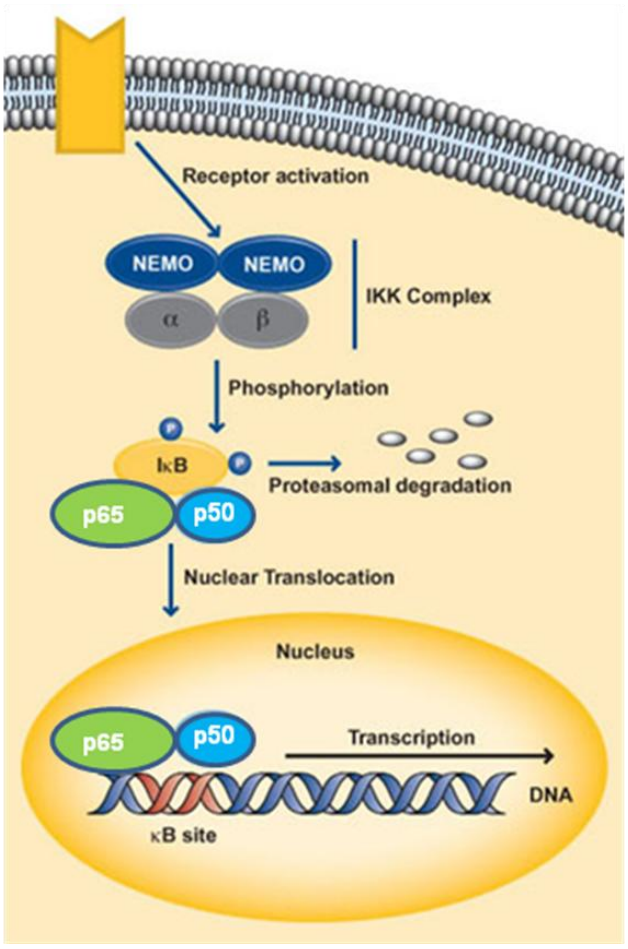
Inhibitory IκB proteins sequestrate NFkB dimers into the cytoplasm and include: Ik-Balfa (IKBA), Ik-Bbeta, Ik-Bepsilon (Baeuerle and Baltimore 1988).

In the canonical signaling pathway, the binding of ligand to a cell surface receptor, such as a member of the the Toll-like receptor superfamily, leads to activation of the multimeric IκB Kinase (IKK) that results in the phosphorylation of IκB, followed by its ubiquitination and degradation (Henkel et al., 1993; Chen et al., 1995), allowing the translocation of NF-kB dimers, composed by RelA, c-Rel o RelB and p50, into the nucleus. (Karin 1999; Tergaonkar 2006; Gilmore 2006; Scheidereit 2006). (Fig. 1)

More recently, additional mechanisms for transcriptional regulation have been recognized, including post-translational modifications of NF-κB

subunits such as phosphorylation (Zhong et al., 1998; Sakurai et al., 1999), acetylation (Chen et al., 2001; Kiernan et al., 2003), prolyl-isomerization (Ryo et al., 2003), and ubiquitination (Saccani et al., 2004; Maine et al., 2007; Tanaka et al., 2007).

Fig. 1 The binding of ligand to a receptor leads to the recruitment and activation of an IKK complex comprising IKK alpha and/or IKK beta catalytic subunits and two molecules of NEMO. The IKK complex then phosphorylates IkB leading to degradation by the proteasome. NFkB then translocates to the nucleus to activate target genes regulated by kB sites.



By Hooper C. "Overview of NFkB signaling"

Activation of the factor stimulates the transcription of many cytokines, adhesion molecules, inflammatory response, stress proteins, and immune system receptors; hence, deregulations of this pathway have been related to a wide array of immune and inflammatory disorders , but also to apoptosis and delay of cell growth.

1. NF-kappaB and NFAT pathway in Down syndrome

Down syndrome, caused by the presence of three copies of chromosome 21, is characterized by many typical phenotypes, such as immune deficiency, characteristic facial features, mental retardation, congenital heart disease, and early onset of Alzheimer disease. Overexpression of a number of chr21 genes is thought to be responsible, directly or indirectly, for some of these clinical features.

Among them, DSCR1 (Down syndrome candidate region 1) gene, located in the Down syndrome critical region of chromosome 21 (Fuentes et al., 1997; Pfister et al., 2002) appears be linked to mental retardation, inflammation, angiogenesis, and cardiac development (Harris et al., 2005; Hesser et al., 2004; Ryeom et al., 2003; Yao et al., 2004).

DSCR1 is highly expressed in the brain, heart, and skeletal muscles of DS fetuses (Fuentes et al., 1995) and it is known to interact physically and functionally with Ca2+/calmodulin-dependent protein phosphatase 2B (also known as calcineurin A), suppressing its signaling (Fuentes et al., 2000; Rotherme et al., 2000). Recently, Arron and co-workers (2006) found that, together with DYRK1A, another critical chr 21 gene, DSCR acts to control the nuclear localization of the NFAT family of transcription factors, key regulators of vertebrate development, and associated to cardiovascular, neurological, skeletal and immune phenotype strikingly similar to DS.

In immortalized hippocampal cells as well as in primary cortical neurons, it was found that NF-kappaB-inducing kinase (NIK) selectively binds to and phosphorylates the C-terminal region of DSCR1 increasing its protein

stability and blocking its proteasomal degradation leading to an increase in soluble and insoluble DSCR1 levels with cytotoxic effects (Lee et al., 2007).
Other findings showed that DSCR1 overexpression decreases the basal turnover rate of IκBα and consequently attenuates the steady-state and stimulus-induced transcriptional activity of NF-κB in human glioblastoma cells, inhibiting the induction of genes involved in the inflammatory response (Kim et al., 2006)
These evidences demonstrated a link between the NF-kappaB and the NFAT pathway and suggest new hypotheses for the involvemet of these two pathways in the DS pathogenesis.

OBJECTIVE

GO analysis of our previous microarray study from DS lymphoblastoid cell lines revealed a significant down-regulation of the NF-kappaB cascade and of ubiquitin mediated proteolysis. Since NF-kappaB activity is regulated by ubiquitin dependent degradation of its inhibitor and published evidences (Lee et al., 2007). Kim et al. (2006) linked the NF-kappaB to the NFAT pathway, a recent candidate player in the DS phenotype, functional analyses were performed to investigate alterations of this pathway and its association to DS.

MATERIALS AND METHODS

Samples

Lymphoblastoid cell lines from karyotipically confirmed full trisomy 21 children and controls were obtained, after informed consent, collecting peripheral blood samples, isolating lymphocytes by using Ficoll-Hypaque (Gibco), and then immortalizing them by Epstein-Bar Virus (EBV). All subjects were recruited at the Department of Pediatrics, Federico II University

of Naples, the regional referral center for DS.

Cells were grown in RPMI-1640 (Cambrex) supplemented with 20% fetal bovine serum, 5000U/ml penicillin-streptomycin, 2 Mm glutamine (Gibco) and 0.16 mg/ml gentamicin, at 37°C.

Protein extraction

Total protein extraction was obtained by lysing the cells with the Tropix lysis solution (Applied Biosystems) supplemented with 0.1 M DTT and in presence of protease inhibitor cocktail 1X (Sigma). Cytoplasmic and nuclear fractions from cells were obtained by using celLytic NucLEAR Extraction kit (Sigma). Briefly, cells were lysed in isotonic buffer with 0.1 M DTT and protease inhibitor cocktail 1X and incubated on ice for 15 minutes. IGEPAL CA-630 solution to a final concentration of 0.6% was added to the swollen cells, and cytoplasmic fraction was obtained by centrifugation at 10000 X g for 30 seconds at 4°C. The nuclei pellets were resuspended in extraction buffer with 0.1 M DTT in presence of protease inhibitor cocktail 1X and vortexed for 30 minutes at 4°C. The pellet fraction was collected by centrifugation at 20000 X g for 5 minutes at 4°C.

Western blot analysis

Protein extracts (20-40 µgr) were separated by 8-10% SDS-PAGE and then transferred on PVDF membrane (Millipore). Membranes were then blocked for 1 hour in milk 5% - PBS 1X-0.1% Tween-20 (T-PBS) and then incubated overnight with dilutions of specific primary antibodies: anti-NF-kB p65, anti-p105/p50, anti-IkBα (L35A5) and anti-pIKBA (CellSignalling).

Antibodies IgG anti-mouse and anti-rabbit, horseradish peroxidase conjugated (GE-Healthcare), were used as secondary antibody and the specific bands were visualized by ECL reaction on high performance chemiluminescent film (GE-Healthcare).

Luciferase assay

Lymphoblastoid cell lines from DS subjects and controls were plated in 24-well plates at density of $5x10^5$ cells/well. Transfection of the different

plasmids, the specific pNF-KB-Luc (Path Detect NF-KB Cis-Reporting System, Stratagene) and pCMV β expressing the β-galactosidase (Clontech), was performed 24 hours later with Turbofect Transfection Reagent (Fermentas), according to the manufacturer's instructions. Cells were harvested and processed after 48 hour. Luciferase and β-galactosidase expression was measured with Dual Light Luciferase Assay Kit (Applied Biosystem).

EMSA

For the EMSA the Gel Shift Assay (Promega) was used. Nuclear extracts (20 μg) were incubated for 30 minutes at room temperature with a radioactive labeled ([γ-32P]dATP) oligonucleotide probe containing the specific recognition sequence for NF-kB (AGT TGA GGG GAC TTT CCC AGG C), a Binding Buffer 5x and Poly-dIdC. After binding, the samples were separated on a 6% non-denaturating PAGE gel for 2 hours and bands were detected by autoradiography after being dried and stored at -80^0 overnight.

To check if the observed shifted bands were specific for NF-kB, protein extracts were incubated with an additional nonlabeled ("cold") oligonucleotide for competition test.

Statistical Analysis

The data were reported as the means ± S.E. and the statistical significance of differences between means was assessed by using the two-tail unpaired Student's t test. The differences between the means were accepted as statistically significant at the 95% level ($p = 0.05$).

RESULTS

In the present study, the NF-kappaB pathway was investigated in DS and control samples by Western blot, EMSA and luciferase assays.

Western blot experiments, performed on cytosolic and nuclear fractions, showed that in DS there is a significant increase of IKBα in the cytosolic fractions in both dephosphorilated and phosphorilated forms (Fig. 2), while there is a significant reduction of the NF-kappaB p65 subunit levels in the nuclear fractions (Fig. 3). No difference was observed for the cytosolic p65 levels and for the NF-kappaB p50 subunit levels, both in the nuclear and in the cytosolic fractions of DS compared to controls.

To evaluate the effect of the observed alterations, we performed EMSA experiments that showed a reduction of the NF-kappaB binding activity of the DS nuclear subunits, and luciferase assays that assessed a significant reduction also of the NF-kappaB transcriptional activity in DS cell lines compared to controls (Fig. 4, 5).

Finally, immunoprecipitations of IkBa on cytosolic fractions and subsequent Western blot experiments with specific anti-ubiquitin antibody, showed a reduction of the ubiquitinated levels of this inhibitor, supporting the hypothesis of an impaired degradation that could be cause of the previous observed cytosolic accumulation. (Fig. 6).

Fig. 2 Western blot of IKappaBα and p-IKappaBα cytosolic levels

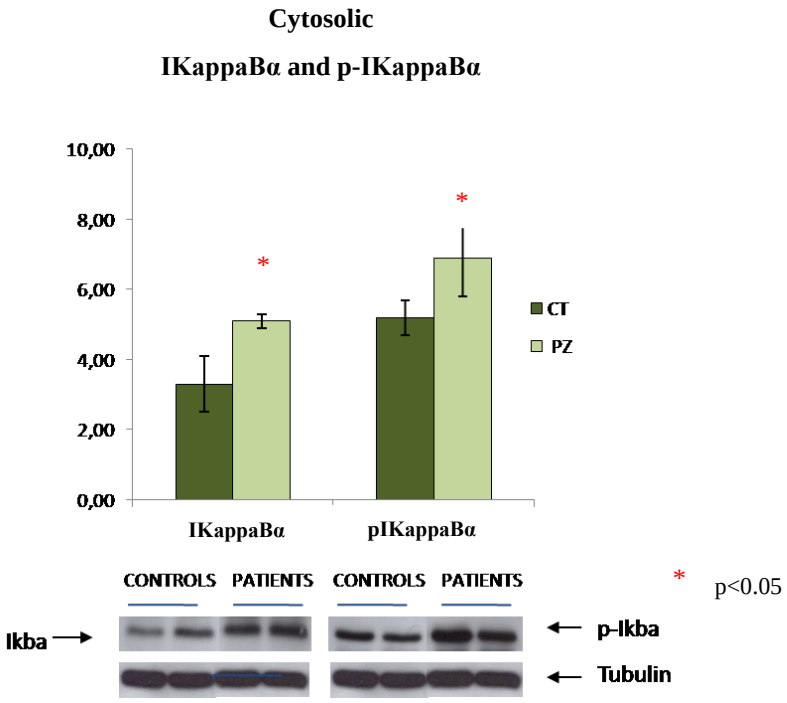


Fig. 3 Western blot of NFkappaB p65 subunit nuclear levels

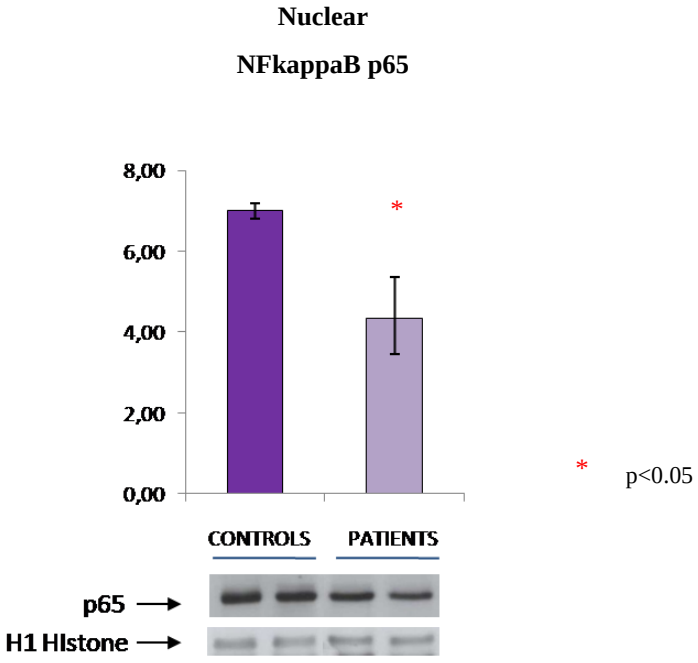


Fig. 4 EMSA of NF-κB DNA-binding activity.

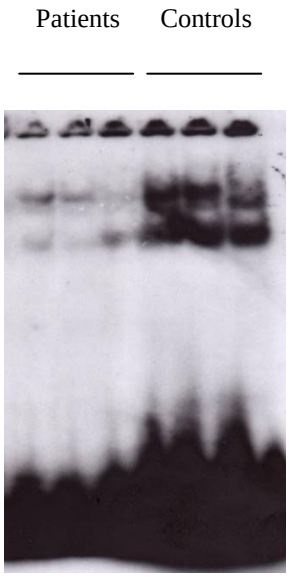


Fig. 5 Luciferase expression under the control of NFkappaB transcriptional activity. Histograms show the relative light units (RLU) of pool of DS subjects versus controls (n=3). The average of the sample activity observed was normalized on the average of the b-galactosidase activity. Experiments were done in duplicate and repeated two times

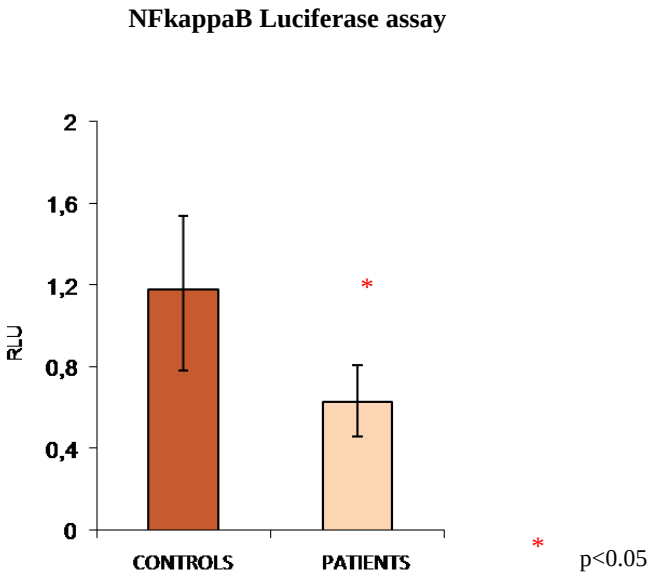
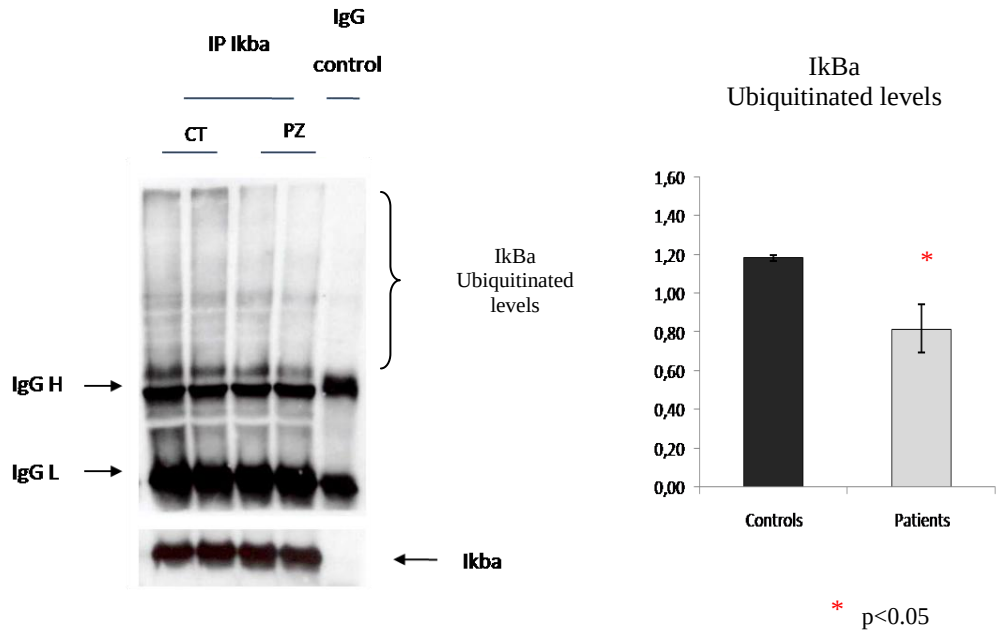


Fig. 6 Ubiquitination of IKBa. A) Cytoplasmic extracts were immunoprecipitated for IKBα and then analyzed by Western blot using an antibody against ubiquitin. IgG H ang IgG L indicates immunoglobulin heavy and light chains in the immunoprecipitates that cross-reacts with the secondary antibody. B) Relative densitometry of ubiquitination levels of NFkappaB inhibitor. Integrated density was evaluated by normalizing ubiquitin density on that of IKBα

A) B)



DISCUSSION

NFkappaB is a dimeric transcription factor activated by the ubiquitin system via a two-step proteolytic mechanism: processing of precursor proteins and signal induced phosphorylation and subsequent degradation of the IKB inhibitors.

GO analysis of differentially expressed genes resulted from our previous microarray data revealed both, NFkappaB cascade and ubiquitin metabolism, among the down-regulated categories.

To confirm that regulation through this transcription factor is affected in

Down syndrome lymphoblastoid cell lines, functional studies were performed.

Our results revealed a significant increase of IKBα, both phosphorylated and not phosphorylated, in the cytosolic fractions of DS subjects and a reduction of the NF-kappaB p65 subunit levels in DS nuclear fractions. In agreement with these results, a significant reduction of the NF-kappaB transcriptional activity was showed in DS cell lines.

Finally, the ubiquitinated levels of IKBα were found to be reduced in DS compared to control subjects.

These results confirmed that in DS lymphoblastoid cell lines there is a down-regulation of the NF-kappaB cascade and suggested that this alteration should be also a consequence of the previously observed ubiquitin proteasome system deregulation.

Since recent evidences demonstrated a link between NF-kappaB and NFAT pathway that was recently proposed as one of the candidate pathway for DS phenotype, and that NF-kappaB itself is connected to a wide array of features also seen in DS such as immune and inflammatory disorders, apoptosis and cell growth delay, our findings suggested that this transcriptor factor might have a possible role in the DS pathogenesis and that further analyses should be carried out.

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Chapter 4

The role of the Dyrk1a/Dscr1/NFAT pathway in Down syndrome

INTRODUCTION

Down syndrome is caused by the presence of full trisomy of chromosome 21 or of only the distal half of Hsa21, band q22.

Therefore, it is presumed that a relatively small region of Hsa21 plays a major role in DS phenotypes, and the concept of a Down syndrome critical region (DSCR) has been proposed (Korenberg et al., 1990; 1994). DSCR was defined with a proximal boundary between markers D21S17 (35.1 Mb) and D21S55 (38.0 Mb) and a distal boundary at *MX1* (41.7 Mb). This region spans 3.8–6.5 Mb and contains up to 25–50 genes (Delabar et al., 1993; Korenberg et al., 1994). Even if many studies revealed that the triplication of DSCR is important but not sufficient for some DS phenotypes (Olson et al., 2007; Korbel et al., 2009), expression deregulations of genes mapping on this region, alone or in cooperation, were suggested as responsible for some clinical manifestations including mental retardation, congenital heart defects, gastrointestinal malformations, immune and endocrine system defects, and early onset of dementia of the Alzheimer type.

Recently, two chromosome 21 genes, Dyrk1A and RCAN1, both mapping on DSCR, have been associated to some of these phenotypes and were associated to Down syndrome by regulating the nuclear factor of activated T cells (NFAT) (Arron et al., 2006).

Dyrk1A is a dual-specificity protein kinase that is activated by autophosphorylation on a tyrosine residue in the activation loop, and it phosphorylates serine or threonine residues in cytosolic and nuclear substrate proteins such as transcription factors, splicing factors, and synapse-associated proteins (Becker et al; 2011; Park et al.; 2009). Dyrk1A also functions as a priming kinase for GSK3 phosphorylation of proteins such as Tau and NFAT (Woods et al., 2001; Gwack et al., 2006). The protein encoded by Dyrk1A is considered a protein potentially responsible for mental retardation (Kentrup et al., 1996; Song et al., 1996; Guinera et al., 1996; Shindoh et al., 1996) and early onset of AD-like symptoms (Park et al.; 2009, Ryo et al., 2008; Ryo et al., 2007; Kimura et. al, 2007; Park et al., 2007; Ryu et al., 2010) in DS patients. Transgenic mice overexpressing Dyrk1A (Dyrk1A TG mice) show

severe hippocampal-dependent learning and memory defects (Smith et al., 1997; Ahn et al., 2006; Altafas et al., 2001). Overexpression of Dyrk1A in DS brains may contribute to early onset of AD through hyperphosphorylation of Tau and enhancement of Aβ production, potentially due to the phosphorylation of APP and Presenilin 1 (Ryo et al., 2008; Ryo et al., 2007; Ryu et al., 2010). Finally, it plays also a critical role in neurodevelopment, including neuronal differentiation and synaptic plasticity (Tejedore et al., 2011).

DSCR1 (Down syndrome critical region 1) also termed RCAN1, is known to act as an endogenous regulator of Calcineurin (Caln), a ubiquitous and multifunctional calcium-activated serine/threonine protein phosphatase and its overexpression results in the inhibition of signaling pathways that are controlled by the nuclear factor of activated T cells (NFAT) (Davies et al., 2011).

DSCR1 is widely expressed in the brain, both during development and in adults (Porta et al., 2007), and DSCR1−/− mice showed impairments in spatial learning and memory (Hoeffer et al., 2007). So, this gene results as a candidate protein responsible for mental retardation in DS.

DSCR1 and DYRK1A, were proposed for the first time as strong candidates for the DS pathogenesis when Arron and coworkers (2006) reported that they act synergistically to prevent nuclear occupancy of the NFATc transcription factors in mice models and showed that knock-out mice for some of the NFAT family members (NFATc1, NFATc2, NFATc3 and NFATc4), displayed cardiovascular, neurological, skeletal and immune phenotypes strikingly similar to DS.

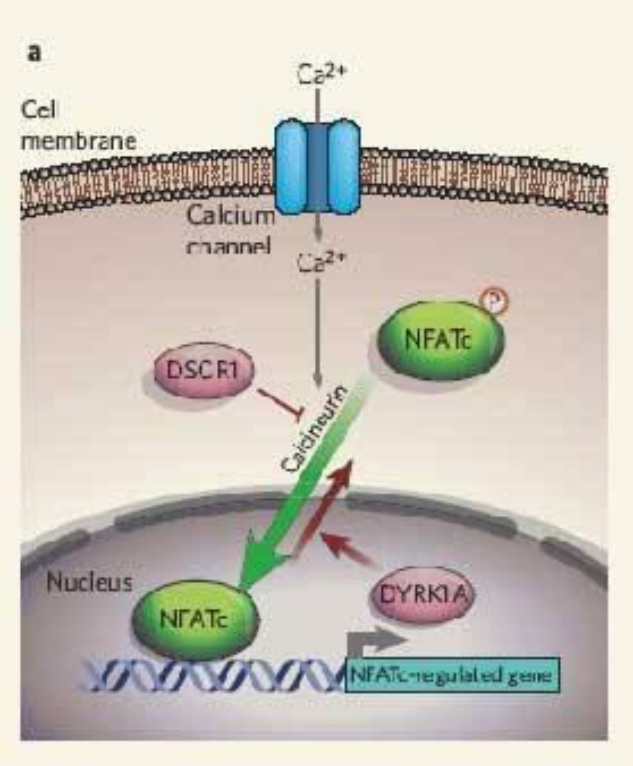
The nuclear factors of activated T cells (NF-ATs) are members of a family of transcription factors that includes five members (NFATc1-5) involved in the expression of cytokines and genes of the immune response. Like their distant relatives, the Rel family which includes NF-κB, NFATc are located in the cytoplasm of resting cells in a phosphorylated form and, except for NFATc5, are all activated by calcineurin, following Calcium signalling. Calcineurin induces their dephosphorylation and translocation into the nucleus, where they cooperate with other proteins (NFATn) to form complexes on DNA.

(Luo et al., 1996). (Fig. 1)

Even if NFATc2 is more expressed in T lymphocytes (80%), the NFATc family is expressed in several cells and tissues, transducing calcium signals also in the, cardiac, muscular and nervous system.(Crabtree 1999)

They can act with a dual function: in differentiated cells they can regulate the early activation of inducible genes while in some tissues can control cell differentiation and adaptation with a role in the regulation of chondrocytes, myocytes, adipocytes and keratinocytes, and in the development of cardiac valves and cardiac muscle cells (Horsley et al., 2002).

Fig. 1. Activation of NFAT. Calcium influx leads to calcineurin-mediated NFATc dephosphorylation, nuclear entry, and activation of NFATc target genes. *DSCR1*, an inhibitor of calcineurin, and DYRK1A, a nuclear serine/threonine kinase, act synergistically to prevent NFAT nuclear export.



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OBJECTIVE

Since DYRK1A and DSCR1, two genes mapping on chromosome 21 within the Down syndrome critical region, are associated to mental retardation and early onset of Alzheimer's disease, and to the negative regulation of the the NFAT transcription factors leading to phenotypes similar to DS (Arron et al. 2006), we analyzed the DYRK1A/DSCR1/NFAT pathway to evaluate the effect of these two trisomic genes in human DS lymphoblastoid cell lines and their involvement in the DS pathogenesis.

MATERIALS AND METHODS

Cell culture and treatment

Lymphoblastoid cell lines (LCLs) were generated from peripheral blood samples, after informed consent, from controls and kariotipically confirmed full trisomy 21 children, recruited at the Department of Pediatrics, Federico II University of Naples, the regional referral center for DS.

Lymphocytes were isolated by Ficoll-Hypaque (GIBCO), immortalized by Epstain-Bar virus (EBV) and grown in RPMI 1640 (Cambrex) supplemented with 20% fetal bovine serum (FBS), 2mM L-glutamine, 0.16 mg/ml of gentamicin and 5000U/ml of penicillin-streptomycin (Gibco)

For the induction of Calcium flows, cells were incubated with 10 ng/ml PMA and 1□g/ml Ionomycin (Sigma) for 4 hours at 37° C.

Quantitative Real-Time PCR

Total RNA from 6 subjects with full trisomy 21 and 6 controls was obtained using TRIzol reagent (Gibco/BRL Life Technologies, Inc., Gaithersburg, MD). Quality and amount of starting RNA were confirmed using spectrophotometry and agarose gel electrophoresis. cDNA was synthesized with random hexamer primers starting from 2 μg of total RNA using the High Capacity cDNA Archive Kit (Applied Biosystems). Real-time PCR was

performed using 2 μg of cDNA and TaqMan Universal PCR MasterMix 2X on the Applied Biosystems 7300, according to the manufacturer's protocols. PCR reactions for each sample were performed in triplicate.
Genes analyzed and reference genes are reported in Table 1 and 2

Table 1. Assay used in the RT-PCR experiments

GENE	ASSAY ID	REF SEQ	EXON JUNCTION	AMPLICONE LENGTH
NFATC2	Hs00234855_m1	NM_173091.2	1 - 2	74
NFATC1	Hs00190037_m1	NM_172390.1	2 - 3	98
NFATC3	Hs00190046_m1	NM_173163.2	2 - 3	59
NFATC4	Hs00232342_m1	NM_004554.3	2 - 3	99
DYRK1A	Hs00176369_m1	NM_130438.1	8 - 9	98
DSCR1	Hs00231766_m1	NM_203418.1	3 - 4	88

Table 2. Reference genes used in the RT-PCR experiments

GENE	ASSAY ID	REF SEQ	GIUNZIONE ESONI	AMPLICONE LENGTH
RNF111	Hs00405638_m1	NM_017610.6	6-7	98
B2M	Hs99999907_m1	NM_004048.2	2-3	75

Protein extraction

Total protein extraction was carried out lysing the cells with the Tropix lysis solution (Applied Biosystems) supplemented with 0.1 M DTT and in presence of protease inhibitor cocktail 1X (Sigma). Cytoplasmic and nuclear fractions from cells were obtained by using celLytic NucLEAR Extraction kit (Sigma) according to the manufacturer's instructions.

Western blot analysis

Protein extracts (20-40 µgr) were separated by 8-10% SDS-PAGE and then transferred on PVDF membrane (Millipore). Membranes were then blocked for 1 hour in milk 5% - PBS 1X-0.1% Tween-20 (T-PBS) and then incubated overnight with diluitions of specific primary antibodies: anti-NFATc2 (4G6-G5), anti-NFATc3, anti-NFATc4, anti-NFATc1, anti-DYRK1A (H-143) (SantaCruz), anti-DSCR1 (N-20) (sigma); anti- Anti-mouse and anti-rabbit IgG horseradish peroxidase conjugated (GE-Healthcare) was used as secondary antibody and the specific bands were visualized by ECL reaction (GE-Healthcare).Anti-tubulin and anti-H1 histone were used to normalization.

Statistical Analysis

The data were reported as the means ± S.E. and the statistical significance of differences between means was assessed by using the two-tail unpaired Student's t test. The differences between the means were accepted as statistically significant at the 95% level ($p = 0.05$).

RESULTS

Real-time PCR and Western blot experiments were performed to evaluate some of the genes involved in the DYRK1A/DSCR1/NFAT pathway. In particular, by Real-time PCR we observed a significant upregulation of DYRK1A, DSCR1 and NFATc4 (about 2 fold increase) and a downregulation of NFATc2 (69%) and NFATc1(49%) expression profiles in DS compared to controls (Fig. 2). No different expression was observed for NFATc3. To evaluate the levels of their relative proteic products, we performed Western blot experiments that confirmed the significant reduction of NFATc2 and the increase of DYRK1A and DSCR1 also in DS total protein levels (Fig. 3), while no difference was observed for NFATc1, NFATc4 and NFATc3 proteins.

To assess if the overexpression of DYRK1A and DSCR1 could influence the NFAT nuclear localization, we performed Western blot experiments on cytosolic and nuclear fractions after induction of lymphoblastoid cell lines with Calcium flows through PMA / ionomycin. Results showed a reduction of the NFATc2 levels in DS nuclear fractions following stimulation, suggesting the presence of mechanisms acting to inhibit its translocation (Fig. 4 A, B).

Fig. 2 Relative quantification of genes involved in the NFAT pathway. Histograms show mRNA levels of pool of DS subjects versus controls (n=3).

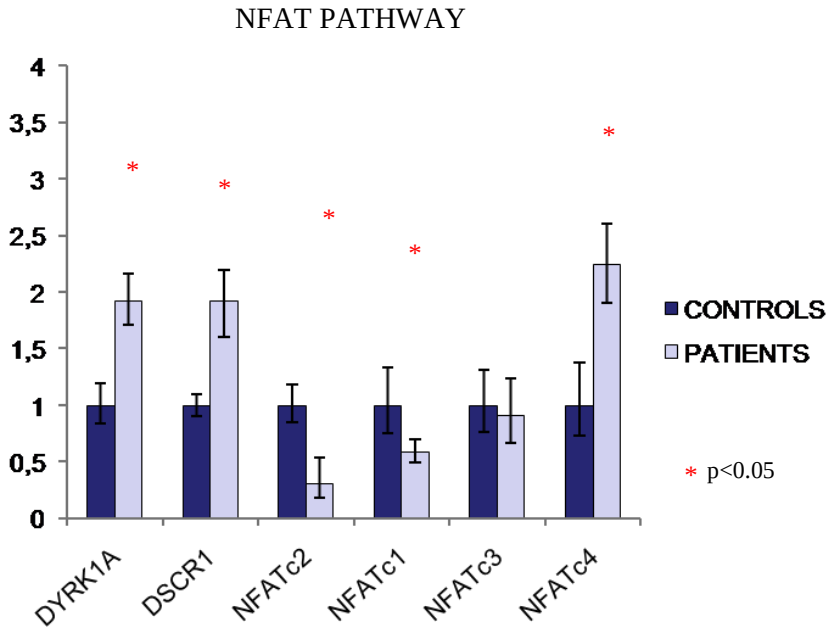


Fig. 3 Western blot of DYRK1A, DSCR1 and NFATc2 total protein levels and their relative densitometry

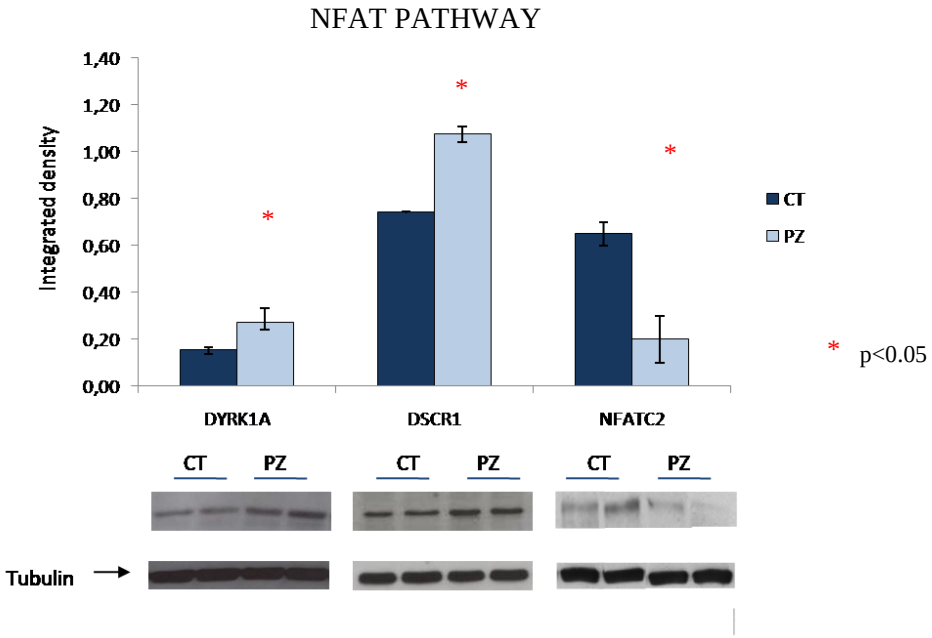
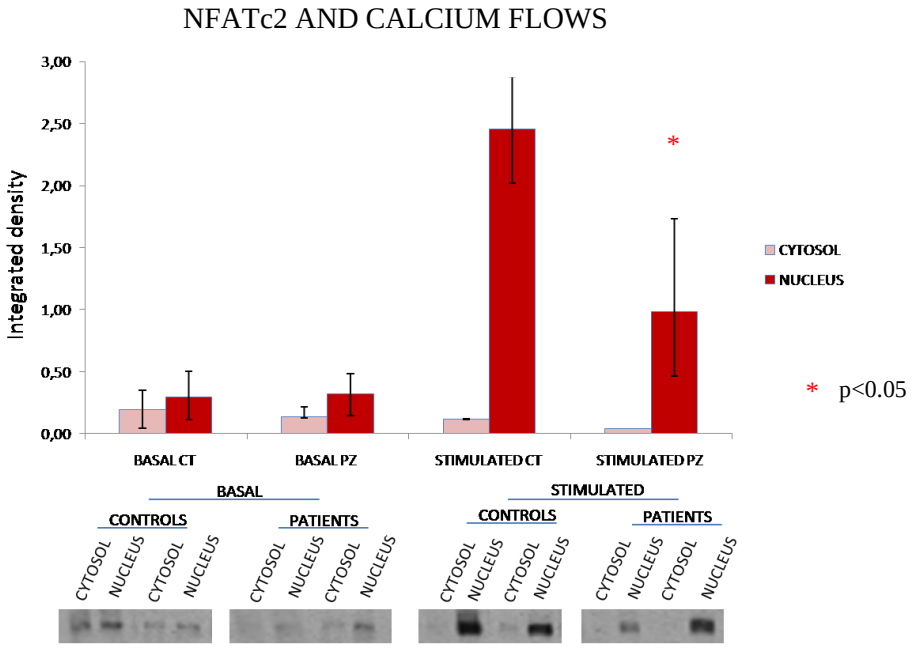


Fig. 4 Western Blot of NFATc2 cytosolic and nuclear fractions with and without PMA and Ionomycin (10 ng/ml and 1g/ml respectively)



DISCUSSION

The nuclear factors of activated T cells (NF-ATs) are member of a family of transcription factors that transduce calcium signals in the immune, cardiac, muscular, and nervous systems
(Crabtree, 1999). NF-ATs are located in the cytoplasm of resting cells and are activated by calcineurin following calcium signalling that induces their movement into the nucleus. (Zhou et al., 2000).

DYRK1A and DSCR1, located on the DS critical region and involved in mental retardation, were associated to the nuclear translocation of the NFAT factors in mice models of DS (Arron et al., 2006) and the DYRK1A/DSCR1/NFAT pathway was suggested as a candidate to have a role in the DS phenotype.

In the present study we analyzed this pathway, in human lymphoblastoid cell lines from DS and controls, by Real Time PCR and Western blot experiments.

As expected, results indicated that DYRK1A and DSCR1 genes are overexpressed of about 2-fold in DS cell lines compared to controls. For the NFATc transcriptor factors we found a downregulation of the NFATc2 and the NFATc1 gene and an up-regulation of the NFATc4 gene in DS subjects. Further analyses confirmed the significant increase of DYRK1A and DSCR1 and the reduction of NFATc2 also at protein levels, while no differences were observed for the other NFATc members.

When analyzed the cytosolic and the nuclear fractions after Calcium flows stimulation, we found a reduction of the nuclear translocation of NFATc2 in DS subjects.

These results suggested that there is a deregulation of the NFAT pathway also in human DS lymphoblastoid cell lines, and confirmed that the overexpression of the two ch21 genes, DYRK1A and DSCR1, leads to the reduction of the NFATc2 nuclear export.

Therefore the DYRK1A/DSCR1/NFAT pathway remains a strong candidate for the DS pathogenesis.

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Chapter 5

MCPH1 gene, a key regulator of brain development, is down-regulated in DS but is not mutated in primary microcephaly

INTRODUCTION

The human brain develops from a swelling at the rostral end of the neural tube. Rapid and sustained cellular proliferation during fetal development is required to generate the brain, and especially the cerebral cortex whose surface area has increased over a thousand fold during mammalian evolution, to accomodate the brain within the skull. The cerebral cortex must fold to assume an invaginated, convoluted appearence with gyri and sulci.

Microcephaly is a term denoting a reduced head circumference greater than 3 standard deviations below the mean (-3 s.d.) having multiple aetiologies, both genetic (nonsyndromic, syndromic or chromosomal) and environmental (eg. perinatal asphyxia, and intrauterine infection).

Microcephaly is a common feature observed in more complex genetic disorders that additionally confer growth delay (Down syndrome and Di George syndrome) but could be also present also in patients that exhibit solely a reduced cerebral cortex, without other developmental abnormalities or neurological deficits, aside from mental retardation (Autosomal recessive Primary Microcephaly). In this case brains, although very small (typically -4 to -12 s.d.), exhibit normal cortical architecture, suggesting that microcephaly is a consequence of reduced cell number either as a result of reduced neuroprogenitor division or increased apoptosis during neurogenesis. (Mochida and Walsh 2001).

1. *MCPH1 gene*

MCPH1 was the first causative gene of primary microcephaly identified (Jackson et al., 2002). It was described in 2 linked Pakistani families, as a gene mapping on chromosome 8 (8p22-pter), consisting of 14 exons encoding a protein of 835 amino acids termed microcephalin. The antisense strand of intron 12 contains the angiopoietin 2 gene.

Microcephalin, contains 3 BRCT domains (C-Terminal domain of a Breast

cancer susceptibility protein), known to be present in several key proteins involved in the cell-cycle regulation and DNA repair (Huyton et al., 2000), and it is mainly expressed in brain, kidney and fetal liver and at low levels in other fetal and adult tissues . In situ hybridization study of mRNA from fetal mouse sections have revealed the expression of microcephalin in the developing brain.

As predicted by the presence of the BRCT domains, Xu et al., (2004) showed, in human embryonic kidney cells, that MCPH1 is involved in the response to DNAdamage, such as double-strand breaks (DSBs, double strand breaks) induced by ionizing radiation, and control of cell cycle progression, particularly at the transition G2/M, by adjusting the BRCA1 and Chk1 (Checkpoint Kinase 1) levels. In addition, Alderton et al., (2006) found a defective G2/M checkpoint arrest, nuclear fragmentation after DNA damage, and supernumerary mitotic centrosomes in human lymphoblastoid cell lines with different truncating mutations in MCPH1. By coordinating the input stage of mitosis during the cell cycle, MCPH1 has an important effect in the process of proliferating neural cells.

2. MCPH1 and Down syndrome

Microcephaly is a common features oserved in subjects with Down syndrome (DS), the most frequent genetic cause of mental retardation.

DS brain is characterized by a reduced cerebellar volume and number of granular cells, defective cortical lamination and reduced cortical neurons, malformed dendritic trees and spines, and abnormal synapses. These neurological alterations, cold result both from the dosage effect of some Human Chromosome 21 (HC21) genes and from their infuence on the expression of critical developmental genes.

A previous transcriptome analysis permormed by our group on DS lymphoblastoid cell lines by using DNA microarray (Affymetrix HU133 plus 2.0 oligonucleotide arrays), allowed us the screening of about 38,500 well characterized human genes with the aim to identify molecular changes

associated to some DS phenotypes.

Interestingly, finding deregulations of genes that may lead to defective neuronal circuits in DS pathogenesis, the MCPH1 gene was found strongly down-regulated. ($p<0.01$ FC 0.69).

3. MCPH1 and autosomal recessive microcephaly

Autosomal recessive primary microcephaly (MCPH, OMIM 251200) is a rare non-syndromic congenital microcephaly inherited as an autosomal recessive trait. The incidence of MCPH ranges between 1:30,000 and 1:2,000,000 in non-consanguineous populations, as compared to 1:10,000 in consanguineous northern Pakistani families (Woods et al., 2005). Distinct subtypes of MCPH have been described: microcephaly vera (MV), microcephaly with simplified gyral pattern (MSG) and microlissencephaly (MLIS) (Barkovich et al.,2005). Before the discovery of the genetic bases of MCPH, these disorders were phenotypically defined by strict diagnostic criteria such as (1) congenital microcephaly at least 4 SD below age and sex means; (2) mental retardation; (3) absence of other neurological findings including spasticity, seizures or progressive cognitive decline; (4) normal height and weight; (5) absence of chromosomal aberrations; (6) no dysmorphisms and brain malformations (Jackson et al., 2002; Roberts et al., 2002).

To date, seven loci (MCPH1-MCPH7) have been mapped with seven corresponding genes ((MCPH1, WDR62, CDK5RAP2,CEP152, ASPM, CENPJ,and STIL) identified from different world populations.

The *ASPM* gene at MCPH5 locus and the and *WDR62* gene at MCPH2 locus are the most frequent genetic cause of MCPH being mutated in about 55-60% of the MCPH families jointly (Roberts et al., 2002; Nicholas et al., 2009; Nicholas et al., 2010; Mahmood et al., 2011); while MCPH1 mutations account for 4% of cases in consanguineous Pakistani families (Roberts et al., 2002; Woods et al., 2005). Mutations of other genes are even rarer. For *MCPH1*, the following homozygous gene mutations have been described so far: a nonsense mutation in Pakistani consanguineous families (c.74 C > G; p.S25X) (Jackson

et al., 2002), an insertion in a Lebanese family (c.427_428insA) (Neitzel et al., 2002; Trimborn et al., 2004) and a missense mutation in a Caucasian patient likely descending from consanguineous parents (c.80 C > G; p.Thr27Arg) (Trimborn et al., 2005). Studies in unrelated patients are scanty. *MCPH1* is also rich in polymorphisms, c.940G > C representing the most frequent one. Among patients with *MCPH1* mutations, mild microcephaly, short stature, and periventricular neuronal heterotopias have been reported (Neitzel et al., 2002; Trimborn et al., 2004; Trimborn et al., 2005; Garshasbi et al.,2006). Seizures have been rarely described (Woods et al., 2005).

OBJECTIVE

MCPH1, the first causative gene of primary microcephaly identified, was found significantly down-regulated in a population of subjects with Down syndrome, in whom syndromic microcephaly has been described.

Since *MCPH1* mutations are associated to autosomic recessive primary microcephaly and account for 4% of cases in consanguineous Pakistani families (Roberts et al., 2002; Woods et al., 2005) but studies in unrelated patients are scanty, we analyzed the sequence of the entire gene to identify the presence of molecular changes in a population of eighty-one unrelated patients with primary micricephaly from different ethnic groups.

MATERIALS AND METHODS

Patients

Eighty-one unrelated patients (78% Caucasian, 16% Arabs, 6% otheI) were enrolled in the study (81.5% MV, 17.3% MSG, 1.2% MLIS). Patients were divided in two groups: group 1 including 34 patients meeting the strict MCPH criteria (Woods et al., 2005); and group 2 including 47 patients meeting the

expanded MCPH criteria (microcephaly ≤−2 SD at birth and <−3 SD at inclusion (after 1 year of age) and brain malformations) (Passemard et al., 2009b). All patients had microcephaly at enrollment, with an OFC ranging from −3 to −8 SD, and had no ASPM mutations. Written informed consent was obtained from all patients or their guardians and ethics approval was given by the host institutions.

DNA extraction and sequencing

DNA was extracted from peripheral blood samples by salting out according to Miller and coworkers instructions (1998) or by using Qiagen kit (Qiagen, Hilden, Germany) and a DNA pre-amplification Genomiphi kit (GE Healthcare Biosciences AB, Uppsala, Sweden) according to the manufacturer's recommendations. Sequence analysis of the 14 exons and exons/introns boundaries of the MCPH1 gene was performed using 19 primer pairs (table 1), exon 8 was divided into 6 fragments (AF). PCR conditions were variables depending on the primer sequences and the relative products were first purified, by using QIAquick PCR purification Kit (Qiagen) or Exo-SAP-purification system (Roche), and then sequenced by using Big Dye Terminator Ready Reaction Kit (PE Biosystem). Sequences were purified by Centri-Sep Columns and loaded on ABI PRISM 377 (Applied Biosystems, Foster City, CA). Sequences were compared with a control DNA and a reference cDNA sequence (GenBank accession: AX087870).

Statistical analysis

For statistical analysis, chi-square test was used to compare categorical data (EpiInfo version 3.3, 2004). To correct for inflated alfa error, the modified Bonferroni test provided by Keppel was used (Keppel, 1991). The adjusted *P*-value was considered as the new cut-off to determine statistical significance (adjusted *P*-value: 0.04). *Z*-score was used to compare the proportion of *MCPH1* mutations in the 81 MCPH patients (zero mutations) to the proportion of mutations in consanguineous families (4%).

Table 1 : Primers used for sequence analysis of the MCPH1gene

EX	FORWARD	REVERSE
1	5'-GAGAAATCCCGGAAACGCGG-3'	5'-CGAGTGCGCCCGCGACGAGG-3'
2	5'-GCGTGAGCCCATGTGCCGGC-3'	5'-CGCTTACCCATTGCTTCGTCCAG-3'
3	5'-CGAGCGTTATGCATTCCTTTGAG-3'	5'-GCTTCAGGACCTGCAGGCTCC-3'
4	5'-CCTGCCTCTTGCTTCTGTTAT-3'	5'-GCTATCGAAGAAGTCATAGG-3'
5	5'-GCGAAAGGGCTTTTTCTCCTGCC-3'	5'-GAACCCCACTAGTCATCATCATGC-3'
6	5'-GCAGGAGTAGGTAATGATGTTG-3'	5'-CCATAATTGAATCGGCTATTTGC-3'
7	5'-CTTCCTGCTGGCTTCATCTGC-3'	5'-CTCATCAGAAGTTGCTACATG-3'
8A	5'-GGAAATGAGAAGAACTCAAGTGTGG-3'	5'-CGAGGTGAGTGAAAGATGGTG-3'
8B	5'-GGAAAGGAAGTTGGAAGGATCC-3'	5'-CTTCGGAGGTGAATGGGAGC-3'
8C	5'-CCTTATCTTCAACAAAAGGCC-3'	5'-CCTTCTTAGAAAGACTTCTGCAGC-3'
8D	5'-GCTGTGGGCAGTCTTCATATG-3'	5'-GCTTCTTCAGGGGCAGAAGTCACG-3'
8E	5'-GCAAAAACCATCTCCAGTCC-3'	5'-GCTCATGTTCACTCTGGGCTTCGC-3'
8F	5'-GCGGTTGGTCTGAAAAGCAC-3'	5'-CCACAAGACAGCAAGTCATCTATTCC-3'
9	5'-GCTTAAGTTGTATTTGGTCCATG-3'	5'-GGTTTATGTTTCATTGACCC-3'
10	5'-GCTTTGGGGACAGTATCTGAG-3'	5'-GACAAGTTGCCTAAAGGCACCC-3'
11	5'-GCTTTGATGGGCATGTGCAAC-3'	5'-GCTGATGTCGGCACCTCAGG-3'
12	5'-GCGGAGTGTATCACTTTTTGC-3'	5'-GGGCATAATTGTGCTTGACTGG-3'
13	5'-CTGCAACAGTTCGCCTACGC-3'	5'-CTGGAAACCTGTCCACAGATCTGG-3'
14	5'-GCCTATGGACAACACAGCTCTTGG-3'	5'-CCTATGAGTTCTTAAGAACC-3'

RESULTS

As expected, the molecular analysis of *MCPH1* revealed that this gene is highly polymorphic with exon 8 and 13 particularly rich in known genetic variants. In particular, the c.790A > G, c.863C > A, c.911G > T, c.940G > C, c.1175A > G, c.1428C > T, and c. 1782G > A polymorphisms were found in exon 8, while the c.2226C > T, c.2282C > T, and c.2418C > A polymorphisms were found in exon 13. Other polymorphisms: c.228G > T, c.513G > T, c.2045C > A, c.2466G > A, and c.2482C>T were found in exons 3, 6, 11, and 14 respectively. (Table 2).

Table 2.: Results of the molecular analysis of the *MCPH1* gene on 74 patients.

Gene	Protein	Exon
c.228 G>T	p.V76V	3
c.513 G>T	p.R171S	6
c.783 T>A *	**p.D261E**	**8**
c.790 A>G	p.I264V	8
c.859 A>C *	**p.S287R**	**8**
c.863 C>A	p.P288H	8
c.911 G>T	p.R304 I	8
c.940 G>C	p.D314H	8
c.989 A>G *	**p.Y330C**	**8**
c.1175 A>G	p.D392G	8
c.1782 G>A	p.T594T	8
c.1428 C>T	p.F476F	8
c.2045 C>A	p.T682N	11
c.2226 C>T	p.S742S	13
c.2282 C>T	p.A761V	13
c.2418 C>A	p.A806A	13
c.2466 G>A	p.Q822Q	14
c.2482 C>T	p.P828S	14

***** new mutations

No pathogenetic mutations were found in both groups analyzed, but three novel sequence variations in the heterozygous state were found in exon 8: the c.783T > A transition, causing a synonymous substitution of an aspartic with a glutamic acid residue at position 261 (p.D261E); the c.859A > C transversion, causing the change of a serine with an arginine at position 287 (p.S287R); the c.989A > G mutation, causing the substitution of a tyrosine with a cysteine at position 330 (p.Y330C).

The new mutation c.783 T>A was found in a patient and his sister in

combination with the polymorphism c.940 G>C in the homozygous state, while the other two new mutations were found in one patient associated to the c.940 C>G polymorphism in the heterozygous state.

DISCUSSION

Microcephaly is a congenital reduction in brain size greater than three standard deviations (S.D.) below the age-related mean, that could be syndromic, in association to other features of more complex pathologies such as Down syndrome; or nonsyndromic, in which patients exhibit solely a reduced cerebral cortex without other developmental abnormalities or neurological deficits aside from mental retardation.

Primary microcephaly (MCPH, OMIM 251200) is a rare non-syndromic congenital microcephaly inherited as an autosomal recessive trait and MCPH1 (microcephalin) was the first causative gene identified.

Microcephalin is implicated in DNA damage induced cellular responses, chromosome condensation and cell-cycle regulation, and was suggested as a key player of neural proliferation and brain development.

In agreement, our previous transcriptome analysis revealed that in a population of DS subjects, affected by a syndromic form of microcephaly, the MCPH1 gene was significantly down-regulated.

The sequence analysis of the entire gene in a non-consanguineous heterogeneous population instead, did not identified causative mutations of MV. Only, three new gene variants were found, two of wich in the same patient and associated to another yet known polymorphism.

In conclusion, MCPH1 may be associated to brain development in DS subjects, but it is not involved in the pathogenesis of MV in a large cohort of Caucasian patients and from NorthAfrica .

A possible role of a combination of certain gene variants in the pathogenesis of microcephaly vera remains to be determined.

APPENDIX 1

RESEARCH LETTER

AMERICAN JOURNAL OF medical genetics PART A

Absence of Microcephalin Gene Mutations in a Large Cohort of Non-Consanguineous Patients With Autosomal Recessive Primary Microcephaly

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TO THE EDITOR:

Autosomal recessive primary microcephaly (MCPH, OMIM 251200) is a rare non-syndromic congenital microcephaly inherited as an autosomal recessive trait. The incidence of MCPH ranges between 1:30,000 and 1:2,000,000 in non-consanguineous populations, as compared to 1:10,000 in consanguineous northern Pakistani families [Woods et al., 2005]. Distinct subtypes of MCPH have been described: microcephalia vera (MV), microcephaly with simplified gyral pattern (MSG) and microlissencephaly (MLIS) [Barkovich et al., 2005]. Before the discovery of the genetic bases of MCPH, these disorders were phenotypically defined by strict diagnostic criteria such as (1) congenital microcephaly at least 4 SD below age and sex means; (2) mental retardation; (3) absence of other neurological findings including spasticity, seizures or progressive cognitive decline; (4) normal height and weight; (5) absence of chromosomal aberrations; (6) no dysmorphisms and brain malformations [Jackson et al., 2002; Roberts et al., 2002].

To date, seven loci (MCPH1-MCPH7) have been mapped and five of the corresponding genes characterized: Microcephalin at *MCPH1* (OMIM 251200); Cyclin dependent kinase 5 regulatory associated protein 2 (*CDK5RAP2*) at MCPH3, abnormal spindle-like, microcephaly associated (*ASPM*) at MCPH5; Centromeric protein J (*CENPJ*) at MCPH6; and SCL/TAL1-interrupting locus (*STIL*) at MCPH7 [Passemard et al., 2009a]. *ASPM* is the most frequent genetic cause of MCPH being mutated in about 40% of both consanguineous and non-consanguineous MCPH families [Roberts et al., 2002; Nicholas et al., 2009]; *MCPH1* mutations account for 4% of cases in consanguineous Pakistani families [Roberts et al., 2002; Woods et al., 2005]. Mutations of other genes are even rarer. After MCPH genes discovery, MCPH diagnostic criteria have been revisited. Among patients with MCPH due to *ASPM* mutations, Nicholas et al. [2009] showed that the classic MCPH criteria are suitable discriminators for *ASPM* mutations.

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However, a 7% improvement of the detection rate was achieved when criteria were relaxed to microcephalic children with mental retardation. More recently, some of us reported the highest mutation rate (75%) among patients defined by expanded MCPH criteria, including occipito-frontal head circumference (OFC) at birth between −2 and −3 SD, seizures, spasticity, and brain malformations such as partial agenesis of corpus callosum, cerebellar hypoplasia, and focal cortical defects. Borderline-normal intellectual efficiency was also reported in three patients [Passemard et al., 2009b]. Only one patient over 16 (6%) met the classical MCPH criteria. Accordingly, another recent study reported brain malformations in three siblings with *ASPM* mutations [Saadi et al., 2009]. As for *MCPH1*, the following homozygous gene mutations have been described so far: a nonsense mutation in Pakistani consanguineous families (c.74 C > G; p.S25X) [Jackson

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et al., 2002], an insertion in a Lebanese family (c.427_428insA) [Neitzel et al., 2002; Trimborn et al., 2004] and a missense mutation in a Caucasian patient likely descending from consanguineous parents (c.80 C > G; p.Thr27Arg) [Trimborn et al., 2005]. Studies in unrelated patients are scanty. *MCPH1* is also rich in polymorphisms, c.940G > C representing the most frequent. Among patients with *MCPH1* mutations, mild microcephaly, short stature, and periventricular neuronal heterotopias have been reported [Neitzel et al., 2002; Trimborn et al., 2004; Trimborn et al., 2005; Garshasbi et al., 2006]. Seizures have been rarely described [Woods et al., 2005].

Here, we report on the first study of *MCPH1* in a large cohort of non-consanguineous patients with MCPH. Eighty-one unrelated patients (78% Caucasian, 16% Arabs, 6% other; Table I) were enrolled in the study (81.5% MV, 17.3% MSG, 1.2% MLIS). They all lived in Europe at enrollment. Patients were divided in two groups: the first set of 34 patients (Table I, group 1) included those meeting the strict MCPH criteria [Woods et al., 2005]; the second set of 47 patients (Table I, group 2) met the expanded MCPH criteria [Passemard et al., 2009b]. All patients had microcephaly at enrollment, with an OFC ranging from −3 to −8 SD, and had no ASPM mutations. Inclusion and exclusion criteria, brain imaging and developmental assessment were those previously described [Passemard et al., 2009b]. Written informed consent was obtained from all patients or their guardians and ethics approval was given by the host institutions.

For *MCPH1* gene sequencing, DNA was extracted using Qiagen kit (Qiagen, Hilden, Germany) and DNA pre-amplification was performed using Genomiphi kit (GE Healthcare Biosciences AB, Uppsala, Sweden) according to the manufacturer's recommendations. Sequence analysis of the 14 exons and exons/introns boundaries of the MCPH1 gene was performed using 19 primer pairs. PCR conditions and primer sequences are available on request. PCR products from triads of patients were pooled, sequenced using the BigDye Terminator Ready Reaction Mix and analyzed on ABI PRISM 377 (Applied Biosystems, Foster City, CA). Sequences were compared with the reference cDNA sequence (GenBank accession: AX087870). In the case of a new sequence variation, each patient of the pool was re-analyzed in a second round of sequence analysis using native DNA. For statistical analysis, chi-square test was used to compare categorical data (EpiInfo version 3.3, 2004). To correct for inflated alfa error, the modified Bonferroni test provided by Keppel was used [Keppel, 1991]. The adjusted *P*-value was considered as the new cut-off to determine statistical significance (adjusted *P*-value: 0.04). *Z*-score was used to compare the proportion of *MCPH1* mutations in the 81 MCPH patients (zero mutations) to the proportion of mutations in consanguineous families (4%).

The molecular analysis of the *MCPH1* gene in both groups 1 and 2 showed no pathogenetic mutations, indicating that the frequency of *MCPH1* mutations in this cohort of unrelated MCPH patients is lower than the 4% previously reported in consanguineous families (z-value = −1.84, $P = 0.03$). As expected, the *MCPH1* gene was highly polymorphic, exon 8 and 13 being particularly rich in known genetic variants: the c.790A > G, c.863C > A, c.911G > T, c.940G > C, c.1175A > G, c.1428C > T, and c. 1782G > A polymorphisms were found in exon 8, the c.2226C > T, c.2282C > T, and c.2418C > A polymorphisms in exon 13, and the c.228G > T, c.513G > T, c.2045C > A, c.2466G > A, and c.2482C > T polymorphisms in exons 3, 6, 11, and 14, respectively. In addition, three

TABLE I. Clinical Features of MCPH Patients

Patients	Group 1: strict MCPH criteria (N = 34)[a]	Group 2: expanded MCPH criteria (N = 47)[b]
Ethnic/geographic origin	25 Caucasian/8 Arab/1 other[c]	38 Caucasian/5 Arab/4 other[d]
Sex	18 M/16 F	21 M/26 F
Phenotype	28 MV/6 MSG	38 MV, 8 MSG, 1 MLIS
Head circumference at birth (SD)	−4 to −8	−2 to −8
Age at enrollment (years; M ± SD)	11.1 ± 8.8	7 ± 4
Head circumference at enrollment (SD)	−4 to −8	−3 to −8
Mental retardation	Borderline 6 (18%) (6 MV) Mild 7 (20%) (5 MV, 2 MSG) Moderate 16 (47%) (14 MV, 2 MSG) Severe 5 (15%) (3 MV, 2 MSG)	Borderline 9 (19%) (8 MV, 1 MSG) Mild 14 (30%) (12 MV, 2 MSG) Moderate 16 (34%) (13 MV, 3 MSG) Severe 8 (17%) (5 MV, 2 MSG, 1 MLIS)
Epilepsy	8 (23.5%) (8 MV)	11 (23.4%) (8 MV, 2 MSG,1 MLIS)
Short stature	No	No
Extra CNS malformations	No	No
CNS features other than microcephaly	No	Mild ventricle enlargement (n = 11) Corpus callosum hypoplasia (n = 9) Mild cerebellar hypoplasia (n = 2) Mild cerebellar vermis hypoplasia (n = 2)

MV, microcephalia vera; MSG, microcephaly with simplified gyral pattern; MLIS, microlissencephaly; CNS, central nervous system.
[a]Group 1 includes patients with congenital microcephaly at least 4 SD below age and sex means, mental retardation and no evidence of progressive cognitive decline, absence of other neurological findings, chromosomal aberrations, dysmorphisms or brain malformations, normal height and weight.
[b]Group 2 includes patients with microcephaly ≤−2 SD at birth and <−3 SD at inclusion (after 1 year of age) and brain malformations previously described in MCPH patients with *ASPM* mutations.
[c]One patient from Senegal.
[d]Two patients from Ivory Coast, one from Tahiti; one from Caribbean Islands.

novel sequence variations in the heterozygous state were found in exon 8: the c.783T > A transition, causing a synonymous substitution of an aspartic with a glutamic acid residue at position 261 (p.D261E); the c.859A > C transversion, causing the change of a serine with an arginine at position 287 (p.S287R); the c.989A > G mutation, causing the substitution of a tyrosine with a cysteine at position 330 (p.Y330C). The pathogenetic role of these variants was ruled out on the basis of the autosomic recessive pattern of inheritance of MCPH due to *MCPH1* mutations.

The main clinical features of the enrolled MCPH subjects are reported in Table I. Among patients with strict MCPH criteria (Table I, group 1), borderline intellectual disability (IQ 70–80) was observed in 18% of cases and epilepsy in 23.5% of cases. No association between the degree of mental retardation and the occurrence of epilepsy was observed ($\chi^2 = 3.59$, $P = 0.3$; χ^2 for trend 1.93, $P = 0.16$). No cases of short stature were reported. Compared with this first group, patients with extended MCPH criteria (Table I, group 2) showed a similar frequency of borderline mental performance (19%) and epilepsy (23.4%). Epilepsy was associated with severe mental retardation ($\chi^2 = 9.4$, $P = 0.009$) with a linear trend among different classes of mental performance (χ^2 for trend 7.5, $P = 0.006$). Seizures were not associated with brain anomalies ($\chi^2 = 0.59$, $P = 0.4$).

Taken together, results from this study suggest that *MCPH1* mutational screening is not indicated for diagnostic purposes in populations with low prevalence of consanguinity. In this setting, the molecular analysis of MCPH patients will find ASPM mutations in less than half the cases; in the remaining 50–60%, the diagnosis will still rely on clinical parameters. For this reason, further efforts aimed at the optimal phenotypic characterization of MCPH are needed. To this purpose, we report on clinical and prognostic insights on MCPH based on this cohort of patients, mainly on MCPH patients meeting the classical MCPH diagnostic criteria (Table I; group 1). It is known that an OFC of −3 SD can be occasionally associated with normal intelligence but individuals with OFC of −4SD or less with normal intelligence are rare [Teebi et al., 1987; Roberts et al., 2002]. In this study, borderline mental performance (IQ 70–80) was observed in 18% of cases fulfilling the strict MCPH criteria, including an OFC at birth ≤ -4 SD. They all had a MRI finding of MV and an age at evaluation ≥ 3 years, when possible motor/mental deficits would have been detected. The first report of a large Arab kindred with MCPH and normal intelligence dates back to 1987 [Teebi et al., 1987]. Interestingly, borderline-normal intelligence was observed also in 3 out of 16 patients with ASPM mutations [Passemard et al., 2009b]. Moreover, the Caucasian MCPH patient carrying the *MCPH1* p.Thr27Arg gene mutation had a full-scale IQ of 84 at the age of 6 years [Trimborn et al., 2005]. Hence, the frequency of borderline-normal IQ appears to be higher than expected on the basis of previous reports [Dolk, 1991; Woods et al., 2005] and may be likely due to the early educational programs available in many western countries. Epilepsy is not rare, being present in 23% of cases. In 2005, the first case of *ASPM* mutations in a patient with microcephaly and epilepsy was reported [Shen et al., 2005]. Subsequent studies described epilepsy in 10–20% of MCPH patients [Nicholas et al., 2009; Passemard et al., 2009b], indicating that it is not rare in MCPH, should not preclude the diagnosis, and should be considered in genetic counseling.

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