University of Siena

Ph.D. in Medical Genetics

Molecular bases of the clinical variability in Rett syndrome

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Thesis suitable for the title of "Doctor Europeus"

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The first version of this thesis has been revised according to the following attached reviewers' comments.

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Dr. Gérard HILAIRE

To whom it may concern

Comments on the Ph.D. manuscript of Elisa Scala

The manuscript submitted by Elisa Scala for defending her Ph.D. in Medical Genetics is dealing with Rett Syndrome, a severe neurodevelopmental disorder that mostly affects females and that is characterised by a wide spectrum of clinical manifestations. The manuscript focuses on the molecular bases that determine the phenotypic variability observed in Rett Syndrome.

The manuscript is constituted by five different sections. The first section introduces the clinical features of Rett Syndrome, clearly highlighting the differences between the classic Rett Syndrome and five distinct categories of variant forms of Rett Syndrome. Thereafter, the author summarises the molecular knowledge on Rett Syndrome, noting that MECP2 mutations are responsible for about 90 % of the classic cases of Rett Syndrome but for a significantly lower percentage of the variant forms (only 20-40 %). Thus, additional modifier genetic factors could contribute to Rett phenotype modulation. The author discusses the different functions of MECP2 gene, presents the different mouse models of Rett Syndrome and analyses the target genes of MeCP2. The question is raised whether Rett pathogenesis is caused by prenatal developmental disorder or postnatal dysfunctions. The author notes that the principle of reversibility has been recently demonstrated for MECP2 gene.

The second section briefly presents the rationale and objectives of the work that are to identify genetic defects in variant Rett patients without MECP2 mutations and to identify modifier factors involved in phenotype modulation of Rett patients with MECP2 mutations. In the third section of the manuscript (Result section), the author presents four published articles where she was co-author (among which two articles where she was first author) and different works in progress that also deserve publication. Clearly, these publications convincingly demonstrate that CDLK5 is mutated in patients with variant forms of Rett syndrome (infantile seizure onset variants) and that a molecular link may exist between MECP2 and CDKL5. As discussed in the fourth section (Future Perspectives), these results may have a broad impact for the search of therapeutic strategies for Rett Syndrome since this work is the first attempt to identify genetic modifier factors of Rett Syndrome. The manuscript is ended by a reference list of 126 publications.

As a whole, the manuscript is well written, easy to read and clearly presented. The author convincingly demonstrates important results in the field of the Rett Syndrome and more precisely on the molecular basis determining the phenotypic variability of Rett Syndrome. To conclude, from the quality of the manuscript and results, Elisa Scala deserves the Ph.D. degree and the title of “Doctor Europeus”.

Marseille, 30 May 2007,

Gérard Hilaire

hospital
sant joan de déu
UNIVERSITAT DE BARCELONA

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Dear Alexandra

This is a wonderful thesis, I really enjoyed reading it.

I knew many of the works, but thanks God she realized that there was a part missing on page 52 that I received a few days ago.

My comment are:

Over the evaluation score on clinical grounds, I think that the weight depends on age of the child (when they are young they are much slimmer, and does not contribute on the severity of the disease.

Another point is that walking unsupported and sitting unsupported at the age of 5 is quite abnormal, I think that walking before 30 months is since then pathologic.

On Intellectual disability, she should say which scale has been used.

I am in accord that large deletions correspond to classical forms

Finally I have found very nice the chapter 3.4.2 and I hope you go on studying the regulatory elements on MeCP2.

Congratulations for you and Elisa

Kind regards

M. Pineda

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Acknowledgments

During this long journey I have met many people, each of them have contributed in a different way to my personal and professional growth.

First of all, I would to thank prof. Alessandra Renieri because she allowed to me to live this important experience.

I would to thank all my actual female colleagues and the obstinate few males that still resist.

Thanks to who has gone, but it is always in my mind…

Thanks to Rossella, historical companion in adventures….

A special thanks to Francesca, for her precious help, her patience and for…her innumerable quality.

Like in an obstacle-race, it is important to remember who extended help to climb the difficulty, and who was your fan.

Thanks “in ex aequo” to my parents and to Francesco.

1. Introduction

1. Introduction

Rett syndrome (RTT, OMIM#312750) was originally described as a clinical entity by Dr. Andreas Rett, an Austrian paediatrician, in 1966 [1]. After a long period of silence, Hagberg and colleagues increased awareness of the syndrome with a further description of the disorder in a group of 35 girls [2]. RTT is now recognised as a debilitating neurological disorder with an estimated prevalence of 1 in 10.000 to 20.000 female births and represents the second most common cause of mental retardation in girls [3]. In the classic form, RTT is characterized by regression of speech and purposeful hand movements after 6-18 months of apparently normal development. RTT patients develop stereotypic hand movements and neurological symptoms, including seizures, irregular breathing patterns and autonomic instability [4]. In addition to the classic form, there are five recognised atypical RTT variants [5] [6].

The earliest hypothesis on the inheritance of RTT was funded on the seemingly exclusive appearance of the disorder in females. These observations suggested an X-linked dominant inheritance with possible male lethality. In 1999, it was firstly demonstrated that RTT is caused by mutations in the gene encoding the methyl-CpG binding protein 2 (*MECP2*), located in Xq28 [7]. This gene encodes a protein that is expressed in many mammalian tissues and acts as a repressor of transcription [8] [9] [10]. *MECP2* mutations are found in about 90% of classic RTT patients and in a lower percentage of variants cases [11], [12] [13] [14]. These data suggest locus heterogeneity, expecially for RTT variants. In accordance with this hypothesis, recently mutations in another gene, *CDKL5* (cyclin-dependent kinase-like 5) have been found in patients with a phenotype overlapping the infantile seizure onset variant of RTT [15] [16] [17] [18] [19] [20] [21] [22] [23]. Moreover, even among patients classified as classic or variant RTT, there is a high level of phenotypic variability in type and age of onset, severity of impairments and profile of clinical course. To date, it has been hypothesized that this phenotypic heterogeneity could be due to differences in the type of *MECP2* mutation or X-chromosome inactivation. However, these studies have yielded conflicting results and have left unresolved the issue of genotype-phenotype correlations, suggesting that additional modifier genetic factors can be involved [24].

1.1 Clinical features of Rett syndrome.

Classic Rett syndrome.

The diagnosis of RTT is based on the use of established diagnostic criteria, that have been recently revised to clarify previous ambiguities in interpretation of clinical feature [25] [26] [6]. These criteria for classic RTT include a normal prenatal and perinatal period with normal developmental progresses. The neurological development is then arrested and begins to regress according to a predictable course that comprises four stages. During **Stage I** (6-18 months), RTT girls cease to acquire new skills. The head growth undergoes deceleration that usually leads to microcephaly. During **Stage II** (1-4 years), girls lose the ability to speak and the purposeful use of the hands accompanied by a reduction in interpersonal contact and the appearance of autistic features. During this stage, involuntary movements and the classic stereotypic hand activities (tortuous hand wringing, hand washing, clapping, patting), EEG abnormalities and microcephaly become evident. In **Stage III** (4-7 years), girls become more alert and interested both in people and their surroundings; however inability to speak, hand apraxia and the stereotypic hand activities persist. Other somatic and neurological handicaps, such as severe scoliosis, reduced somatic growth and epilepsy, become evident. **Stage IV** (5-15 years and older), is characterized by further somatic and neurological deterioration resulting in end-stage spastic quadriparesis (Fig.1a and c).

Variant forms of Rett syndrome.

In addition to the classic form of RTT, five distinct categories of variants have been delineated on the bases of clinical criteria [27] [6]. These variants show some, but not all diagnostic features of RTT and can be milder or more severe. They include: i) **the preserved speech variant (PSV)**, in which girls recover the ability to speak in single word or third person phrases and display an improvements of purposeful hand movements at stage III of disease progression [28] (Fig.1b); ii) **the infantile seizure onset variant**, characterized by seizure onset before the regression [29]; ii) the **"forme fruste"** with a milder, incomplete and protracted clinical course [30] [31]; iii)

the congenital variant, lacking the normal perinatal period [32]; iv) **the late regression variant**, which is rare and still controversial [33].

Furthermore, in our cohort of RTT patients, it has been described a "highly functioning PSV" associated with acquisition of more complex language function including the use of first person phrases [34] (Fig.1c and d). In this variant, girls acquire a better control of their hands and they are able to draw figures and write simple words [34]. The degree of mental retardation is milder then in PSV with the I.Q that can be as high as 50.

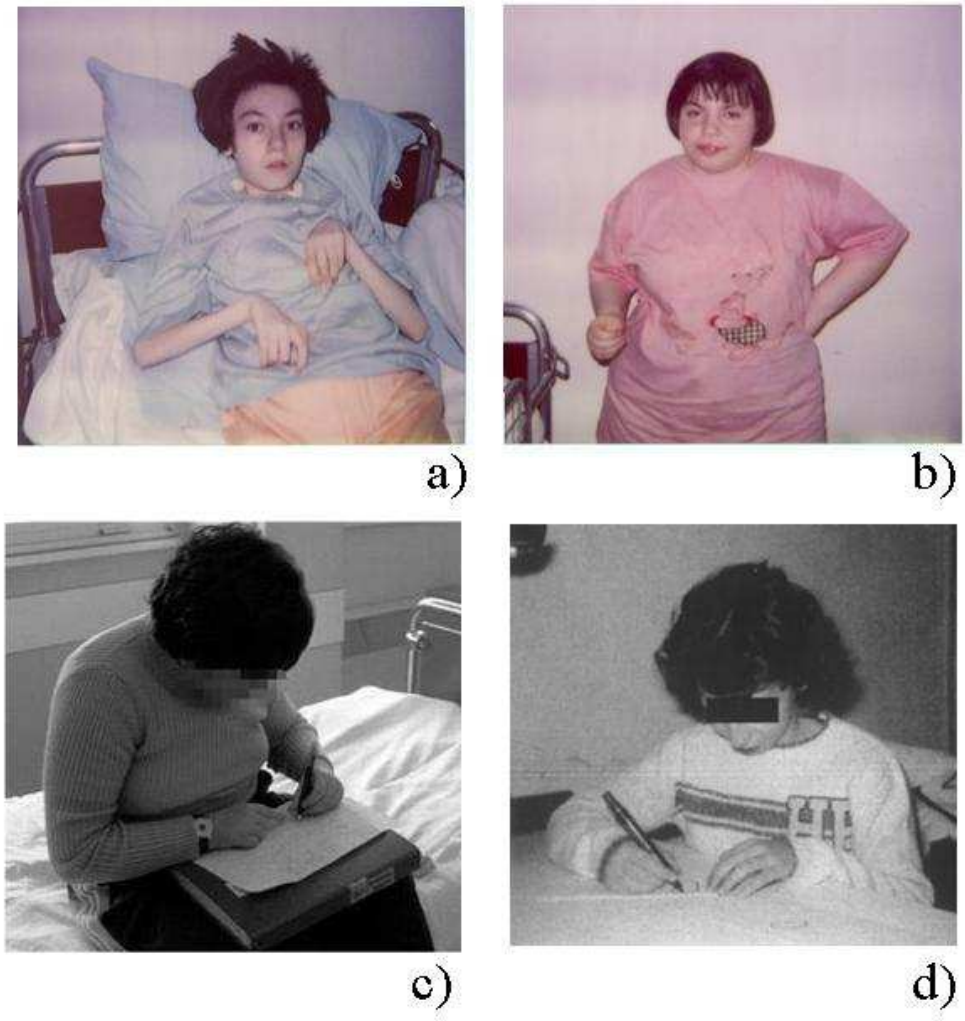

Fig.1. RTT phenotype. In a) is represented a RTT patient with classic phenotype, while in b) a preserved speech variant (PSV) RTT patient. The two patients are a couple of sister. In panels c) and d) are represented two "highly functioning PSV" RTT patients.

1.2 Molecular knowledge on Rett syndrome

MECP2 mutations in RTT females

MECP2 mutations account for up to 90% of classic RTT, 50% of PSV cases and a lower percentage of the other variants patients [11], [13] [12]. The spectrum of *MECP2* mutations causing RTT includes missense, frameshift, nonsense mutations, and intragenic deletions. About 70% of the mutations arise from a C-T transition at eight CpG dinucleotides, whereas carboxy-terminal deletions are estimated to occur in 10-15% of patients [35]. A number of studies aimed to establish a genotype/phenotype correlation have yielded discordant results [36] [37] [38] [39] [40]. Whereas some investigators found no significant correlation between mutation type and clinical features, we and others have found a partial correlation [41] [37] [14] [38] [12] [40] [13] [39] [24]. In particular, we have preliminary evidence that missense, early and late truncating mutations are found in classic patients, whereas only missense and late truncating mutations are identified in PSV cases [24]. The discrepancies in results among the different studies can be due to variation in the degree of accuracy in phenotype definition. Alternatively, the influence of X-chromosome inactivation (XCI) status or modifier genes can contribute in modulating the phenotype. In support of the XCI hypothesis, skewed XCI (presumably favouring inactivation of the mutant allele) has been observed in healthy carrier mothers of RTT patients and partially skewed XCI in less severe RTT phenotypes [13] [39] [42] [37] [43]. However, such a role was not confirmed in other studies showing random XCI in healthy carrier females and highly skewed X inactivation in classic RTT patients [13] [44] [45]. Furthermore, it has been demonstrated that XCI may vary remarkably between tissues [46] [47]. Thus, the extrapolations of results based on sampling peripheral tissues, such as lymphocytes, to other tissues, such as brain, may be misleading. Studies performed on RTT brain tissues suggest that balanced XCI patterns are prevalent [48] [49]. However, XCI has been investigated in a limited number of regions in few RTT brain samples and no definitive conclusions can be drawn. Taken together, these studies suggest that additional modifier genetic factors can be involved in RTT phenotype modulation.

***MECP2* mutations in males.**

Although *MECP2* mutations were initially thought to be prenatally lethal in males, it has been shown that *MECP2* mutations actually cause a variable phenotype in male patients [50]. The phenotypes can be divided in three categories: i) presence of severe neonatal encephalopathy. Soon after birth there is severe neurodevelopmental delay and male patients die during early childhood. These male patients carry *MECP2* mutations that are also found in RTT girls; ii) presence of symptoms that are highly similar to classic RTT in females [50] [51]. This phenotype results from somatic mosaicism for specific *MECP2* mutations or occur in cases of Klinefelter syndrome (47,XXY) [50]; iii) presence of mental retardation. In this case, the phenotype ranges from mild to severe mental retardation [52] [53] [54] [55]. These patients carry *MECP2* mutations that have never been found in RTT females [52]. More recently, duplication in the Xq28 region that involve *MECP2*, have been reported in males as cause of mental retardation and progressive neurological symptoms [56] [57] [58] [59]. Altogether, these findings indicate that qualitative and quantitative *MECP2* defects might therefore account for a significant proportion of mentally retarded male patients.

1.3 *MECP2* gene and its functions

The *MECP2* is encoded by a four-exon gene located at q28 on the X chromosome [60] (Fig.2a). Two MeCP2 isoforms that differ in the N-terminus are generated by alternative splicing of exon 2 [61] [62]. The first identified isoform, MeCP2A, uses a translational start site within exon 2, whereas the other isoform, MeCP2B drives from an mRNA in which the exon 2 is excluded, and a new in-frame ATG located within exon 1 is used (Fig.2b) [61] [62]. Interestingly, MeCP2B is predominantly expressed in brain, while MeCP2A may be abundant in other tissue, such as fibroblast and lymphoblast cells [62].

MeCP2, in addition to the nuclear localization signal, has two functional domains: the methyl-CpG binding domain (MBD), that bind the symmetrically methylated CpGs islands and the transcriptional repression domain (TRD), which is able to recruit co-repressor complexes, that mediate gene silencing through deacetylation of core histones [63] [64] (Fig.2a). MeCP2-mediated gene silencing occurs through chromatin modification mediated by MeCP2 interaction with Sin3A/HDACI or Ski/NcoR/HDACII repression complexes [9]. These enzymes remodel chromatin, which become inaccessible to the transcriptional machinery [63]. Furthermore, the interaction of MeCP2 with the basal transcriptional machinery suggest its involvement in a chromatin-independent transcriptional repression [65]. In a more recent study, MeCP2 has been also implicated in maintaining imprinting through chromatin looping [66]. *MECP2* is almost ubiquitously expressed; therefore it has been considered to be a transcriptional repressor [67]. However, in 2005, has been demonstrated that MeCP2 in addition to its role as a global repressor, acts as a splicing regulator [68]. The authors identified the RNA-binding protein Y box-binding protein 1 (YB1), a principal component of messenger ribonucleoprotein particles that controls multiple step of mRNA processing, as a MeCP2 binding partner [68]. The functional significant of this interaction was investigated by determining whether the MeCP2-YB1 complex affects mRNA processing and splice-site selection. It has been shown that in MeCP2-deficient neurons, the splicing is altered, and aberrantly spliced trancripts can be produced [68]. In conclusion, MeCP2-related neurodevelopmental disorders might therefore be the results of misregulation of both transcription and splicing processes.

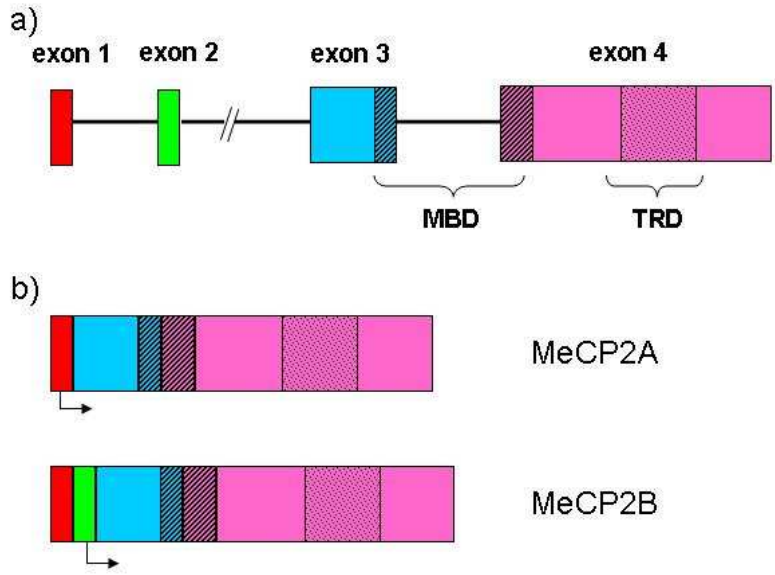

Figure 2. The *MECP2* gene and its splicing isoforms. a) Structure of the *MECP2* gene with its 4 exons. The region encoding the MBD (Methyl-CpG Binding Domain) and the TRD (Trascriptional Repression Domain) are indicated. b) The two alternatively spliced *MECP2* trancripts, excluding or including exon 2, corresponding to MeCP2A and MeCP2B protein isoforms, respectively. The arrows show the position of the transduction initiation codons.

1.4 Rett syndrome and mouse models.

In order to clarify the molecular mechanisms of RTT, different mouse models have been generated. *Mecp2* heterozygous female mice are viable, fertile and appear normal even in early adulthood [69] [70]. However, at about 6 months of age, they begin to show neurological symptoms reminiscent of RTT . Hemizygous null male or homozygous null female mice appear healthy at birth, but develop clinical manifestations similar to RTT between 3 and 8 weeks of age and usually die by 10 weeks of age. Mutation of *Mecp2* restricted to neuronal lineage resulted in a phenotype indistinguishable from that of mice lacking *Mecp2* in all tissues. These results suggested that absence of normal protein function in neurons is sufficient to cause the disease [70] [69]. Inactivation of the gene in post-mitotic neurones of the forebrain, hippocampus and brainstem caused delayed onset of phenotypes similar to those shown by *Mecp2* knockout mice, indicating that *Mecp2* play an important role in these post-mitotic cells [69]. A recent study showed that the postnatal loss of MeCP2 in the forebrain is sufficient to cause many of the behavioural aspects of RTT in mice [71]. Actually, a major unresolved issue in the pathogenesis of RTT is whether the disorder is caused by dysfunction of postnatal neurons at the time when symptoms become manifest or whether it is a prenatal developmental disorder with postnatal phenotypic manifestation in the CNS. Very recently, some authors showed that induction of a *Mecp2* transgene in postnatal mutant animals delayed onset of symptoms and time of death [72] [73]. These findings imply that pathological alterations of neurons may occur only later in life. Moreover, they establish the principle of reversibility in a mouse model raising the possibility for a future therapeutic approach to RTT [72] [73].

1.5 Target genes of MeCP2.

Given the biological characteristics of MeCP2, it was considered a global gene silencer. However MeCP2 deficiency does not appear to cause widespread deregulation of gene expression, but instead might affect specific subset of genes [74]. To date the following genes have been identified as target of MeCP2:

***BDNF*.** In 2003, by candidate gene approach, *Bdnf* has been identified as *Mecp2* target in mammals [75] [76]. BDNF is a neurotrophin essential for survival, growth and maintenance of neurons during development [77]. In addition, *BDNF* has important functions for neuronal plasticity, learning and memory [78] [79] [80]. In MeCP2 deficient neurons, the basal levels of *Bdnf* are two fold higher than those of wild type neurons, while there are no significant changes in activity induced levels of *Bdnf* expression [81]. However, more recently, it has been demonstrated that BDNF protein level are reduced in *Mecp2* knockout mice [81]. These conflicting results can be explained by the observation that *Mecp2* deficient mice show reduced neuronal activity and this might indirectly cause decreased BDNF protein levels. Moreover, it has been demonstrated that *Bdnf* overexpression in the *Mecp2* mutant brain extended the life span, rescued a locomotor deficit and reversed electrophisiological deficit [81]. Recently, Zhou et al. demonstrated that the phosphorylation of a specific amino acid residue S421 of MeCP2 controls the ability of the protein to regulate dendritic patterning, spine morphogenesis and the activity dependent induction of *Bdnf* transcription [82]. These findings suggest that, by triggering MeCP2 phosphorylation, neuronal activity regulates a program of gene expression that mediates neuronal connettivity in the nervous system [82].

***Sgk1* and *Fkbp5*.** In 2005, using cDNA microarrays, Nuber and colleagues found that *Mecp2*-null mice differentially express several genes that are induced during the stress response by glucocorticoids [83]. They observed increased levels of Sgk1 (Serum glucocorticoid-inducible kinase 1) and Fkbp5 (FK506-binding protein 51) mRNAs in *Mecp2*-null mice. Over-expression of these genes occurred both before and after the onset of neurological symptoms. They also found that MeCP2 is bound near the promoters of Sgk1 and Fkbp5 in brain. Given the known deleterious effects of glucocorticoid exposure on brain development, the authors hypothesized that

disruption of *Mecp2*-dependent regulation of stress responsive genes contribute to RTT symptoms.

***ID1*, *ID2*, *ID3* and *ID4* genes.** In 2006, Peddada et al. performed expression microarray analysis to identify novel gene targets of MeCP2 during neuronal maturational differentiation in a human SH-SY5Y neuronal cell culture system [84]. By this approach, they have identified all four known members of the inhibitors of differentiation or inhibitors of DNA-binding (*ID1*, *ID2*, *ID3* and *ID4*) subfamily of helix-loop-helix (HLH) genes as neuronal target of MeCP2. These genes encode inhibitors that block the function of tissue-specific basic HLH transcription factors involved in the regulation of important neuronal differentiation genes such as *NEUROD1*. The authors reported significantly increased protein expression of all four ID genes in both *Mecp2*-deficient mice and RTT human brain tissues [84]. Because of their involvement in cell differentiation and neuronal development, ID genes are likely involved in the molecular pathogenesis of RTT.

FXYD1. Very recently, Deng et al. identified the *FXYD1* (FXYD domain-containing ion transport regulator 1) gene, encoding a transmembrane inhibitor of Na+, K+-ATPase pumps, as a novel MeCP2 target gene that is selectively overexpressed in frontal cortex of RTT patients and *Mecp2*-null mice [85]. Increasing *FXYD1* expression in neuronal cultures is sufficient to recapitulate the loss of dendritic arborization and dendritic spines observed in frontal cortex neurons of RTT patients [85]. Moreover, *Mecp2* null mouse cortical neurons have decreased Na+, K+-ATPase activity, suggesting that aberrant *FXYD1* expression contribute to abnormal neuronal activity in RTT. It has been also shown that *FXYD1* is subjected to a direct MeCP2–mediated trascriptional repression in the frontal cortex neurons [85]. Given these findings, *FXYD1* deregulation may be a factor promoting key features of RTT.

Imprinted genes. Several mammalian genes are imprinted and monoallelically expressed in a parent-of-origin-dependent manner. DNA methylation plays an important role in the establishment and maintenance of genomic imprinting. Given that MeCP2 selectively binds to methylated DNA, such genes represent candidate targets of this protein.

UBE3A (ubiquitin protein ligase E3A) locus is controlled by complex imprinting mechanisms in brain [86]. Mutations or deletions of *UBE3A* affecting the maternally inherited allele cause Angelman syndrome (AS) [87]. The phenotypic overlap between RTT and Angelman syndrome, including microcephaly, seizures, absence of speech, ataxia and mental retardation, led to investigate expression levels of the imprinted *UBE3A* gene in RTT. Regulation of *UBE3A* expression involves the UBE3A antisense transcripts that have been suggested to inhibit transcription from the paternal allele [88 89]. It has been demonstrated that UBE3A mRNA and protein levels are decreased in brains of MeCP2 deficient mice and RTT patients [90] [91]. This correlates with a biallelic production of UBE3A antisense RNA and changes in chromatin structure [91]. The authors concluded that MeCP2 deficiency causes epigenetic aberration at the PWS/AS imprinting centre that results in loss of imprinting of the UBE3A antisense gene, increase in UBE3A antisense RNA level and consequently decrease in UBE3A production [91]. However, most recently Jordan and Francke reported that *Ube3a* expression was not altered in two strains of *Mecp2*-deficient brain by quantitative real-time RT-PCR and immunoblot (Jordan C and Francke U). These conflicting results do not allow drawing definitive conclusions about the role of UBE3A/Ube3a expression levels in the pathophysiology of RTT.

In 2005, convincing evidence emerged that MeCP2 is important for imprinting at the ***Dlx5*** (distal-less homeobox gene 5) locus [92] [66]. Horike et al. carried out chromatin immunoprecipitation experiments to identify MeCP2-binding sites in mouse brain and found several sequences located within an imprinted gene cluster on chromosome 6. The analysis of genes contained in this cluster showed an increased expression of the imprinted *Dlx5* gene and the neighbouring non-imprinted *Dlx6* gene in brains of *Mecp2*-null mice. Interestingly, it was demonstrated that the *DLX5*-imprinting pattern is disrupted in both *Mecp2*-null mice and lymphoblastoid cell lines obtained from RTT patients. MeCP2 was shown to be essential for the formation of a silent chromatin structure at the *Dlx5* locus by histone methylation and through the formation of a chromatin loop. DLX5 has the capability to induce glutamic acid decarboxylase (GAD) expression and the differentiation of GABAergic neurons [93]. Since changes in the density of the GABAergic receptors has been reported in RTT, alteration of GABAergic transmission may play a role in the pathogenesis of the syndrome.

2. Rationale and objectives of this work

2. Rationale and objectives of this work

The Rett syndrome was discovered in 1966. During the subsequent years, an increasing number of reports have described patients who present signs of RTT phenotype but who show considerable variation in type and age of onset, severity of impairment and profile of clinical course. These patients include cases who can be classified as variant RTT or girls who show phenotypic manifestations suggestive of RTT but who do not fulfil the international diagnostic criteria [34]. Moreover, even among patients belonging to the same RTT category (classic or variant), there is a high level of clinical variability.

This work has been concentrated on the identification of the molecular basis that determine the phenotypic variability observed in RTT. To this aim, we searched for: i) the genetic defect in variant RTT patients without *MECP2* mutations, and ii) modifier factors involved in phenotype modulation of *MECP2* mutated RTT patients.

The availability of a large number of RTT samples has been essential for this study. A DNA and cell lines bank has been established in the Medical Genetics Unit of Siena since 1998. At present, the biobank contains a total of 227 RTT probands, including both classic and variant patients, with or without *MECP2* mutations. Given that this collection represents a very important tool for the scientific community, we have connected the biobank to a freely available on-line database containing clinical and molecular data (http://www.biobank.unisi.it) [94] (**Result 3.1**).

In order to explain the fraction of *MECP2* mutation negative RTT variant patients, we hypothesized the existence of an additional RTT locus (**Result 3.2).** Initially, we observed two girls with clinical features typical of the infantile seizure onset variant of RTT. This variant presents a phenotypic overlap with West syndrome, also called "X-linked infantile spasms" (ISSX). ISSX is characterized by the triad of infantile spasms, hypsarrhythmia and severe to profound mental retardation. Since ARX and *CDKL5* genes have been associated with ISSX, we decided to analyzed the two genes. The identification of *CDKL5* deletions in the two girls led us to extend the analysis to the other *MECP2*-negative patients of our collection (http://www.biobank.unisi.it). We found *CDKL5* mutations in three other girls with the infantile seizure onset variant, strengthening the correlation between *CDKL5* and RTT (**Result 3.2.2 and Result 3.2.3**). Considering the similar phenotypes caused by mutations in *MECP2* and *CDKL5*, we hypothesized that the two genes play a role in

common pathogenic processes. To verify this hypothesis we investigated the expression pattern of *Mecp2* and *Cdkl5* in embryonic and postnatal mouse brains, the kind of interaction in vitro and in vivo between the two genes and eventually, the functional activity of CDKL5 (**Result 3.2.2)**.

We also hypothesized that a fraction of mutation-negative RTT cases could be due to the limited sensitivity of the methodology used for *MECP2* mutation analysis. In the last years, several studies have identified *MECP2* large rearrangements, not detectable by PCR-based traditional techniques, in an additional fraction of RTT patients [95] [96] [97] [98] [99] [100]. Moreover, other studies have found a small subset of RTT patients with *MECP2* mutations in exon 1, previously considered non coding and therefore excluded from mutation analysis [101] [102] [62] [103] [104] [105].We investigated the presence of such "missed" *MECP2* defects in our collection of classic and variant RTT patients and we performed genotype-phenotype correlation (**Results 3.3**).

MECP2 mutations are associated with a broad spectrum of clinical phenotypes in RTT. It has been demonstrated that the type of *MECP2* mutation or X-inactivation pattern (XCI) are not sufficient to explain such phenotypic heterogeneity [24]. We hypothesized that this variability could be due to the level of expression/function of additional genetic factors. To test our hypothesis, we have first analyzed a polymorphism in the *CDKL5* gene on the basis of its association with a phenotype overlapping with RTT. In particular, we genotyped the p.Q791P (rs35478150) missense variation, that alters protein sequence and thus might affect *CDKL5* activity. Since *CDKL5* mutations cause RTT variants with precocious and often untreatable seizures, genotyping results have been statistically correlated with the onset and severity of seizures in a large cohort of *MECP2* mutated RTT patients (**Results 3.4.1).**

In order to understand the genetic factors that make dissimilar the phenotypic manifestations in RTT patients with identical *MECP2* mutations, we have searched for differences in segmental genomic variation by array-CGH analysis (**Results 3.4.2).** Gene content of identified rearranged regions has been studied to select new candidate modifiers of RTT phenotype (**Results 3.4.2)**.

This study allowed to determine the genetic cause in a subset of "unsolved" RTT variant cases, and to identify a molecular link between *MECP2* and *CDKL5*. Interestingly, this work represents an initial step towards the identification of genetic modifier factors of RTT. These studies may have a broad impact on the design of

future therapeutic strategies. By acting on those specific genes/proteins that makes the difference between classic RTT and the milder Preserved Speech Variant phenotype, affected girls may improve their language, manual and cognitive abilities.

3. Results

Result 3.1

The Italian Rett database and Biobank

Sampieri K, Meloni I, Scala E, Ariani F, Caselli R, Pescucci C, Longo I, Artuso R, Bruttini M, Mencarelli MA, Speciale C, Causarano V, Hayek G, Zappella M, Renieri A, Mari F.

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DATABASES

Italian Rett Database and Biobank

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Rett syndrome is the second most common cause of severe mental retardation in females, with an incidence of approximately 1 out of 10,000 live female births. In addition to the classic form, a number of Rett variants have been described. *MECP2* gene mutations are responsible for about 90% of classic cases and for a lower percentage of variant cases. Recently, *CDKL5* mutations have been identified in the early onset seizures variant and other atypical Rett patients. While the high percentage of *MECP2* mutations in classic patients supports the hypothesis of a single disease gene, the low frequency of mutated variant cases suggests genetic heterogeneity. Since 1998, we have performed clinical evaluation and molecular analysis of a large number of Italian Rett patients. The Italian Rett Syndrome (RTT) database has been developed to share data and samples of our RTT collection with the scientific community (www.biobank.unisi.it). This is the first RTT database that has been connected with a biobank. It allows the user to immediately visualize the list of available RTT samples and, using the "Search by" tool, to rapidly select those with specific clinical and molecular features. By contacting bank curators, users can request the samples of interest for their studies. This database encourages collaboration projects with clinicians and researchers from around the world and provides important resources that will help to better define the pathogenic mechanisms underlying Rett syndrome. Hum Mutat 0, 1–7, 2007. © 2006 Wiley-Liss, Inc.

KEY WORDS: RTT; Rett syndrome; *MECP2*; *CDKL5*; molecular database; clinical database; biobank; biological samples

INTRODUCTION

Rett Syndrome (RTT; MIM# 312750) is a neurodevelopmental disorder that predominantly affects girls and is the second most common cause, after Down syndrome, of severe mental retardation in females [Hagberg, 1995]. It has an incidence of approximately 1 out of 10,000 live female births [Leonard et al., 1997]. RTT patients show a well-defined clinical course and peculiar characteristics. Clinical criteria for the diagnosis of RTT were defined in the 1980 s [Hagberg et al., 1985; Trevathan and Moser, 1988] and recently revised in 2001 to clarify previous ambiguities in interpretation of clinical features [Hagberg, 2002]. In classical RTT girls, birth and early development appear to be normal, although several investigators consider RTT to be a developmental disorder manifesting very soon after birth [Einspieler et al., 2005; Kerr, 1995]. After this apparently normal period, the clinical course is characterized by a stagnation of development followed by regression lasting for several months and usually occurring between 1 to 3 years of age. The fully developed clinical picture is dominated by mental retardation with autistic features, reduction of communication skills, loss of purposeful hand movements combined with hand stereotypes, progressive postnatal microcephaly, abnormal locomotion, and other various signs such as seizures, breathing abnormalities, and other autonomic dysfunctions. In classic RTT, it is possible to appreciate variability in disease severity. Furthermore, several RTT variants have been described, including the "preserved speech variant" (PSV), characterized by preservation of some degree of speech [Fukuda et al., 2005; Yamashita et al., 2001; Zappella, 1992; Zappella et al., 1998]; the "congenital variant," recognized from birth; the "early onset seizures variant" with seizures onset before regression; the "forme fruste," with a milder, incomplete clinical course (regression between 1 and 3 years); and the "late regression variant." Patients showing a striking preservation of their abilities in comparison with PSV girls, especially concerning language and hand use levels, have been reported and classified as highly functioning PSV [Zappella et al., 2003].

Up to 90 to 95% of classic RTT and 40 to 50% of RTT variants are caused by mutations in the X-linked *MECP2* gene encoding for

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methyl CpG binding protein 2 (MIM# 300005) [Weaving et al., 2005]. Recently some groups [Evans et al., 2005; Mari et al., 2005; Nectoux et al., 2006; Scala et al., 2005; Weaving et al., 2005] identified mutations in the *CDKL5* gene encoding for cyclin dependent kinase-like 5 (MIM# 300203) in patients with the diagnosis of the early onset seizures variant and in other phenotypes overlapping with RTT. While the high percentage of MECP2-positive classic RTT patients supports the hypothesis of a single gene causing this phenotype, the low frequency of "solved" RTT variant patients supports genetic heterogeneity in RTT variants, as already demonstrated by *CDKL5*-mutated cases.

To date, three RTT databases have been developed: one established at the University of Edinburgh and the other two funded by the International Rett Syndrome Association (IRSA). Originally developed as a *MECP2* mutation collection, the first database has been successively improved with clinical data to allow genotype–phenotype correlations (www.mecp2.org.uk). In 2001, RettBASE was established to collect both published and unpublished data about MECP2 pathogenic mutations, benign polymorphisms, and sequence variations of uncertain significance from around the world (mecp2.chw.edu.au) [Christodoulou et al., 2003]. Two years later, an international RTT clinical phenotype database, linked to RettBASE, became available (www.ichr.uwa.edu.au/rett/irsa) [Fyfe et al., 2003]. All these databases are excellent electronic tools that are very useful for genotype–phenotype correlations. However, none of them is connected to a biobank of RTT patients.

Since 1998, the Medical Genetics Unit of the University Hospital of Siena has collected DNA and lymphoblastoid cell lines from a large number of RTT patients. During the last years, the collection of samples has significantly expanded. The rapid enlargement of the samples collection required the establishment of an online database for data management and sharing of resources with the scientific community (www.biobank.unisi.it). At present (September 2006), the site contains 221 entries corresponding to RTT patients included in the bank. For each entry, the site contains clinical and molecular information about each proband and indicates the biological samples available for patients and relatives included in the bank. The database is a useful tool for researchers working on RTT since they can rapidly search for RTT patients with specific clinical and/or molecular features and, by contacting the bank curators, they can request the biological samples for their studies.

RTT Bank

At present (September 2006), the bank contains 631 DNA samples (219 RTT patients and 412 relatives), 49 lymphoblastoid cell lines, and 52 leukocytes in dimethyl sulfoxide (DMSO) medium for a total of 63 probands, and 13 lymphoblastoid cell lines and 61 leukocytes of relatives, also in DMSO medium.

Concerning the phenotype, among the total of 223 probands, 126 are classified as classic RTT and 61 as RTT variants, according to the international diagnostic criteria [Hagberg et al., 2002]. Among RTT variant cases, 41 are PSV, three are "highly functioning PSV," 10 are early onset seizures variants, five are "forme fruste," and two are congenital variants. Among the remaining 36 patients, 18 have been classified as RTT-like, i.e., cases who do not completely fulfill the international clinical criteria for RTT, and 18 as not determinable (ND), when the very young age of the patient does not allow a definitive clinical classification.

MECP2 mutations have been identified in 113 out of 126 classic cases (mutation detection rate: 90%), in 27 out of 61 variant cases (mutation detection rate: 44%), in 17 out of 18 ND cases, and in five RTT-like patients. *CDKL5* mutations have been found in 4 out of 10 early onset seizures variant RTT patients. In the other 57 cases (13 with a classic phenotype, 30 with variants, one ND, and 13 RTT-like patients), *MECP2* and/or *CDKL5* mutation screening failed to identify any pathogenic change.

EDTA peripheral blood samples are used for DNA extraction using a QIAamp DNA blood kit (Qiagen, Hilden, Germany; www.qiagen.com). After the extraction, the quality of the DNA is tested through a spectrophotometer. For each sample an aliquot containing at least 400 µg with an Optical Density (OD) 260/280 ratio of 1,8-2 is stored in dedicated boxes at –20°C for long-term conservation. Heparin peripheral blood samples are treated for leukocyte isolation in DMSO medium or establishment of Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines. Four aliquots of transformed cell lines and primary leukocytes are stored at −135°C. Two additional aliquots of cell line of each patient are conserved in a liquid nitrogen dewar to avoid the loss of samples in case of electronic damage. Cells are frozen at passage 2 in 1.5–2 × 10^7 cells/ml aliquots.

All cases contained in the bank come from all over Italy and all have been clinically evaluated by the Medical Genetics Unit (University of Siena). Patients fulfilling the clinical criteria for RTT and its variants and those who have a mutation in either *MECP2* or *CDKL5* are inserted in the database. *MECP2* and *CDKL5* mutation analysis is performed by a combination of DHPLC for all coding exons (exons 1–4) and real-time quantitative PCR (qPCR).

To protect the patient's privacy, when a biological sample arrives it is stripped of personal identifiers and for each sample an internal code is assigned: RETT followed by a sequential number. Anonymity of the samples is assured in each step of the research. A consent form has to be signed by the patient's guardians in order to insert the sample in the bank, according to international standards.

RTT Database

The RTT database has been available (at www.biobank.unisi.it) since 2004. It is maintained and updated every 3 months on the University of Siena server. The website is written in VBScript and takes advantage of an Internet Information Server (Microsoft IIS; Microsoft, Redmond, WA) with Active Sever Pages (ASP) (Microsoft) technology. The website contains an Access (Microsoft) database to manage the data.

In the general homepage of the database, there are links to four independent databases: X-Linked Mental Retardation (XLMR); Rett syndrome; Retinoblastoma; and Other. All these databases are managed by the Medical Genetics Unit (Fig 1). The first two databases are funded through a Telethon grant. By accessing to the RTT section of the bank, users can see the complete list of patients contained in the bank.

The general homepage contains useful links important for site navigation for all the four databases: 1) Guidelines, containing a description of the procedures to follow in order to store and request biological samples (the first option is only possible for the XLMR database); 2) Contact information, for contacting the bank administrator; 3) Services offered from the bank; in particular, isolation of leukocytes from human peripheral blood samples, establishment of EBV-transformed lymphoblastoid cell lines from human peripheral blood leukocytes, DNA extraction, cryopreservation of transformed cell lines and primary leukocytes at −135°C, storage of DNA and plasma at –20°C, and, finally, distribution of the stored biological samples (the latter is the only service

FIGURE 1. **Home page of the Rett Database and Biobank, available at www.biobank.unisi.it. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]**

available for the Rett database); 4) Bank organization, describing the general organization of the bank and its sections; 5) Forms, containing the forms users have to complete to take advantage of bank services and the informed consent.

Database Organization

The database is organized on two levels: a "public" level freely available to the general public and a "curator" level accessible only to bank curators through the use of a username and password and containing personal data of patients and relatives and their detailed clinical information. All users accessing the main page of the site (Supplementary Fig. S1; available online at http://www.interscience.wiley.com/jpages/1059-7794/suppmat), can visualize a table with the following information: 1) Code: a progressive number that identifies the single families, usually consisting of the affected girl, the parents, and other relatives when available. The system assigns a progressive number automatically every time a new case is inserted, and prevents a double insertion of the same case. 2) Internal code: the personal code assigned to each individual (proband, parents, other relatives) coming to our attention. These codes allow single individuals to be identified without using the personal data. Together with the personal code, the relationship within the family (proband, father, mother, etc.) is visible in this column, so that users can immediately know the individuals with available biological samples. 3) Phenotype category: the phenotypic definition of the proband. Seven phenotypic definitions are available: classic RTT; PSV, which is the most common RTT variant in our series; highly functioning PSV; forme fruste; congenital variant; early onset seizures variant; and Rett-like, including patients with a suggestive phenotype but who can not be framed in one of the specific phenotypes reported above. In addition, for very young patients for whom a definitive clinical classification is not possible the phenotype is indicated as "ND age less than 4 years." 4) Gene name: the name of the mutated gene in each patient (*MECP2* or *CDKL5*). In those patients for whom the causative mutation has not been identified, the gene name is designed as "Unknown." 5) Mutation type: pathogenic mutations are classified in four general categories: a) missense mutations, in which a single amino acid has been substituted with a different one; b) early truncating mutations, which interrupt the protein, eliminating part or all of the methyl-CpG-binding domain (MBD) and/or transcription repression domain (TRD) domain; c) late truncating mutations, which interrupt the protein in the C-terminal portion after the TRD domain; and d) gene deletion, either partial or total. 6) Nucleotide change: the change at nucleotide level is reported according to the standard nomenclature [den Dunnen and Antonarakis, 2000]. 7) Amino acid change: the change at protein level according to the standard nomenclature [den Dunnen and Antonarakis, 2000]. 8) Additional info: an icon in this column links to another page containing additional information about the family. On this page, bank curators can visualize all available information about the family, including personal and clinical data of patients and relatives. At the moment, external users can visualize only information about the X-inactivation status of the patient (skewed or partially skewed, with the cut off being 90% and 70%, respectively) [Sharp et al., 2000] and the mutation inheritance (de novo mutation, apparently sporadic, carrier mother, mosaicism in one parent). 9) Biological sample available: a list of the biological samples available for each family (lymphoblastoid cell line, leukocytes in DMSO medium, DNA, fibroblasts). In addition to the type of biological sample, the internal code of all family members for whom that sample is available is reported. Clicking on each sample, only bank curators, through the use of a protected password, can access to the information regarding the location of the sample. 10)

Reference: contains a link that allows users to view the PubMed reference of articles reporting that specific patient. All information listed above, with the exception of those reported in "Additional info," is available to the general public.

On the main page, users can find links to three additional pages, which focus on patients with mutations in the *MECP2* or *CDKL5* gene. The pages are: 1) List of mutations; 2) Graph of mutations; and 3) Rare variants (see next section for details).

Finally, the main page contains, in the right upper part, the "search by" tool, through which all database users may select patients with specific features.

MECP2 and *CDKL5* Mutations and Variants

On the main page, in addition to the list of all inserted patients, there are three links in the left upper part of the page, having to do with mutations and variants of *MECP2* and *CDKL5* genes (Supplementary Fig. S1).

These links are: 1) "List of Mutations", which provides a table of all identified mutations ordered by their frequency (Supplementary Fig. S2). For each mutation there is the mutation type column, in which the following fields may be present: missense, early truncating, late truncating or gene deletion. The mutations are identified by a systematic name of nucleotide change (GenBank accession number NM_004992 and NM_001037343) and amino acid change (GenBank accession number NP_004983 and NP_003150). One column reports the number of patients in whom the mutation has been found. Another column describes the phenotype of each patient carrying a specific mutation. The last column is dedicated to references, which allows the user to view the PubMed references of papers on the selected patients. Data in this table are automatically updated every time a new mutated patient is inserted in the database. 2) "Graph of Mutations", which contains a dynamic graph showing the position of the pathogenic mutations and their relative frequency (Fig 2). On the X-axis of the graph a scaled schematic representation of the protein with the main identified domains is given. On the Y-axis, the frequency expressed as the percentage of mutated patients bearing a specific mutation is given. Mutations are positioned along the protein scheme according to their amino acid numbering and they are represented as vertical columns; the height of the column represents the frequency of the mutation. Moreover, from this page, a link allows access to a pie chart that reports the frequency of the different types of mutations (missense, early truncating, late truncating) expressed as a percentage of mutated patients bearing a specific mutation type. Both graphs are managed by a dedicated program that reads data in the table of mutations and updates the graphs every time a sample is added. At present, *MECP2* gross rearrangements are not counted in the graph of mutation and in the pie chart. 3) "Rare variants", which contains a table listing all nonpathogenic rare variants identified in the patients of the bank (Supplementary Fig. S3). The table includes the following information: a) ID, a progressive number that identifies the single variant; b) nucleotide change; c) amino acid change; d) number of unrelated samples, i.e. the number of unrelated individuals where the variant has been identified; e) samples, i.e. the internal code of all the individuals in which the variant is present; f) reference, which allows the user to view the PubMed references of papers reporting the specific variant.

"Search by" Tool

The website is interactive, with a user-friendly graphical interface. Users that visit the online database can search for a patient by simply looking through the list of registered cases. Alternatively, the database may be explored using the "Search by" tool located in the upper right part of the main page. The search page allows the user to browse the database selecting for specific fields (Fig 3). The patients of interest can be selected by: 1) mutated gene (*MECP2* or *CDKL5*) or absence of mutation ("Unknown" option); 2) mutation type; 3) nucleotide change; 4) amino acid change; 5) phenotype category; and 6) survival, which allows to search alive patients of specific ages or dead patients. Users can choose to search by one single option or to combine two or more options; e.g. they can search all patients with missense mutations or they can search all patients with missense mutations in the *MECP2* gene and a PSV phenotype. As a result of the search, they will visualize a table containing all the fields present in the general table and listing all patients fulfilling the requested characteristics. This extremely flexible search option allows users to perform an accurate selection of patients so that they can immediately evaluate whether the type of samples they need are present in the bank and can choose the samples to request before contacting the bank curators.

Security and Quality Assurance

The database has been constructed in compliance with the guidelines of the Italian Society of Human Genetics and Telethon Foundation for biobanking [Dagna Bricarelli et al., 2003]. The database design assures patients' anonymity, privacy, and confidentiality, according to international criteria [Godard et al., 2003]. Moreover, the informed consent has been written explicitly, covering all aspects of stored samples and personal data management [Godard et al., 2003].

The RTT bank is available to all users who contact bank curators and fill in and sign a specific form asking for biological samples. Biological samples stored in the bank are distributed only to qualified professionals for research purposes only. The specimen cannot be distributed to other investigators without previous written permission of the bank curator. Researchers are asked to include the name of one of our researchers and/or to acknowledge the bank in any paper that includes results obtained using the bank samples/services. Anonymity of the samples is assured in each step of the research. Only the bank curator has access to the whole information content of the database, including personal data.

DISCUSSION

The Italian RTT database has been developed to share data and samples of our RTT biobank with the scientific community. The database allows to immediately visualize RTT samples contained in the biobank and to rapidly select those with specific clinical and molecular features. Contacting bank curators, users can request the samples of interest, following simple procedures indicated on the homepage of the website. In comparison with the already existing RTT databases, this database is the first one that is connected with a biobank. It is important to underline that all patients inserted in the database are evaluated by the same group of clinicians and the molecular analysis is performed by the same laboratory, allowing a uniform clinical and molecular data collection. The database includes both classic and variant RTT patients and cases who do not completely fulfill the international clinical criteria for RTT, but who have clinical features strongly resembling the RTT phenotype (RTT-like patients). Our cohort of *MECP2*-mutated patients also comprises those patients with a *MECP2* gene deletion. Using the "Search by" tool, users can select

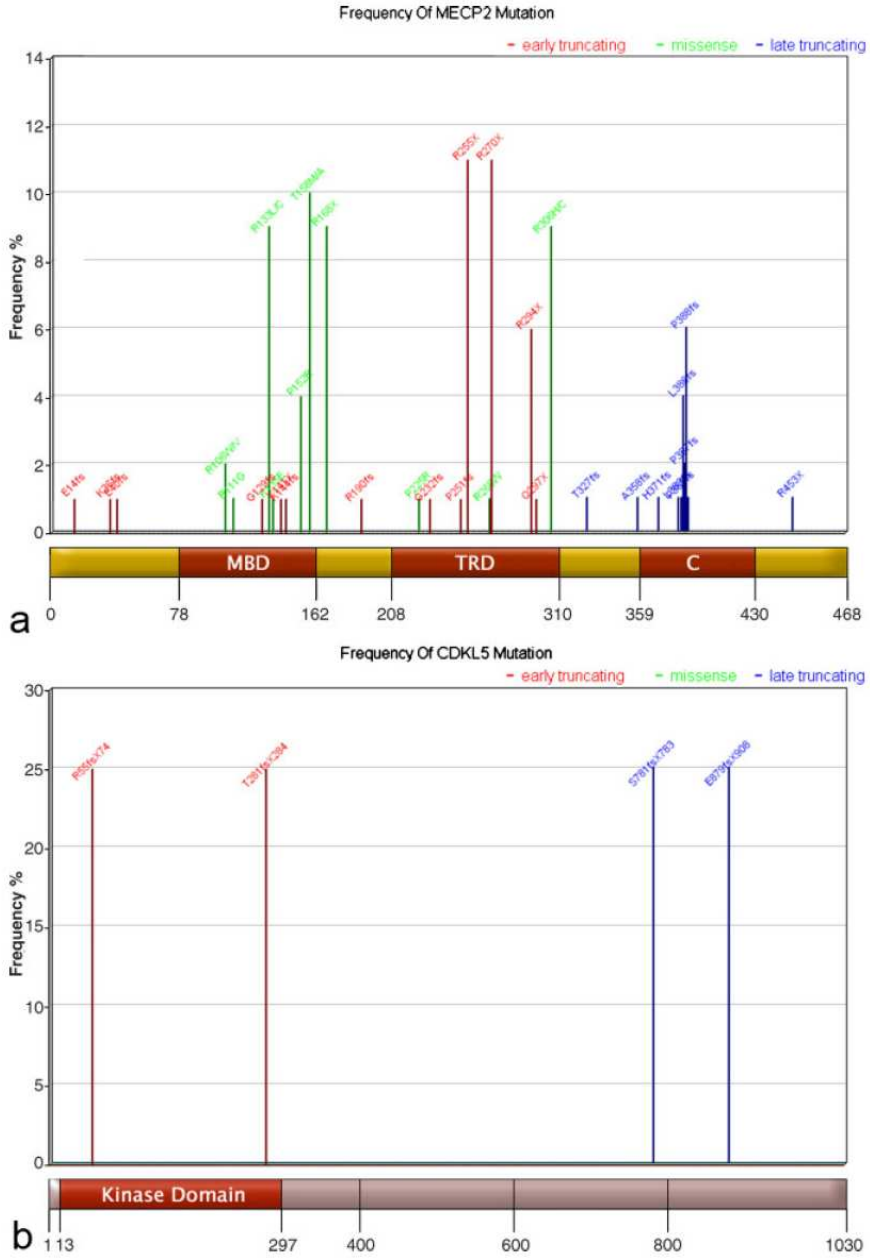

FIGURE 2. **Graphical view of mutation distribution within the *MECP2* (a) and *CDKL5* (b) gene. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]**

for patients with a *MECP2* mutation or a *CDKL5* mutation, or for patients in whom the molecular analysis failed to reveal a pathogenic mutation. The well-characterized collection of mutated patients is very important for researchers working on genetic modifiers of the RTT phenotype or on the functional consequences of a specific mutation, while mutation-negative RTT patients are potentially important for the identification of other genes involved in the syndrome.

During the last years, it has become evident the importance of international data to perform accurate genotype–phenotype studies [Leonard et al., 2005; Robertson et al., 2006]. InterRett, linked to RettBASE, has collected data on a large scale sample of

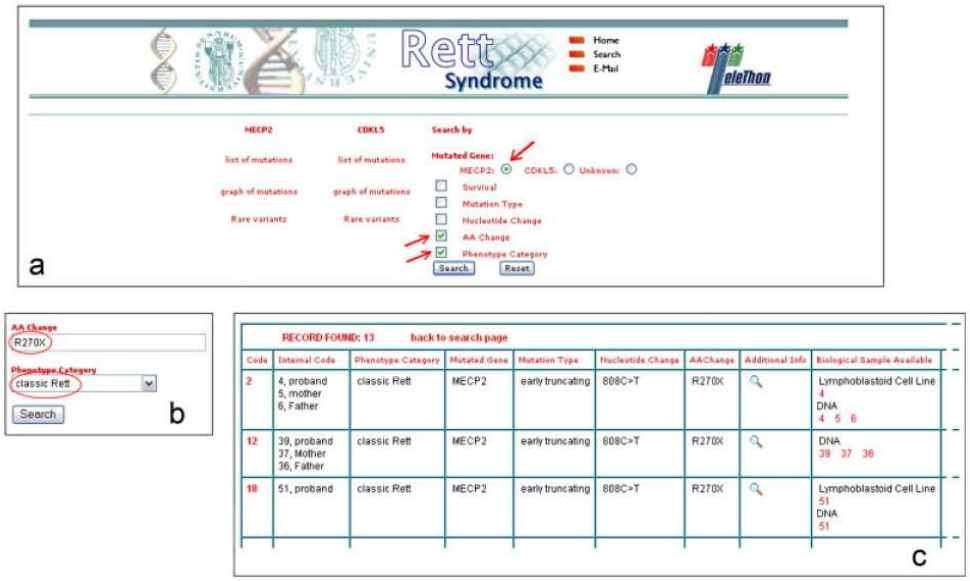

FIGURE 3. **The "Search by" option. This option allows the user to browse the database, selecting for a single field or combine two or more fields. For example, the user could select all patients with the p.R270X mutation in the *MECP2* gene and with a classic phenotype (a,b). c: The user will see the list of patients that show the requested characteristics. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]**

cases (2,089) representing 28 countries around the world and it is the most powerful tool for researchers working on genotype–phenotype correlations. For this reason, since our database contains a collection of RTT patients limited to the Italian population, we will make all effort to support this international project. However, it is important to also maintain and to improve this database to preserve its unique features. In addition to the already-mentioned characteristics, our database contains data on the X-inactivation status for a great portion of patients; these data are freely available for the general public. This has been possible thanks to the collection of parents' DNA samples. Since one of the most important factors in phenotype modulation is the X-inactivation status, this information will allow users to perform more accurate genotype–phenotype correlations once the patients' clinical section is improved (see Future Prospects). In addition, this information is fundamental for researchers who request biobank samples for expression studies.

In a separated section, the database contains tables indicating *MECP2* and *CDKL5* polymorphisms/rare variants with the code for the patients in whom they have been identified. This information, together with detailed clinical data (see Future Prospects), is important to study if these variants contribute in modulating the phenotype. In fact, the recent demonstration that *MECP2* and *CDKL5* interact suggests that the association between mutations in one of the two genes and specific variants in the other can modulate the RTT phenotype [Mari et al., 2005].

Given that for rare disorders such as RTT, the Internet provides an important means of communication, this online database encourages collaborative projects with clinicians and researchers from all around the world. This database, providing the opportunity for researchers to take advantage of this collection of clinically and molecularly well-characterized patients, represents an important resource to accelerate the clarification of the molecular basis of RTT.

FUTURE PROSPECTS

A detailed clinical characterization of each patient is a fundamental step for inclusion in the database. At present, all clinical details except for the general classification in classic, variant, or RTT-like cases, are available only for bank curators on the "Additional info", page. We are planning to create, for each case, a table with a set of clinical features and a related clinical score according to data in the literature [Charman et al., 2005; Colvin et al., 2003; Huppke et al., 2002; Kerr et al., 2001; Monros et al., 2001]. The new schedule will be freely available to the general public. Users will access this information by clicking on "Additional info", which already exists on the main page. This amelioration will make possible a better definition of the clinical phenotype of each patient. All these clinical features will be added in the "Search by" tool, making it possible for all users to sort cases by particular fields in the clinical data.

Regarding the X-inactivation data, we plan to ascertain the X-inactivation status of all patients included in the database and to display this information on the "Additional info" page.

Furthermore, the bank includes RTT patients with chromosomal rearrangements not involving *MECP2* or *CDKL5* [Delobel et al., 1998; Pescucci et al., 2003]. The expanding number of these cases will eventually lead to the identification of patients with overlapping rearrangements. These cases will possibly contribute to the identification of new RTT candidate genes. We plan to add the column "chromosomal rearrangements" to the main page of the database in order to allow users to immediately visualize this information and, eventually, to request the samples.

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Result 3.2

The *CDKL5* gene and early onset seizure variants of RTT

Result 3.2.1

CDKL5/STK9 is mutated in Rett syndrome variant with infantile spasms.

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ORIGINAL ARTICLE

CDKL5/STK9 is mutated in Rett syndrome variant with infantile spasms

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Background: Rett syndrome is a severe neurodevelopmental disorder, almost exclusively affecting females and characterised by a wide spectrum of clinical manifestations. Both the classic form and preserved speech variant of Rett syndrome are due to mutations in the *MECP2* gene. Several other variants of Rett syndrome have been described. In 1985, Hanefeld described a variant with the early appearance of convulsions. In this variant, the normal perinatal period is soon followed by the appearance of seizures, usually infantile spasms. We have observed two patients with signs of Rett syndrome showing acquired microcephaly and stereotypic midline hand movements. The disease started with generalised convulsions and myoclonic fits at 1.5 months in the first patient and with spasms at 10 days in the other, suggesting a diagnosis of the Hanefeld variant. In these patients, *MECP2* point mutations and gross rearrangements were excluded by denaturing high performance liquid chromatography and real time quantitative PCR. The *ARX* and *CDKL5* genes have been associated with West syndrome (infantile spasms, hypsarrhythmia, and mental retardation).
Methods: Based on the clinical overlap between the Hanefeld variant and West syndrome, we analysed *ARX* and *CDKL5* in the two girls.
Results: We found frameshift deletions in *CDKL5* in both patients; one in exon 5 (c.163_166delGAAA) and the other in exon 18 (c.2635_2636delCT). *CDKL5* was then analysed in 19 classic Rett and 15 preserved speech variant patients, all *MECP2* negative, but no mutations were found.
Conclusion: Our results show that *CDKL5* is responsible for a rare variant of Rett syndrome characterised by early development of convulsions, usually of the spasm type.

Rett syndrome (RTT; MIM# 312750) is a neurodevelopmental disorder characterised by a wide spectrum of clinical manifestations. In the classic form, after a period of normal development, patients show growth retardation and regression of speech, along with purposeful hand movements and appearance of stereotyped hand movements. RTT variants have been described, including the preserved speech variant (PSV), characterised by the recovery of some degree of speech; the congenital variant (recognised from birth); the "early seizure variant" (seizure onset before regression); and the "forme fruste", with a milder, incomplete clinical course (regression between 1 and 3 years).[1–6] These variants present some symptoms of RTT, but show considerable variation in type and age of onset, severity of impairment, and clinical course. Among these, the "early seizure variant'' was initially described by Hanefeld in 1985, who reported a girl with infantile spasms with hypsarrhythmia in her early development.[3]

Approximately 80% of patients with classic RTT have a mutation in the methyl CpG binding protein 2 gene (*MECP2*; OMIM #300005).[7–9] *MECP2* mutations have also been identified in about 50% of PSV cases and in a lower percentage of other variants.[8 10–12] In the variant with early development of convulsion described by Hanefeld, *MECP2* mutations have not been published.[3]

The Hanefeld variant of RTT presents a phenotypic overlap with West syndrome, also called infantile spasm syndrome, X linked (ISSX). ISSX is characterised by the triad of infantile spasms, hypsarrhythmia, and severe to profound mental retardation. Some families with ISSX carry mutations in the aristaless related homeobox (*ARX*) gene, which maps to Xp21.3–p22.1.[13 14] *ARX* mutations cause several forms of epilepsy, including infantile spasms, myoclonic seizures, and peripheral dystonia, as well as syndromic and non-syndromic X linked mental retardation.[13 14] Recently, a second gene has been found to be involved in ISSX, the cyclin dependent kinase-like 5 gene (*CDKL5*/STK9; NM_003159).[15] These authors characterised two unrelated female patients with an apparently balanced translocation, 46,X,t(X;7)(p22.3;p15) in one case and 46,X,t(X;6)(p22.3;q14) in the other. The two patients presented a similar phenotype, comprised of severe early onset infantile spasms with hypsarrhythmia and profound global developmental arrest. In both patients, the X chromosomal breakpoints disrupted *CDKL5*. As there is phenotypic overlap between the Hanefeld variant and ISSX, we tested both *ARX* and *CDKL5* for mutations in the two RTT patients with early onset of convulsions. The analysis was subsequently extended to 19 classic RTT and 15 PSV cases.

METHODS

Patients

We investigated two patients, aged 9 and 8 years respectively, with early development of convulsions, who later developed many characteristics of RTT. They both fulfilled the criteria for the early seizure variant of RTT.[4 5 16]

We then investigated 19 classic RTT and 15 PSV patients. The girls with classic RTT were diagnosed according to the international criteria.[17] The PSV girls fulfilled the criteria of Hagberg and Skjedal for RTT variants.[5] In particular, the PSV

Abbreviations: DHPLC, denaturing high performance liquid chromatography; ISSX, infantile spasm syndrome, X linked; PSV, preserved speech variant; RS, retinoschisis; RTT, Rett syndrome

cases show the same clinical features and stages of RTT in the first years of life, but they subsequently improve in fine motor ability and start to speak with an increasing number of words and phrases.

Molecular analysis

Blood samples were obtained after informed consent. DNA was extracted from peripheral blood using a QIAamp DNA blood kit (Qiagen). DNA samples were screened for mutations in *ARX* and *CDKL5* using transgenomic WAVE denaturing high performance liquid chromatography (DHPLC). The *CDKL5* coding portion was entirely analysed using the primers and conditions given in table 1. PCR products resulting in abnormal DHPLC profiles were sequenced on both strands by use of PCR primers with fluorescent dye terminators on an ABI Prism 310 genetic analyser (PE Applied Biosystems, Foster City, CA, USA). PCR products of exon 5 and exon 18 were separated on 6% polyacrylamide gel to define exactly the deleted bases. Normal and mutant alleles were cut from the gel and sequenced individually. X inactivation studies were performed using the assay of Pegoraro *et al*. Intensity of silver stained bands was measured using the Diversity Database program (Biorad) and the values were corrected for preferential allele amplification.[18] RNA isolation from lymphoblasts and cDNA synthesis were performed according to standard protocols.[19] We used primers designed to form cDNA products spanning exons 4–6 (4RTf: 5′-GAAACA CATGAAATTGTGGCG-3′; 6RTr:5′-GTGAATAGCCTTGATTAG CTG-3′) and 17–18 (17RTf: GAGAAGATCTCAGATCTGCAG; 18RTr: AGCTGGAGGGCTGGCCTG). RT-PCR products were separated by electrophoresis through a 6% polyacrylamide gel and silver stained.

RESULTS

We observed two patients with early development of convulsions who later showed characteristics of RTT. The phenotype of each case is described below.

Patient 1

This patient is a girl, now aged 9 years. Her pedigree is represented in fig 1A (left). The mother had a normal pregnancy and delivery. The child was affected with slight cyanosis after birth. Birth weight was 3600 g and head circumference was 34 cm (50th centile). Generalised convulsions appeared at 1.5 months of age and were barely controlled by various antiepileptic drugs. In the following months she was examined in hospital, and myoclonic fits resembling infantile spasms were noted, although her EEG was not typical of hypsarrhythmia. Her developmental milestones were delayed and she was able to sit unaided at 1 year and to walk unaided at 6.5 years. She was examined again in hospital at the age of 2 years. MRI of the head, evaluations for aminoacidaemia, karyotype, search for Fragile X syndrome, methylation pattern for Angelman syndrome, and *UBE3A* gene sequencing were all negative. The patient was initially examined in our unit at the age of 8.5 years. She was able to briefly hold an object in her hands, dropping it shortly afterwards. She brought her hands frequently to her mouth, or beat them together. She had stereotypic hand-washing activities, reported as occurring since the age of 1 year. Her facial expression varied and she was able to interact at a pre-verbal level. She was able to utter one word and had occasional bruxism and hyperventilation. Her head circumference was 48.5 (<3rd centile). Scoliosis, kyphosis, cold extremities, and constipation were not present. The EEG showed sharp waves in the central and occipital regions.

Table 1 Primer sequences and conditions for PCR reaction and DHPLC analysis of *CDKL5* amplicons

					DHPLC analysis	
Exon	Forward primer	Reverse primer	Product length (bp)	PCR annealing temp (°C)	Temp of elution (°C)	Buffer (%)
2	AGGTAAGATTGGTTACTAGAG	AATAACTAACTGTTCATTGCTC	350	58	55	57
					57	57
3	TGAGAAGCAATGTCAGTATAG	CCTGTACATGCCCACACGC	201	58	55	53
4	CTGGCTTCTTGCTACTCTG	TCCCACTTCTTCCACACTC	242	58	54	53
5	AGTGTTCTTGGAATTCTTTG	GGCAAATGTGCACATTGGC	244	54	57	53
6	CTCTGTATTGGATGAATTATTC	TTCTTAAAGACAGTAACATGTG	303	59	54	55
					56	55
7	TTTATCTTGACACTCCAGA	ACTCCTCCAGCAATCAATT	237	56	53	53
8	GCCCATCGGAGAACAGTCATTAC	GCAAATGACAATAGAATCAGCAG	280	55	56	54
					57	54
9	TTATTCACTTGTGTTCTGATGAT	CAAATACTGCAGTATTGATTCC	410	54	54	58
					56	58
10	TATGAATTTGACTGGGATTGG	CTATGGTCACATGTAGACAC	275	59	57	54
11	TTGATATTCTGCAATGACTGTG	AGCCACCTCCTCCACCTAC	333	62	53	56
					55	56
12_a	TTGTGTGTCAGCTATTGAGG	GGTTCTGCTGAGATCTGCTG	406	60	56	60
					59	60
12_b	CAACAACATACCACACCTTC	TTCTCGTGTCACTGTGTCTG	422	60	57	60
					60	60
12_c	ACTCCAAGTCTGTGAGCAAC	AGATGGACCCTCATCACATC	541	60	57	63
13	GGTTATGGTCCTAGTTCTAC	CACTTCAACTTATTTGTGGG	298	60	57	55
					59	55
14	CAATAGAGTGAGACCCTGTC	CTGAGTCGGTGAAAGCAGTG	279	65	57	55
					60	55
15	AAAAGTCCATCAGTGACTTAC	CCTAGCAGGAGAAAGGACAC	262	60	56	54
16	TATAGGAACCTAGTGTCATGC	CAACTTTGATTGCCAAGTGC	293	59	53	57
17	CTTGGGTGTGGTTGCATATC	CTGTAACATTGAGAGGCTAAG	296	60	59	55
18	CTTGCACATGCTTGCCCTTC	CACCCAGCTGTTCAGAGTAG	418	62	61	58
19	ACTCTGGTCAATGGGATGTG	CATTCAGTAGTCTAGGGTCG	249	60	59	53
20	TTGGCTTCAGCTGGTGTCTG	CATCTGCATTTCTACAGCTC	345	61	61	58
21	CATTAGCCAGAGTGCACCTG	AGGAAAAACTCAACCTCAGCG	290	60	59	55
					63	54

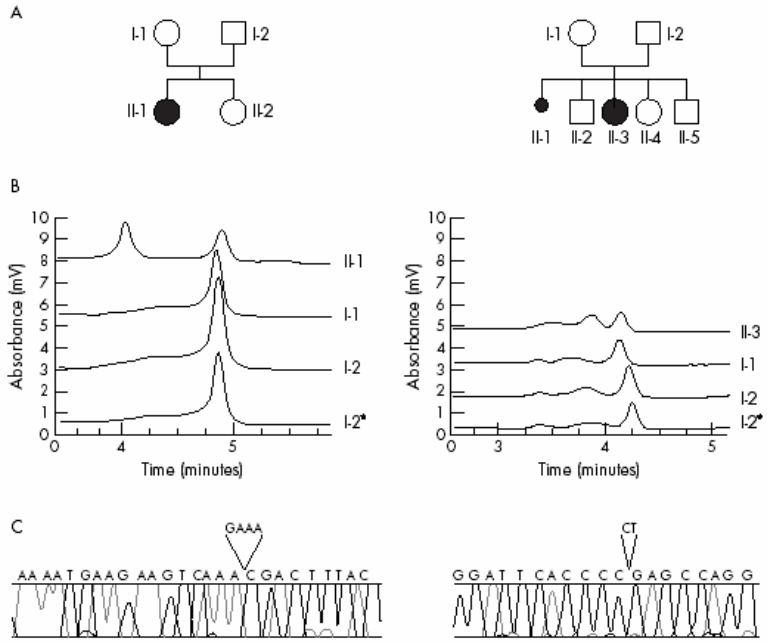

Figure 1 Pedigree (A), DHPLC analysis (B), and DNA sequencing (C). Left side, case 1, right side, case 2. (B) An asterisk indicates that the DNA of the father is 1:1 mixed with a control male. (C) The chromatograms represent the mutated sequences separated from normal alleles on 6% polyacrylamide gel. Deleted bases are indicated above.

Patient 2

This patient is a girl, now aged 8 years. Her pedigree is represented in fig 1A (right). The mother had a normal pregnancy and delivery, and the child was affected with slight cyanosis at birth. Her birth weight was 3800 g but no data concerning her head circumference are available. At 10 days, generalised convulsions, lasting only a few seconds, appeared, which were difficult to control with various antiepileptic drugs and persisted during the following years. Myoclonic seizures, when her arms suddenly opened, possibly infantile spasms, were also reported by her parents, although clinical records concerning these details were not available. Her MRI was normal. Her development was delayed; in the first years of life she did not respond to interactions, she had been found hypotonic by medical professionals, and she was unable to hold an object in her hands. She was able to sit alone at 1.5 years of age. She was examined in our unit when 4 years 10 months of age. Her head circumference was 49 cm (tenth centile), she had a moderate degree of generalised hypotonus, and was unable to hold an object in her hands, which were brought frequently to her mouth and occasionally twisted together. Furthermore, she had myoclonic epileptic fits occurring two or three times a day, and occasional generalised convulsions. An EEG showed generalised discharges of sharp and slow waves and focal sharp waves in the right centrotemporal region. She was evaluated at the age of 7 years 10 months, when a slight improvement in the use of her hands was noted. She could hold a biscuit and some bread in her hands and eat them. Her facial expression varied notably and she was capable of reciprocal modulations. Generalised hypotonus was still present: she was able to sit unaided but unable to walk without support. The stereotypic activities of her hands were still present. She occasionally uttered one or two words. Her head circumference was 50 cm (tenth centile). Scoliosis, kyphosis, cold extremities, and constipation were absent. Myoclonic fits and generalised convulsions were still present in spite of various treatments, including phenobarbital, valproate, lamotrigine, and carbamazepine. An EEG showed the persistence of generalised sharp and slow waves and the presence of additional multifocal abnormalities.

Molecular analysis

MECP2 point mutations, including the recently identified exon 1, and gross rearrangements were excluded by DHPLC and quantitative PCR, respectively.[20–22] Based on the partial clinical overlap between the Hanefeld variant and ISSX, we screened the two RTT variant patients for point mutations in *ARX* and *CDKL5*. *ARX* analysis did not identify any mutation. DHPLC analysis of *CDKL5* revealed a different frameshift mutation in the two patients, absent in the DNA of the parents in both cases (fig 1B).

In case 1 we identified a 4 bp deletion in exon 5 (c.163_166delGAAA) (fig 1C; left) leading to loss of most of the CDKL5 protein (NP_003150). The frameshift mutation creates a stop codon in position 74, after a short stretch of abnormal amino acids (fig 2A, 2B; top). This deletion interrupts the catalytic domain covering the first 300 amino acids, and creates a non-functional CDKL5 product.

Case 2 showed a 2 bp deletion in exon 18 (c.2635_2636delCT) (fig 1C; right) leading to protein truncation in position 908, after a short stretch of incorrect amino acids (fig 2A, 2B; bottom). This deletion eliminates a putative signal peptidase I serine active site, as predicted by the ScanProsite program (http://ca.expasy.org/prosite) (fig 2A).

We then investigated whether mutated and normal alleles were expressed in the two patients. We studied X inactivation in DNA from blood cells of both patients. The assay showed balanced X inactivation in both cases (not shown). Band intensities were measured: the ratio between the two alleles was 52:48 in case 1 and 67:33 in case 2. We then performed RT-PCR to test if mutated mRNA alleles were degraded by nonsense mediated RNA decay. RT-PCR products, separated by electrophoresis and silver stained, demonstrated the

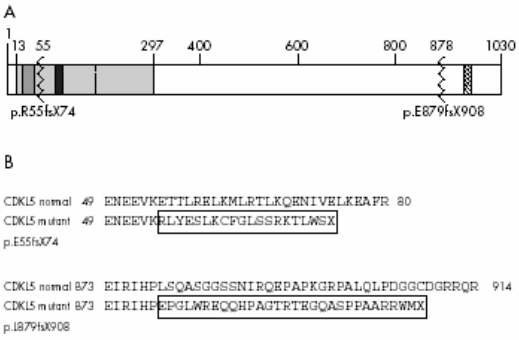

Figure 2 CDKL5 protein with mutation positions (A) and alignment between the normal and the mutated amino acid sequences (B). (A) The catalytic domain (light grey) contains an ATP binding site (dark grey) and the serine–threonine protein kinase active site (dark box). The conserved Thr-Xaa-Tyr motif is indicated with a sketch line. The signal peptidase I serine active site is represented by the reticulated box. The two frameshift deletions are indicated by zigzag lines. The numbers at the top refer to the amino acid positions. (B) In patient 1 (top), the deletion creates a stop codon in position 74. In patient 2 (bottom), the deletion leads to protein truncation in position 908. Stretches of incorrect amino acids are boxed.

expression of both mutated and normal alleles in the two patients (fig 3).

We then extended the *CDKL5* analysis to 19 classic RTT and 15 PSV cases, all *MECP2* negative; no mutation was found.

DISCUSSION

The two girls reported above had a similar clinical course: they showed convulsions very early in life, respectively at 1.5 months and at 10 days, drug resistant in both cases. They otherwise fulfilled the criteria for RTT, including acquired microcephaly, hand apraxia, generalised hypotonus, and stereotypic hand activities.[4 5 16] Hyperventilation was present in one girl. Scoliosis, constipation, and cold feet were absent in both, suggesting a possibly better preserved autonomic system. It was difficult to retrospectively assess whether they had had the typical stage sequence of RTT. When examined at ages close to 8 and 5 years, they were expressive and interacted in a manner adequate to their reduced general abilities, and they fulfilled the criteria for the early seizure variant of RTT.[4 5 16] It should be noted that only a few cases of this disorder have been reported[4 5] and, with the exception of the first case described by Hanefeld (personal communication), no *MECP2* mutations have been described. In the two

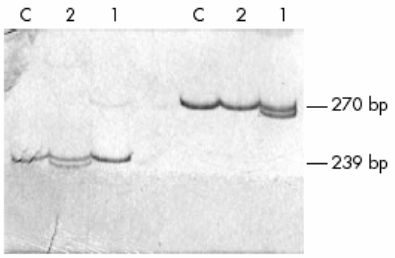

Figure 3 RT-PCR analysis of patients (1 and 2) and a control individual (C). The polyacrylamide gel shows RT-PCR products. RT-PCR products spanning exons 17–18 (left) and exons 4–6 (right). In both cases, the mutant allele is evident as a lower band. Size is indicated on the right.

RTT variant patients, *MECP2* point mutations and gross rearrangements were excluded by DHPLC and qPCR.

Our results indicate that RTT variant with infantile spasms may be due to inactivating *CDKL5* mutations. CDKL5 is a member of the serine–threonine kinase gene family.[23] Kinase proteins are a large superfamily of homologous proteins, characterised by a highly conserved kinase domain (250–300 amino acids). The CDKL5 kinase domain is most closely related to human KKIALRE and KKIAMRE and their orthologues.[23] The sequence alignment of the CDKL5 protein with these homologues showed two kinase signatures in the catalytic domain: an ATP binding region (amino acids 14 to 47) and a serine–threonine protein kinase active site (amino acids 127 to 144).[23] In addition, a Thr-Xaa-Tyr motif was identified,[23] and the dual phosphorylation of these Thr and Tyr residues has been shown to be essential for activation of the MAP kinase group.[24]

The frameshift mutation found in patient 1 is located in the conserved kinase domain of the CDKL5 protein, causing loss of both the serine threonine active site and the Thr-Xaa-Tyr motif. Thus, the deletion abolishes the catalytic function of the protein. Furthermore, it has been reported that lack of functional CDKL5 protein causes severe ISSX.[15] This finding was derived from a study of two severely affected ISSX female patients with apparently de novo balanced X;autosome translocations, both disrupting the *CDKL5* gene in the kinase domain. Additional studies are needed to further elucidate why different truncating mutations in *CDKL5* cause different but overlapping phenotypes.

Except for the kinase domain, the function of the CDKL5 protein is unknown (fig 2A). The region between positions 300 and 1030 is not conserved in different species and does not share a homology with other human proteins, making it difficult to predict its function.[23] The deletion found in case 2 falls in the C terminus of the protein. We could speculate that this region contains a functional domain specific for human CDKL5. Using the ScanProsite program we identified a signal peptidase I serine active site (GTSMCPTL), located between positions 971 and 978, abolished by the deletion in patient 2. This domain is one of the five conserved domains present in all known signal peptidases.[26] CDKL5 probably does not have a peptidase activity, as it lacks other conserved residues necessary for the catalytic activity.[26 27] However, we cannot exclude that CDKL5 might form a complex with other proteins that harbour the other essential domains.

A C terminal deletion of CDKL5 has already been reported by Huopaniemi *et al*[28] in a family with X linked juvenile retinoschisis (RS). No additional phenotype was reported in these patients with the exception of epilepsy in one. This deletion, spanning from the 5′ region of the RS1 gene to intron 3, disrupts two other genes, *PPEF-1* and *CDKL5* (exon 20). It is possible that this truncation, more 3′ than that reported here, generates a milder phenotype.

Finally, it is important to understand why *MECP2* and *CDKL5* mutations lead to a similar phenotype. MeCP2 and CDKL5 could belong to the same signalling pathway. As it has demonstrated that MeCP2 is subjected to phosphorylation and that CDKL5 has a kinase domain, it is possible that MeCP2 is directly phosphorylated by CDKL5.[25 29] However, at least in patient 1, CDKL5 kinase activity is abolished, and a reduced degree of MeCP2 phosphorylation would lead to a reduction in its dissociation from methylated DNA and to a gene silencing increase.[29] However, is known that *MECP2* mutations presumably cause a reduction of gene silencing.[9 30] Alternatively, CDKL5 might phosphorylate a second protein that could dephosphorylate MeCP2. Additional studies are necessary to determine whether MeCP2-CDKL5 interaction really exists and to unravel the complex mechanisms underlying the above phenotypes.

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Result 3.2.2

CDKL5 belongs to the same molecular pathway of MeCP2 and it is responsible for the early-onset seizure variant of Rett syndrome

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CDKL5 belongs to the same molecular pathway of MeCP2 and it is responsible for the early-onset seizure variant of Rett syndrome

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Rett syndrome (RTT) is a severe neurodevelopmental disorder almost exclusively affecting females and characterized by a wide spectrum of clinical manifestations. Most patients affected by classic RTT and a smaller percentage of patients with the milder form 'preserved speech variant' have either point mutations or deletions/duplications in the *MECP2* gene. Recently, mutations in the *CDKL5* gene, coding for a putative kinase, have been found in female patients with a phenotype overlapping with that of RTT. Here, we report two patients with the early seizure variant of RTT, bearing two novel CDKL5 truncating mutations, strengthening the correlation between *CDKL5* and RTT. Considering the similar phenotypes caused by mutations in *MECP2* and *CDKL5*, it has been suggested that the two genes play a role in common pathogenic processes. We show here that CDKL5 is a nuclear protein whose expression in the nervous system overlaps with that of MeCP2, during neural maturation and synaptogenesis. Importantly, we demonstrate that MeCP2 and CDKL5 interact both *in vivo* and *in vitro* and that CDKL5 is indeed a kinase, which is able to phosphorylate itself and to mediate MeCP2 phosphorylation, suggesting that they belong to the same molecular pathway. Furthermore, this paper contributes to the clarification of the phenotype associated with *CDKL5* mutations and indicates that *CDKL5* should be analyzed in each patient showing a clinical course similar to RTT but characterized by a lack of an early normal period due to the presence of seizures.

INTRODUCTION

Rett Syndrome (RTT, OMIM 312750) is a progressive neurological disorder primarily affecting females with an incidence of approximately 1:15 000 born females (1,2). The disorder is characterized by a wide spectrum of phenotypes. In the classic form, after 6–18 months of almost normal development, patients display a developmental arrest, followed by a regression with loss of speech and purposeful hand use and appearance of postnatal microcephaly, stereotypic hand movements, ataxia, hand-apraxia and abnormal breathing. At this stage, similarities with autistic behavior are present. Later, there is a limited amelioration followed, in older girls, by a final somatic and neurologic deterioration. Up to 80% of patients experience epileptic episodes (1,3). In addition to classic RTT, some variants have been described presenting some features of the classic form but displaying differences in disease onset and severity.

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A very few cases of familial RTT made it initially difficult to determine the mode of inheritance of this disorder, but the virtual absence of affected males suggested an X-linked dominant inheritance pattern (1). Consistent with this, it was shown that mutations in the methyl-CpG-binding protein 2 gene (*MECP2)* located in Xq28 are the primary cause of RTT (4–6). As a matter of fact, ~80% of patients with classic RTT carry mutations within *MECP2*, whereas only 20–40% of patients affected by RTT variants are mutated within *MECP2* (7–9). Recently, mutations in another X-linked gene, cyclin-dependent kinase-like 5 (*CDKL5)* located in Xp22, have been identified in patients affected by an RTT-like phenotype or the early-onset seizures variant of RTT (Hanefeld variant) (10–12).

MeCP2 is a broadly expressed nuclear protein binding to DNA methylated at CpG dinucleotides through a conserved methylated CpG-binding domain (MBD) (13). Through its ability to recruit chromatin-remodeling complexes containing histone deacetylase activities (HDAC) as well as histone methyltransferase activities, MeCP2 is able to abrogate gene expression by modifying chromatin structure (14,15). Furthermore, by interacting directly with a component of the basal transcriptional machinery, TFIIB, MeCP2 seems to be able to repress transcription in a chromatin independent manner (16). Eventually, this methyl-binding protein is able to compact a nucleosomal array on its own (17), suggesting that altogether MeCP2 exerts a number of effects on chromatin structure and gene expression.

Mice null for Mecp2, both male hemizygotes and female homozygotes, manifest phenotypes resembling that of RTT (18,19). Importantly, the conditional deletion of Mecp2 in postmitotic neurons recapitulates these features, demonstrating that neuronal dysfunction is the cause of the deficiencies in these mice (19). Furthermore, the rescue of the RTT-like phenotype in Mecp2 knock-out mice by expression of Mecp2 only in postmitotic neurons underscores the importance of this protein for proper brain function (20). In accordance with this, Mecp2 expression is particularly high in neurons and its timing of expression correlates with neuronal maturation (21). Initially, mutations in the *MECP2* gene were proposed to cause RTT because of a defect in preventing unscheduled transcription throughout the genome (22), but only minor changes in gene expression are observed in microarray studies using mRNAs from Mecp2 mutant mice as well as RTT patients (23,24). The recent demonstration that *Hairy2A* in *Xenopus* and *Bdnf* (brain-derived neurotrophic factor) are direct MeCP2 target genes suggests that only specific loci may be deregulated in RTT (25–27). It is important to note that an ~2-fold derepression of *Bdnf* was observed in Mecp2-deficient cells (26) indicating that only subtle changes in gene expression may be present in RTT patients. Importantly, Chen *et al.* (26) demonstrated that *Bdnf* repression is regulated by MeCP2 phosphorylation; in particular, upon membrane depolarization of cultured neurons, MeCP2 becomes phosphorylated and specifically detach, together with the Sin3A/HDAC complex, from the *Bdnf* promoter, thus permitting transcriptional activation. However, the upstream events regulating the observed phenomena and the involved kinase(s) remain unknown.

CDKL5 is a hitherto rather uncharacterized protein containing a conserved serine/threonine kinase domain in its N-terminal, sharing homology to members of the mitogen-activated protein (MAP) kinase and cyclin-dependent kinase (CDK) families (28). However, the kinase activity of CKDL5 has never been demonstrated. The recent discovery that *CDKL5* mutations can cause a phenotype overlapping RTT might indicate that the two proteins belong to the same genetic pathway (10).

In this paper, we report the identification of additional *CDKL5*-mutated patients, reinforcing the link between the gene and RTT. Given the apparent importance of MeCP2 phosphorylation and the evident involvement of CDKL5 in RTT, we found it challenging to deeply investigate the CDKL5 protein concerning its developmental expression pattern, protein interaction and functional activity.

Here, we demonstrate that Cdkl5 expression in developing mice significantly overlaps with that of Mecp2. The possibility that these two proteins belong to the same molecular pathway is reinforced by their capability to interact *in vitro* and *in vivo.* Importantly, the interaction surface on the methyl-binding protein is given by a region including the last residues of the transcriptional repression domain (TRD) and the C-terminal portion of the protein. Interestingly, this region includes residues frequently mutated in RTT patients; moreover, a missense mutation associated with an atypical variant of RTT has been reported (see the MeCP2 mutation frequency at the URL: http://mecp2.chw.edu.au/). Eventually, we demonstrate that CDKL5, according to its primary structure, harbors a kinase activity, which mediates MeCP2 phosphorylation *in vitro*, further reinforcing the idea that they are associated in the same molecular pathway.

RESULTS

Identification of two novel *CDKL5* mutations leading to the early seizure RTT variant

We observed two female patients, aged 7 and 2, who show many characteristics of RTT. Both patients have a normal head circumference and lack the characteristic first normal period due to the presence of seizures. A detailed clinical description is reported subsequently.

Patient 1. This patient is the first child, presently aged 7. The mother has a second child, a male, who is normal. The mother had a normal pregnancy and delivery. From the first days of life, her parents noticed the occurrence of flexion spasms involving the entire body, lasting a few seconds and followed by relaxation. An electroencephalogram (EEG) conducted at 1 month was reported as normal, but a subsequent EEG at 3 months showed the presence of paroxysmal spike-wave activities, more evident in the left temporal region, and biparietal sharp waves, made more active by sleep. A brain magnetic resonance imaging (MRI) showed the presence of an arachnoid cyst in the left temporal region. An anti-epileptic treatment was subsequently instituted but epileptic fits remained in time. She was able to sit alone at 18 months and she has never been able to walk alone. She has never uttered a word and she was always unable to use her hands to take objects. Hand-mouthing and clapping activities are present since the

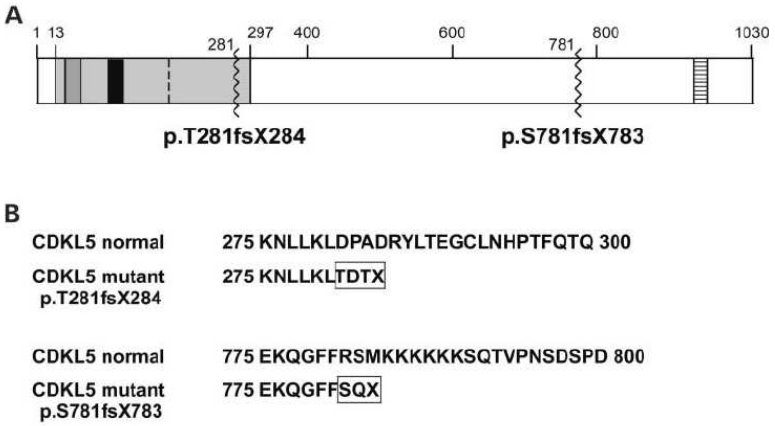

Figure 1. Schematic representation of the CDKL5 protein with mutation position and alignment between the normal and the mutated amino acid sequences. (**A**) The predicted human CDKL5 protein and the positions of the new identified mutations are represented. The catalytic domain (light gray box) contains an ATP binding site (dark gray box) and the serine–threonine protein kinase active site (black box). The conserved Thr–Xaa–Tyr phosphorylation sites are indicated with a sketch line. The signal peptidases I Serine active site, located in the C-terminal region of the protein, is represented by the striped box. The positions of the two frame-shift deletions (p.T281fsX284 and p.S781fsX783) are indicated by zigzag lines. Numbers at the top refer to the amino acid positions. (**B**) Alignment between the normal and the mutated CDKL5 sequences. In patient 1, the deletion led to protein truncation in position 284, after a short stretch of incorrect amino acids (boxed). The deletion creates a protein lacking the final portion of the kinase domain. In patient 2, the frame-shift deletion creates a stop codon in position 783, after a short stretch of abnormal amino acids (boxed). The deletion falls in the C-terminal portion of the protein outside the predicted catalytic domain.

second year of life. Her head circumference is presently 51.3 cm (25–50°). She is able to interact at a preverbal level. Generalized convulsions characterized by tonic–clonic jerks, eyes revulsion and chewing movements of the mouth are now present once a week in spite of various treatments including valproate and benzodiazepin. She has gastroesophageal reflux and she is treated accordingly. In addition, she has stypsis, cold extremities and bruxism.

Patient 2. This patient is a female, now aged 2. The mother had a normal pregnancy and delivery. Birth weight, length and head circumference were in the normal range. Parents referred a sudden awakening at 3 months of age. They also referred that at 5 months of age, she had some episodes, in which she suddenly opened her eyes remaining a few moments with staring eyes, during her sleep. The psychomotor development was normal in the first 6 months, when the first episodes diagnosed as seizures were noted. The seizures resembled infantile spasms, although the EEG did not show hypsarrhythmia. They were difficult to control with various anti-epileptic drugs and they persisted during the following months. In the first months of age, she had gastroesophageal reflux. She was able to utter some words at 12 months and to walk alone at 20 months. A brain MRI performed at 10 months, ophthalmologic examination, biochemical analyses and screening for metabolic disorders were normal. She presently shows autistic features according to DMSIV. She is able to hold an object in her hands. She has hand-mouthing and clapping stereotypic activities. Her head circumference is still in the normal range (48.5 cm, 50°cnt). She can occasionally utter one word. Partial convulsions, characterized by a sudden opening of the arms, are still present, in spite of various treatments. The EEG shows paroxistic activities in the frontal region. In addition, constipation is referred, whereas scoliosis, kyphosis and cold extremities are not present.

Considering the phenotype of the two patients, we decided to analyze both *MECP2* and *CDKL5*. In particular, we excluded the presence of *MECP2* point mutations (including exon 1) and gross rearrangements by DHPLC and qPCR, respectively. DHPLC analysis of *CDKL5* revealed the presence of two different '*de novo*' frame-shift mutations (Fig. 1A). Case 1 showed a 10 bp deletion in exon 11 (c.838_847del10, p.T281fsX284) leading to the loss of almost 800 amino acids of the protein, after a short stretch of incorrect amino acids (Fig. 1B). In case 2, we identified a 1 bp deletion in exon 16 (c.2343delG, p.S781fsX783) leading to protein truncation in position 783 (Fig. 1B).

MeCP2 and CDKL5 do not interact at transcriptional level

Even though, by whole-mount *in situ* hybridization performed on brain of Mecp2-deficient mice, it has been demonstrated that Cdkl5 expression is independent of MeCP2 expression (12), we tested whether the two genes interact at a transcriptional level in humans by real-time qPCR. For this purpose, we analyzed both *CDKL5* and *MECP2* mRNA levels in lymphoblastoid cell lines from patients with *MECP2* early truncating mutations and *CDKL5* mutations, respectively. The expression levels were quantified by the ddCt method. Figure 2 shows the columns corresponding to the mean values of the ddCt ratios obtained for control and mutated samples in both *MECP2* and *CDKL5* assays. The statistical analysis of the expression levels of both the genes indicated that there is

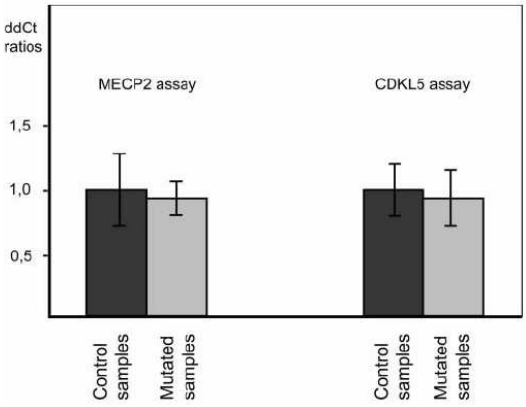

Figure 2. Real-time qPCR analysis. On the left, *MECP2* assay. Mean values of *MECP2* ddCt ratios and standard deviations (SD) of five control samples (dark gray column) and three *MECP2* mutated samples (light gray column). On the right, *CDKL5* assay. Mean values of *CDKL5* ddCt ratios and SD of five control samples (dark gray column) and three *CDKL5* mutated samples (light gray column).

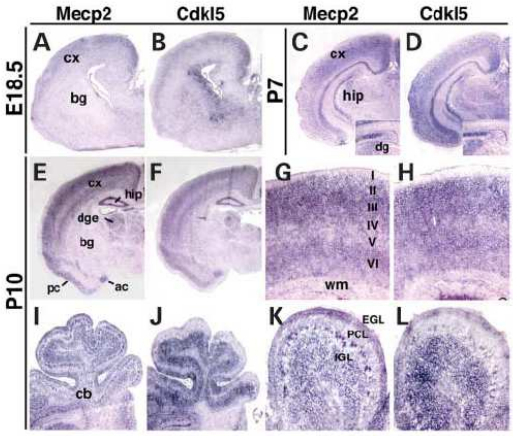

Figure 3. Mecp2 and Cdkl5 expression patterns during neural differentiation. Mecp2 (**A**) and Cdkl5 (**B**) expressions are first detected in E18.5 brains in the neurons migrated into the cerebral cortex (cx). (**C** and **D**) Sections of posterior forebrain hybridized for Mecp2 and Cdkl5, respectively. Note the strong expression in the hippocampus (hip) of both genes. Inserts in (C) and (D) show gene expression in the dentate gyrus (dg). (**E**–**H**) Mecp2 and Cdkl5 expressions in medial forebrain sections (E and F) and in cerebral cortex (G and H) of mouse at P10. Expression of both genes is enriched in all the six layers of the cortex, piriform cortex (pc), amygdala complex (ac), hippocampus (hip) and dorsal geniculate thalamic nucleus (dge). (**I**–**L**) Mouse P10 cerebellum labeled for Mecp2 and Cdkl5 expressions. Note that although both genes are expressed in external granular layer (EGL), Purkinje cellular layer (PCL) and internal granular layer (IGL), their expression level in any specific layer is differently modulated (compare Mecp2 and Cdkl5 staining in PCL in K and L). bg, basal ganglia; wm, white matter.

not a significant difference between control and mutated samples ($P > 0.05$). In conclusion, these data, together with the results obtained in mouse brain (12), suggest that there is not an epistatic relationship between CDKL5 and MeCP2.

Mecp2 and Cdkl5 expression patterns are significantly overlapping in embryonic and postnatal mouse brains

As already published, Mecp2 has a widespread expression throughout the mature brain specifically confined to differentiated neurons (29). However, some heterogeneity in Mecp2 expression levels has been observed in early postnatal stages where neurons that were generated early and are more mature have a stronger expression (21). Hence, Mecp2 expression gradually broadens and gets enhanced in the brain during early postnatal stages. We investigated Cdkl5 expression during neuronal maturation and compared it with that of Mecp2. As for Mecp2, Cdkl5 expression is weakly detectable at late stages of embryogenesis while strongly enhanced from P1 onwards (compare Fig. 3A with E and Fig. 3B with F). Cdkl5 expression is first observed in neural cells that have reached their final position in the cortical plate (Fig. 3B and data not shown). In the early postnatal days, a high enhancement of Cdkl5 expression is observed, reaching a stable peak at P10 (Fig. 3F and H). At this stage, the regions with the highest Cdkl5 expression are the neocortex (Cx), the piriform cortex (pc), the hippocampus (hip), the amygdala complex (ac) and the dorsal geniculate nucleus (dge) as similarly found for Mecp2 (Fig. 3E and F). At this stage, in particular, the majority of the cortical neurons are highly expressing both Cdkl5 and Mecp2 (Fig. 3G and H). Interestingly, the observed general increase of Cdkl5 expression in the first postnatal stages may be closely correlated with neural maturation and synaptogenesis as already proposed for Mecp2 (21,29). On the same line, the delay in the increase of Cdkl5 expression in the dentate gyrus (dg) with respect to the hippocampal area may follow the different phases of neurogenetic maturation of these two fields (inserts in Fig. 3C and D). However, not all the brain regions show a comparable levels of Cdkl5 and Mecp2 expression. For instance, at P10 in the cerebellum, the two genes show different levels of expression in specific cerebellar domains. In fact, a strong Mecp2 but weak Cdkl5 staining is detected in the Purkinje neurons; whereas high Cdkl5 and low Mecp2 expression levels were found in granular cells (Fig. 3I–L). This indicates that different Cdkl5 and Mecp2 expression levels may coexist in the same cells suggesting independent mechanisms of gene regulation in such tissues.

MeCP2 and CDKL5 are directly interacting *in vitro* and *in vivo*

To understand whether MeCP2 and CDKL5 belong to the same molecular pathway, we went ahead analyzing the possibility that the two proteins may even be directly interacting. We addressed this point performing a classical glutathione *S*-transferase (GST) pull-down assay in which a GST–MeCP2 fusion protein, expressed in *Escherichia coli*, was immobilized on a Glutathione–Sepharose resin and challenged with hCDKL5 translated *in vitro*. As shown in Figure 4A, CDKL5 is retained on the GST–MeCP2 resin (lane 4), whereas no CDKL5 is

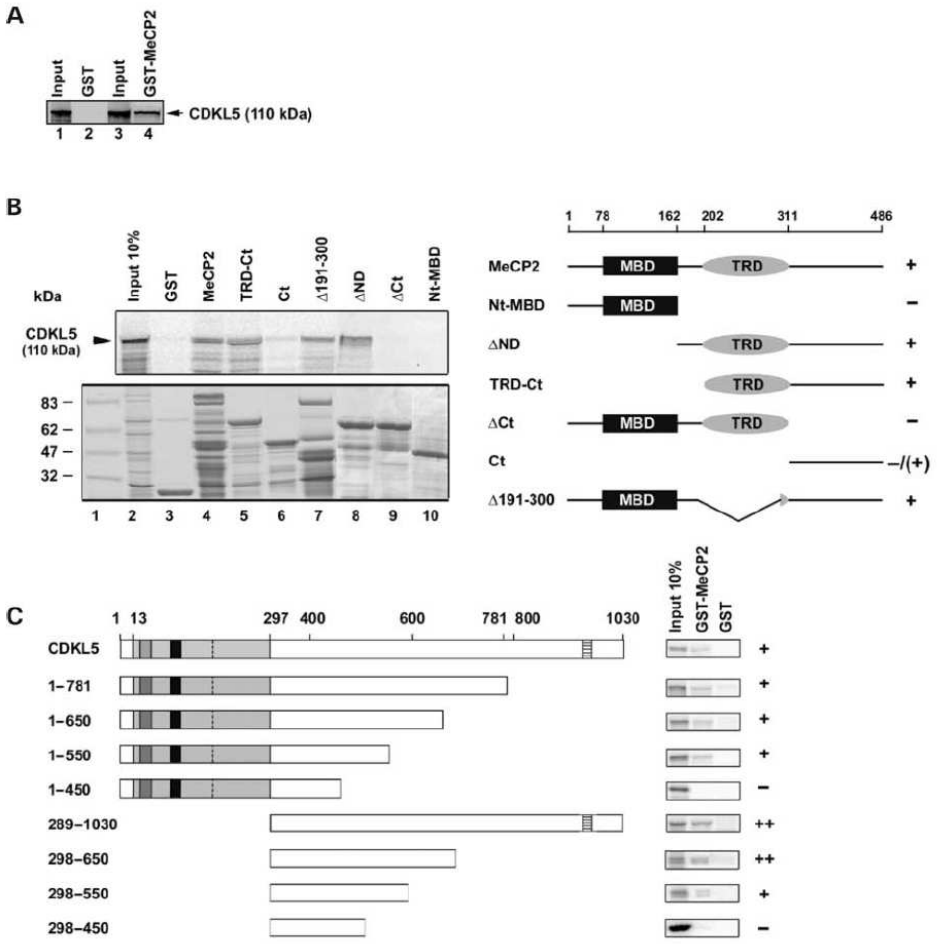

Figure 4. CDKL5 and MeCP2 interact *in vitro*. (**A**) GST pull-down assay in which *in vitro* translated [^{35}S]methionine labeled CDKL5 was incubated with immobilized recombinant GST and GST–MeCP2. Retained proteins were separated on an 8% SDS–PAGE and CDKL5, indicated to the right, were visualized by autoradiography. 'Input' (lanes 1 and 3) corresponds to 10% of *in vitro* translated CDKL5 used in the binding reactions (lanes 2 and 4). (**B**) *In vitro* translated full-length ^{35}S-labeled hCDKL5 was incubated with immobilized recombinant GST and the GST–MeCP2 derivatives schematically illustrated on the right. Retained CDKL5 is visualized in the autoradiogram shown in the upper panel, whereas resin coupled GST and GST–MeCP2 derivatives were detected by Coomassie staining (lower panel). The obtained results are schematized on the right indicating with + and − the presence and absence, respectively, of interaction. (**C**) Full-length CDKL5 and different truncated derivatives were translated *in vitro* and incubated with resins containing GST or GST–MeCP2. The panels in the right part illustrate the amount of CDKL5 retained on the resins and the strength of the interaction is indicated with + and −. By Coomassie staining, it was verified that comparable amounts of GST and GST–MeCP2 were present in the pellets (data not shown).

seen on the GST resin (lane 2). Approximately 5% of CDKL5 used in the binding reaction was found to bind GST–MeCP2 as estimated by comparing the retained protein (lane 4) with the 10% loaded in 'Input' (lane 3). To understand which regions of MeCP2 are engaged in the identified interaction, the pull-down assay, described in Figure 4A, was repeated using deletion derivatives of the methyl-binding protein (Fig. 4B). First, MeCP2 was divided into N- (1–162) and C-terminal (163–486) portions. The N-terminal part contains the well-known MBD (13), whereas the linker region, the TRD and the last residues of the protein, including a new structural domain (amino acids 359–430) common with regulatory factors belonging to the forkhead gene family (30), are contained in the C-terminal portion.

The figure indicates that the N-terminal region is unable to associate with CDKL5; accordingly, the C-terminal portion

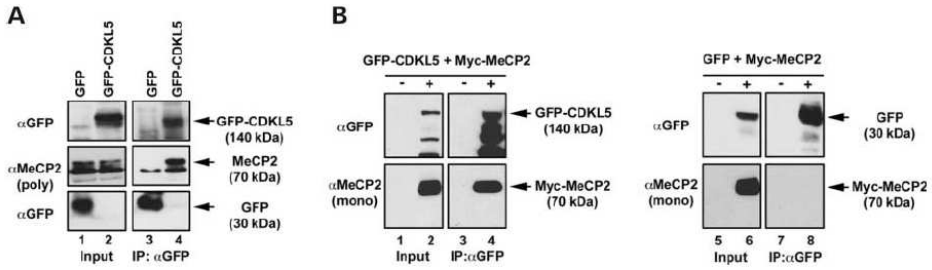

Figure 5. CDKL5 and MeCP2 interact also *in vivo*. (**A**) Coimmunoprecipitation of overexpressed GFP–CDKL5 and endogenous MeCP2. The left panels show a western blot on 10% of the GFP (lane 1) and GFP–CDKL5 (lane 2) human 293T cell extracts used for the immunoprecipitation. To the right, the figure shows GFP (lane 3) and GFP–CDKL5 (lane 4) immunoprecipitated with monoclonal anti-GFP antibody (GFP). Immunoblotting was performed using GFP and anti-MeCP2 polyclonal antibodies (MeCP2). (**B**) Immunoprecipitation performed with the GFP antibody on overexpressed GFP–CDKL5 and Myc-MeCP2 (left panel). As control, the immunoprecipitation was also performed on cells cotransfected with GFP and MeCP2 (right panel). The input corresponding to 10% of the cell extracts is shown in lanes 1, 2 and 5, 6. Immunoblotting was performed using GFP and anti-MeCP2 monoclonal antibodies.

shows an interaction comparable with the full-length protein. The experiment performed with an MeCP2 derivative containing only the TRD and the C-terminal (202–486) demonstrates that this peptide is perfectly able to pull down CDKL5, therefore excluding the linker region as the main interacting surface. Moreover, the ΔCt derivative containing amino acids 1–311 was significantly impaired in its association with the putative kinase, indicating that the TRD is not sufficient for the association. Eventually, as the isolated C-terminal portion (312–486) is unable to interact with the putative kinase, we reasoned that the main interaction surface might include the residues connecting the TRD with the C-terminal portion. Accordingly, an MeCP2 derivative missing most of the TRD domain, excluding the last 11 amino acids, demonstrates its capability to interact with the kinase.

To identify the interaction surface on CDKL5, we *in vitro* translated the deletion mutants schematically illustrated in Figure 4C and used them in classical GST pull-down assays. By progressively deleting the C-terminal of the kinase, we could show that the region containing amino acids 450–550 is required for the interaction with the methyl-binding protein. However, by comparing the autoradiographic signals, we assume that residues included in the 551–650 region reinforce the association. Furthermore, the catalytic domain is not involved in the interaction because a CDKL5 derivative missing the N-terminal 298 amino acids (298–1030) is still able to associate with MeCP2. Actually, this region seems to negatively influence the CDKL5–MeCP2 interaction, because we reproducibly observed a stronger interaction when the kinase domain was missing.

To summarize, we conclude that MeCP2 and CDKL5 are directly interacting *in vitro* and that a portion of MeCP2, containing the last residues of the TRD and the C-terminal region, is the main surface responsible for this association. Regarding the CDKL5 region interacting with MeCP2, so far we have not been able to reveal neither any significant homology to other factors nor the presence of an already known structural motif.

In order to reveal whether the two proteins physically associate also *in vivo*, coimmunoprecipitation experiments were performed (Fig. 5). We transiently transfected human 293T cells with green fluorescent protein (GFP)–CDKL5, or as a control with GFP, and precipitated the overexpressed proteins from the cell extracts with anti-GFP antibodies (Fig. 5A). A subsequent immunoblotting with polyclonal anti-MeCP2 antibodies revealed that endogenous MeCP2 coprecipitates with overexpressed GFP–CDKL5 (lane 4) but not when GFP alone is overexpressed (lane 3). The capability of the two proteins to interact *in vivo* has also been confirmed by coimmunoprecipitation experiments performed on transfected cells overexpressing both GFP–CDKL5 and Myc-MeCP2 (Fig. 5B). The absence of the MeCP2 signal in untransfected cells (lanes 1, 3 and 5) is due to the fact that in this case, we chose to follow the methyl-binding protein with a commercial monoclonal antibody that increased the specificity of the signal, although reducing its sensibility; it is worthwhile to note that 293T cells have been chosen due to their high efficiency of transfection, even though they are low in abundance of MeCP2.

In conclusion, we have been able to demonstrate that CDKL5 and MeCP2 interact also *in vivo*, a result that is coherent with the fact that transfected GFP–CDKL5 is localized, as MeCP2, in the nucleus (data not shown).

CDKL5 is a kinase mediating MeCP2 phosphorylation

Given the direct interaction between MeCP2 and CDKL5 and the fact that the two proteins are coexpressed in different brain regions, it was important to understand the functional role of this interaction. CDKL5 is a putative kinase due to the presence of a conserved kinase domain within the protein, but its catalytic activity has never been proven. To reveal a catalytic activity, we exploited the fact that many kinases are able to autophosphorylate and we incubated *in vitro* translated ^{35}S-labeled CDKL5 in the presence of [γ-^{32}P]ATP. In order to discriminate between ^{35}S- and ^{32}P-signals, the dried SDS–PAGE was exposed to a series of X-ray films of which the one closest to the gel receives the ^{35}S- and ^{32}P-signals, whereas the one farthest away picks up only the signal from ^{32}P. As seen in Figure 6A, the signal derived from incorporated [^{35}S]methionine does not reach the most distant X-ray-film

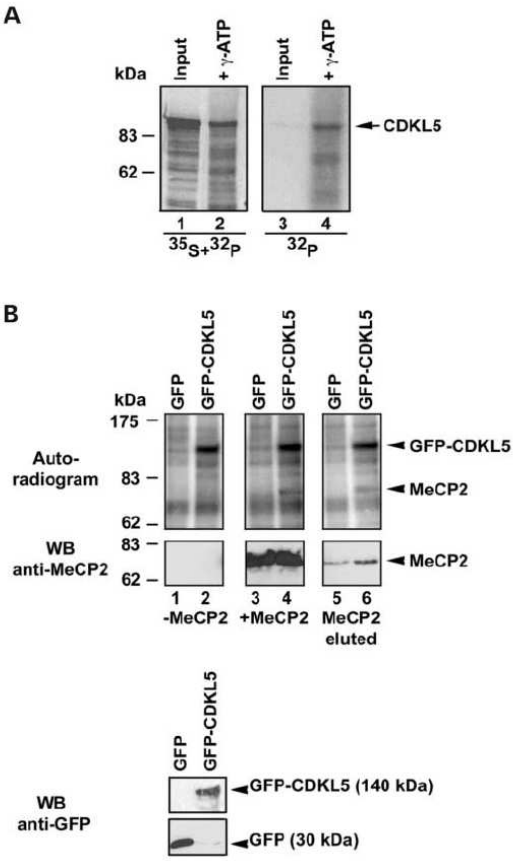

Figure 6. CDKL5 possesses a kinase activity and mediates MeCP2 phosphorylation. (**A**) Autophosphorylation assay in which CDKL5 translated *in vitro*, in the presence of [^{35}S]methionine, was incubated with [γ-^{32}P]ATP for 30 min at 30°C. Proteins were fractionated on an 8% SDS–PAGE, the gel dried and exposed to a stack of three X-ray films, so that radioactivity with different β-emission potentials would impress different layers of the films stack. The left panel shows the X-ray film in the stack in contact with the dried gel picking up radioactivity from both ^{35}S and ^{32}P, whereas the right panel shows the autoradiogram of the third X-ray film (further from the dried gel), which picks up only the β-emission from ^{32}P. 'Input' shows *in vitro* translated ^{35}S-labeled CDKL5 (lanes 1 and 3) whereas '+ γ-ATP' shows CDKL5 incubated with ^{32}P (lanes 2 and 4). The ^{32}P band in lane 4 indicates that CDKL5 can autophosphorylate (indicated with an arrow). (**B**) *In vitro* kinase assay in which GFP–CDKL5 or GFP was purified from overexpressing cells and incubated with immobilized Myc-MeCP2 (lanes 3 and 4), immunopurified FLAG-MeCP2 (lanes 5 and 6) or without exogenously added MeCP2 (lanes 1 and 2) in the presence of [γ-^{32}P]ATP. After 30 min of incubation, the reaction was loaded on SDS–PAGE and the phosphorylated proteins were revealed by autoradiography (autoradiogram panels). Bands corresponding to phosphorylated GFP–CDKL5 and MeCP2 are indicated to the right. The amount of exogenously added MeCP2 present in each lane was evaluated by western blot using a monoclonal antibody anti-MeCP2 (WB anti-MeCP2 panels). The WB anti-GFP panels represent an aliquot of the immunocomplexes identical in amount to those used for the kinase assay (for details see Materials and Methods).

(lane 3), whereas the addition of [γ-^{32}P]ATP leads to a signal corresponding to CDKL5, which is able to penetrate the stack of X-ray films (lane 4), demonstrating that indeed CDKL5 has kinase activity directed against itself.

We next wanted to analyze whether the interaction of MeCP2 with CDKL5 results in the phosphorylation of the methyl-binding protein. To this end, we overexpressed GFP–CDKL5 in mammalian cells, immobilized it on a resin and incubated it with immunopurified-MeCP2 in the presence of [γ-^{32}P]ATP. Labeled proteins were separated by SDS–PAGE and visualized by autoradiography. As can be seen in Figure 6B, the incubation of immobilized Myc-MeCP2 with the resin containing the kinase results in its phosphorylation (lane 4). The control performed with purified GFP alone did not cause any phosphorylation of MeCP2 (lane 3). To confirm the result, an identical assay was performed using an immunopurified FLAG-MeCP2. Once again, only in the presence of CDKL5, the methyl-binding protein gets modified (lanes 5 and 6). Moreover, by comparing the amounts of MeCP2 present in each lane (see the western blot in the lower panels), it appears that phosphorylation of the eluted MeCP2 is significantly more efficient (compare lanes 4 and 6). A parallel experiment was performed without the addition of MeCP2 (lanes 1 and 2) to show the specificity of the reaction. The autophosphorylation of GFP–CDKL5 is also confirmed in this experiment, as visualized in lanes 2, 4 and 6. Eventually, to further analyze the specificity of the reaction, we transfected and purified from mammalian cells an unrelated kinase, the CRIK kinase (31), and assayed its capability to mediate MeCP2 phosphorylation. The obtained results indicated that in our experimental conditions, this kinase maintains a strong autophosphorylation activity but is unable to cause MeCP2 labeling (data not shown).

DISCUSSION

RTT is one of the leading causes of mental retardation and developmental regression in females. RTT patients, after an apparently normal development during the first months of life, show characteristic clinical features including microcephaly, hand wringing, autism, seizures and loss of speech. Besides the classical form, several RTT variants have also been described. The phenotypic spectrum of RTT varies from the most severe cases, including the congenital form and the early onset seizure variant (Hanefeld variant), to the milder forms, comprising the 'forme fruste', the preserved speech variant (PSV) and the late regression variant (32).

Mutations in the *MECP2*, located in Xq28, have been identified in almost 80% of classical RTT cases (4–6). On the contrary, only 20–40% of patients affected by RTT variants, mostly PSV, bear mutations in *MECP2* (7–9). The existence of RTT patients without *MECP2* mutations suggests that additional genetic factors might determine this disorder. According to these data, mutations in another X-linked gene, *CDKL5* located in Xp22, have been recently identified in patients with a phenotype overlapping with that of RTT (10–12). These cases showed a strikingly similar clinical course: they had seizures in the first months of life and subsequently developed recognizable RTT features. In particular,

the phenotype of the two patients described by Scala *et al.* (10) meets the criteria for the diagnosis of the RTT early-onset seizure variant.

In the present work, we report the identification of two additional patients with mutations in *CDKL5*. These girls came to our attention for the presence of epileptic seizures. Later on, they developed characteristics typical of RTT such as stereotypic hand movements and hand apraxia. These features were more evident in the older patient (patient 1), in whom a clinical diagnosis of early-onset seizure variant of RTT was promptly suspected. In the younger patient (patient 2), the phenotype was less characteristic and clinical features were intermediate between the early-onset seizure variant of RTT and autism. Considering the phenotype of these two girls, we expected to find *CDKL5* mutations and we indeed identified two different '*de novo*' frame-shift mutations. The identification of these two novel mutations reinforces the link between the *CDKL5* gene and the pathogenesis of RTT and suggests that *CDKL5* mutation screening should be performed in patients with the early-onset seizure variant.

CDKL5 is a hitherto rather uncharacterized protein originally identified as a serine/threonine kinase gene from sequence similarity searches. Sequence comparisons have indicated that CDKL5 shares homology with members of the MAP kinase family and with cyclin-dependent protein kinases (28). However, its kinase activity has never been demonstrated. *CDKL5* mutations reported so far vary from substitutions in the putative N-terminal catalytic domain to frame-shift mutations in the N- or in the C-terminal portion of the protein (10–12). Whereas it is easy to hypothesize that mutations hitting the kinase domain might influence the catalytic activity of the protein, late truncating mutations could have several effects, such as influencing its stability, cellular localization, protein/protein interactions and/or response to upstream signaling events. Future work will reveal the molecular effects of the identified mutations; however, it is important to note that transfected CDKL5 seems quite unstable, perhaps indicating that its metabolism is specifically regulated.

Given that *MECP2* and *CDKL5* mutations cause a similar phenotype, it was challenging to investigate whether they belong to the same molecular pathway. We compared the expression patterns of Mecp2 and Cdkl5 in embryonic and postnatal mouse brains and we demonstrated that the expression of both proteins increases as neuronal maturation progresses after neurons have reached their final positions inside the cortical plate. Importantly, the two genes generally show a spatial and temporal overlapping expression that is simultaneously activated according to the morphogenetic program specific to each neural district. The obtained results are in favor of a possible involvement of the two proteins in the same developmental pathway. Regarding the cerebellum, Mecp2 and Cdkl5 have common areas of expression, although with different expression levels. This may indicate that in some circumstances, the two genes are regulated independently and/or have specific transduction machineries.

We then investigated whether the two proteins interact at the transcriptional level in human lymphoblastoid cell lines, and we excluded this possibility by performing expression studies by real-time qPCR. These results are in accordance with a previous publication showing that in mouse brain, the absence of MeCP2 does not modify Cdkl5 expression (12).

We then decided to analyze whether the two proteins directly interact. By classical pull-down assays, we have been able to demonstrate that MeCP2 associates with CDKL5 *in vitro*. The same result has been confirmed *in vivo* by means of coimmunoprecipitation experiments. Importantly, a region of MeCP2 including the last residues of the TRD and residues belonging to the C-terminal domain represents the main interacting surface. It is worthwile to note that an analysis of the MeCP2 mutation database (http://mecp2.chw.edu.au/) reveals that residues 301, 302, 305 and 306, belonging to the most C-terminal part of the TRD, are frequently mutated in RTT. Furthermore, the C-terminal contains a hot-spot for Rett mutations and some of them occur in the amino acids close to the TRD, as the 311, 314, 318, 322, 328 and 330. In the future, it will be also important to analyze if any of the RTT mutations affecting this protein domain has lost its capability to interact with CDKL5.

Because CDKL5 is considered a kinase on the basis of sequence homologies, we proceeded analyzing its catalytic activity. To this aim, we performed an autophosphorylation assay and we showed that CDKL5 is able to phosphorylate itself. This result appeared to be of significant relevance, because it has recently been suggested that MeCP2 is not only involved in long-term gene silencing, but also in the regulation of dynamic promoters, modulated by extracellular signals (25–27). In particular, in mammals, phosphorylation of MeCP2 is required for the selective release of the methyl-binding protein from the *Bdnf* promoter, and for its subsequent transcriptional activation (26,27). These findings highlight the importance of MeCP2 phosphorylation in regulating its activity and indicate the relevance of the disclosure of signaling pathways converging on MeCP2. For this reason, we decided to test whether CDKL5 may exert a kinase activity on MeCP2. Our results have shown indeed that an immunopurified CDKL5 mediates the phosphorylation of an exogenously added MeCP2, unraveling a possible function of the interaction. However, further studies are necessary to firmly establish whether MeCP2 is the main target of CDKL5 *in vivo*, and whether the biological significance of the interaction between the two proteins is limited to phosphorylation.

To conclude, we have demonstrated that CDKL5 is the first known kinase capable of mediating MeCP2 modification *in vitro*: its expression pattern, together with its kinase activity, offers a molecular explanation to its involvement in RTT. However, as it is always the case, the same results pave the ways to more studies. In fact, in the future, it will be interesting to understand which RTT mutations in MeCP2 abolish the capability of the methyl-binding protein to interact with its kinase and which one modifies residues that are specifically targeted by the enzyme. Moreover, it will be important to characterize the molecular effects of the *CDKL5* mutations associated with RTT as well as the signaling pathways converging on this enzyme.

From a clinical point of view, the features of the two girls reported in this paper and those described in our previous work stress the idea that *CDKL5* mutations are responsible for a specific phenotype, largely overlapping the phenotype previously described as early-onset seizure variant of RTT (10).

Usually in the classic RTT phenotype, seizures and epileptic signs appear in the 80% of cases and only in the third pseudo-stationary stage which starts at 3–10 years of age; on the contrary, in this variant, these symptoms appear early and blur the characteristic onset symptomatology of RTT. Therefore, it will be interesting to clarify why mutations in *CDKL5* generate a phenotype in which seizures develop earlier than in patients with *MECP2* mutations.

It is important to note that other authors have described slightly different phenotypes associated with *CDKL5* point mutations (11,12,33,34). Because these patients are seen by different clinicians, part of this variability may be due to different clinical sensitivity. Moreover, *CDKL5* may cause both the early-onset seizure variant of RTT and a less defined phenotype ranging from autism and mental retardation.

In conclusion, our results contribute to the clarification of the phenotype associated with *CDKL5* and trace out a molecular link between MeCP2 and CDKL5. In addition, this paper indicates that the *CDKL5* gene should be tested in each patient showing a clinical course similar to RTT but lacking of an early normal period due to the presence of seizures.

MATERIALS AND METHODS

Patients

We investigated two patients aged 2 and 7 with early development of convulsions, who later developed many characteristics of RTT.

Molecular analysis of the identified patients

Blood samples were obtained after informed consent. DNA was extracted from peripheral blood using a QIAamp DNA Blood Kit (Qiagen). DNA samples were screened for mutations in the four exons coding for *MECP2* using Transgenomic WAVE denaturing high performance liquid chromatography (DHPLC). The analysis of the *MECP2* gene for deletions/duplications was performed as previously described (35). DNA samples were screened for mutations in *CDKL5* by DHPLC. The *CDKL5* coding portion was entirely analyzed using primers and conditions as previously indicated (10). PCR products resulting in abnormal DHPLC profiles were sequenced on both strands using PCR primers with fluorescent dye terminators on an ABI PRISM 310 genetic analyzer (PE Applied Biosystems, Foster City, CA, USA).

Analysis of *CDKL5* and *MECP2* mRNA levels

RNA was isolated from EBV-transformed lymphoblasts of probands and control individuals following the TRIZOL procedure (Life Technologies). cDNA was synthesized in a 100 μl reaction containing total RNA (1–2 μg), specific primers (1 μM each), dNTP (500 μM), RNase inhibitor (0.4 U/μl), 1× TaqMan RT buffer, magnesium chloride (5.5 mM), Random Examer (2.5 μM) and Multi Scribe Reverse Transcriptase kit (1.25 U/μl) (Applied Biosystems). The reaction was incubated at 25°C for 10 min, 48°C for 30 min and finally at 95°C for 5 min.

Real-time qPCR assays were performed with the fluorescent TaqMan method and an ABI Prism 7700 Sequence Detection System. Primers and probes for *CDKL5* gene were designed using the Primer Express software (Applied Biosystems), following the criteria indicated in the program:

EX9-CDKL5-F: CTGAGCAGATGAAGCTTTTCTACAGT
EX10-CDKL5-R: TGAGGATGGTTAACAGCTGGAA
PROBE: 6-FAM-TCCTCGCTTCCATGGGCTCCG-TAMRA

The CDKL5 probe contained a fluorophore 5′-FAM as reporter and a 3′-TAMRA as quencer. The GAPDH kit, used as an internal reference, was provided by Applied Biosystems. The GAPDH probe contained a fluorophore 5′-VIC as reporter. We performed separate and multiplex preruns varying the concentrations of primers and probe in order to obtain the highest intensity and specificity of reporter fluorescent signal. PCR was carried out using an ABI prism 7000 (Applied Biosystems) in a 96-well optical plate with a final reaction volume of 50 μl. All reactions were prepared from a single PCR Master Mix consisting of: 2× TaqMan Universal PCR Master Mix, 300 nM CDKL5 forward primer, 300 nM CDKL5 reverse primer, 200 nM CDKL5 probe, 20× GAPDH and HPLC pure water. A total of 100 ng of RNA was dispensed in each of the four sample wells for quadruplicate reactions. Thermal cycling conditions included a prerun of 2 min at 50°C and 10 min at 95°C. Cycle conditions were 45 cycles at 95°C for 15 s and 60°C for 1 min according to the TaqMan Universal PCR Protocol (ABI).

CDKL5 gene is present in two different isoforms: CDKL5 isoform II which is transcribed at a very low level in human fetal brain and testis but not in lymphoblastoid cells and CDKL5 isoform I which is expressed in a wide range of cells including lymphoblastoid cells. We specifically tested the isoform I in lymphoblastoid cell lines of patients.

In order to analyze *MECP2* expression, a commercial assay was purchased from Applied Biosystems (assay code Hs 00172845_m1, the supplied probe and primers were designed across exons 2 and 3). All reactions were prepared from a single PCR Master Mix consisting of: 2× TaqMan Universal PCR Master Mix, 2× MECP2 kit, 2× GAPDH and HPLC pure water. A total of 100 ng of RNA was dispensed in each of the four sample wells for quadruplicate reactions. Thermal cycling conditions included a prerun of 2 min at 50°C and 10 min at 95°C. Cycle conditions were 40 cycles at 95°C for 15 s and 60°C for 1 min according to the TaqMan Universal PCR Protocol (ABI). The TaqMan Universal PCR Master Mix and Microamp reaction tubes were supplied by Applied Biosystems.

MeCP2 is present in two different isoforms: the transcript MeCP2A (or MeCP2β) comprising four exons, with translation start site in exon 2 and the transcript MeCP2B (or MeCP2α) lacking exon 2, with translation start site in exon 1. We have tested only the isoform MeCP2A (or MECP2β) in lymphoblastoid cell lines of patients, because we used a probe and primers designed across exons 2 and 3.

For these assays, we selected three classic RTT patients with early truncating *MECP2* mutations (two with p.R255X and one with p.R270X) and three *CDKL5*-mutated patients, two previously reported by Scala *et al.* (10) (one with p.R55fsX74 and the other with p.E879fsX908), and one,

described here (with p.S781fsX783) (6). Five known control samples were tested in each assay. All samples were run in quadruplicate. A comparative Ct method, as previously described by Livak (36)(ABI Prism 7700 Sequence Detection System, PE Applied Biosystems) was used to calculate the expression levels of the two genes. Using this calculation, a ddCt ratio of about 1 was arbitrarily assigned to one of the control samples. The non-parametric test of Mann–Whitney with a significance level of 95% was used for the comparison between control and mutated samples.

Plasmid construction

The cDNA encoding hCDKL5 was obtained from RZPD, Germany (IRATp970G1233D). The entire cDNA was PCR amplified and cloned into pSP65 (Promega) in frame with a C-terminal Myc-tag. The presence of an SP6 promoter in this vector allowed the coupled *in vitro* transcription/translation reactions. pGFP–CDKL5 was cloned by inserting the entire CDKL5 cDNA into *Bgl* II and *Eco*RI sites in pEGFP-C1 (Clontech). pGST–hMeCP2 and pGST-Nt-MBD, containing the cDNAs encoding the entire coding sequence (486 amino acids) or the N-terminal 162 amino acids of the hMeCP2A, respectively, were cloned by insertion of PCR amplified cDNAs into the *Bam*HI site of pGEX-4T-1. pGST-Ct was obtained by PCR cloning the whole C-terminal domain (residues 311–486) into the *Bam*HI site of pGEX-4T-1; pGST-TRD-Ct was produced inserting a PCR fragment coding for the human residues 201–486 into the *Eco* RI/*Sal* I sites of pGEX-4T1. pGST-ΔND and pGST-ΔC1 have been described elsewhere (37). pSG5-FLAG-MeCP2 was cloned by inserting a *Bam*HI-digested PCR fragment, amplified from the human cDNA, in frame with an N-terminal FLAG tag in pSG5 (Stratagene). All constructs based on PCR were verified by PCR. The pCDNA3 vectors encoding for the CRIK kinase and the human Myc-MeCP2 were a kind gift of Dr Ferdinando Di Cunto and Dr Berge Minassian, respectively. The GST–MeCP2 derivative missing amino acids 199–300 (pGST–MeCP2Δ199–300) was generously given by Dr Ian Marc Bonapace.

GST pull-down assays

To map the interacting domain on the methyl binding protein, GST and GST–MeCP2, or its derivatives, were purified from DH5α using Glutathione–Sepharose 4B (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Immobilized GST proteins (~2 μM) were incubated with 10 μl of *in vitro* translated ^{35}S-labeled CDKL5 for 2 h at 4°C in PBS; 1 mM PMSF. Five washes were performed with PBS; 0.1% Triton X-100; 0.1% NP-40 and retained proteins resolved by SDS–PAGE and detected by autoradiography of the dried gel. GST fusion proteins were visualized afterwards by staining the rehydrated gel with Coomassie blue. *In vitro* translated CDKL5 was obtained using the TNT SP6 Coupled Reticulocyte Lysate System (Promega) with hCDKL5 as template.

The surface of CDKL5 involved in the association with MeCP2 was investigated incubating immobilized GST and GST–MeCP2 with 15–20 μl of *in vitro* translated ^{35}S-labeled CDKL5 derivatives, produced using the TNT T7 Quick for PCR DNA (Promega) using hCDKL5 as template. Incubation conditions, washes and analysis of the interacting proteins and the GST fusion proteins utilized were as described earlier.

In situ hybridization

In situ hybridizations on frozen sections were performed as previously described (38), with the following modifications. Slides were fixed for 30 min at room temperature in 4% paraformaldehyde in PBS and treated for 5 min with 1 μg/ml Proteinase K in 1 mM EDTA and 20 mM Tris–HCl (pH 7.0). Before hybridization, the slides were washed twice in 2× SSC for 15 min, and incubated in 0.1 M Tris, 0.1 M glycine for at least 30 min. Hybridization solution (60 μl/slide) contained 50% formamide, 5× SSC (pH adjusted with citric acid to 6.0), 5% dextran sulfate, 2 mg/ml heparin, 100 μg/ml tRNA and a 1:100 or 1:50 dilution of the riboprobes. Hybridization occurred overnight at 65°C under coverslips. Following hybridization, slides were washed for 1–2 h in 0.5× SSC, 20% formamide at 65°C. Sections were treated with 10 μg/ml RNase A for 30 min at 37°C in NTE, then washed for 4 h in 0.5× SSC, 20% formamide at 65°C and for 30 min in 2× SSC and blocked for 1 h at room temperature in 1% blocking reagent (ROCHE) in MABT. A 1:5000 dilution of anti-digoxigenin-AP conjugate (ROCHE) was preincubated for at least 1 h in 1% blocking reagent in MABT at 4°C. Slides were incubated with the antibody overnight at 4°C, washed for 6 h in TBST, for 30 min in NTMT and stained using centrifuged BM purple AP substrate (ROCHE) in 0.3% Tween-20 for 12–36 h at 4°C and/or room temperature. Slides were washed in NTMT, then in distilled water and embedded in Aqua PolyMount (Polysciences, USA). Cdkl5 and Mecp2 probes were obtained by *in vitro* transcribing the two full-length murine cDNAs containing untranslated regions.

Coimmunoprecipitation experiments

For coimmunoprecipitation, HEK 293T cells were plated on 150 mm Petri dishes (Corning) and transiently transfected with pGFP–CDKL5 or pEGFP-C1 with calcium phosphate method. At 36 h after transfection, total cell extracts were prepared with lysis buffer (Tris–HCl 50 mM pH 8.0, NaCl 150 mM, 1% NP-40, 1 mM dithiothreitol, PMSF and a mix of protease inhibitors from SIGMA). Equal amounts of protein were incubated for 1 h with 10 μl of anti-GFP monoclonal antibody (Roche); 50 μl of Protein G-agarose beads (Amersham) were then added and the immunoprecipite was further incubated for 4 h at 4°C. Immunocomplexes were collected by centrifugation, washed five times with lysis buffer, separated on a 8% SDS–PAGE and blotted to nitrocellulose membrane (Amersham). Filters were blocked in PBS-0.2% Tween plus 5% dried milk and incubated with anti-hMeCP2 rabbit polyclonal antibody or anti-GFP monoclonal antibody. The MeCP2 antibody was a rabbit polyclonal derived from bacterially expressed full-length human cDNA encoding MeCP2 (Fabrizio Bolognese, unpublished data).

In vitro phosphorylation assays

CDKL5 autophosphorylation was revealed incubating10 μl of *in vitro* translated CDKL5 in 30 μl of kinase buffer (20 mM HEPES pH7.4, 10 mM $MgCl_2$, 0.5 mM DTT, 200 μM sodium orthovanadate) in the presence of 50 μM ATP; 5 μCi [γ-^{32}P]ATP for 30 min. at 30°C and separated by SDS–PAGE. The dried gel was exposed to a stack of three X-ray films of which the first receives the mixed ^{35}S- and ^{32}P-signals, whereas the last picks up only the ^{32}P signal.

To detect MeCP2 phosphorylation, total cell extracts were prepared and immunoprecipiated with anti-GFP monoclonal antibody as previously described. The immunocomplexes were divided into four aliquots. One was used to control the quality of the precipitation by western blot using the anti-GFP monoclonal antibody. The remaining aliquots were used for the phosphorylation assay. Immobilized Myc-MeCP2 was obtained transfecting pcDNA3-Myc-Mecp2 into HEK 293T cells. After transfection, total cell extracts were prepared with the following lysis buffer: 20 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.5% NP-40, 1 mM DTT, PMSF and a mix of protease inhibitors (SIGMA). Myc-MeCP2 was immobilized using the anti-C-Myc agarose conjugate (SIGMA) following the manual's instructions. Eluted FLAG-MeCP2 was obtained transfecting pSG5-FLAG-MeCP2 into HEK 293T cells; total cell extracts were prepared as described earlier. FLAG-MeCP2 was immunopurified using E2 view Red ANTI-FLAG M2-agarose (SIGMA) and eluted with the specific tripeptide from SIGMA. Immobilized Myc-MeCP2 or the eluted FLAG-MeCP2 was added to the GFP-immunocomplexes equilibrated in kinase buffer. An aliquot corresponding to 10% was used in a western blot assay performed with the monoclonal anti-MeCP2 antibody. About 7 μCi of [γ-^{32}P]ATP and 25 μM of unlabeled ATP were added to the remaining sample and the reaction incubated for 30 min at 30°C. The reaction was stopped by the addition of Laemmli buffer and directly loaded onto an 8% SDS–PAGE; ^{32}P-labeled protein was detected by autoradiography.

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Result 3.2.3

Case report of a 5th case with CDKL5 mutation

Case report of a 5th case with CDKL5 mutatio

We report here the clinical and neurophysiological course of a female infant with epilepsy, autistic features and severe psychomotor delay associated with a *CDKL5* mutation.

The patient, without antecedent risk factors, presented at age 40 days with tonic seizures followed by a series of symmetrical spasms lasting several minutes. In addition, she had brief sporadic tonic-clonic seizures. Neurological examination was normal and head circumference was at the 75° centile. Seizures rapidly increased up to 20 episodes per day. EEGs were obtained using the10-20 international system and included synchronized EMG of deltoids, ECG and breathing. First EEGs were normal. Video-EEGs recorded several tonic seizures followed by a cluster of spasms without expected EEG changes. At age 4 months, the EEG background activity was poorly organized, lacking interictal epileptiform activity. A tonic seizure was recorded with a discharge over the left central region followed by a prolonged slow activity intermixed with spikes over the left frontal and central regions. She continued to suffer from tonic seizures followed by clusters of spasms. Serum and CSF lactate and aminoacids, urine organic acids, liver function tests, and mutational analysis of the *SLC2A1* gene were unrevealing. Pyridoxine, adrenocorticotropic hormone, carbamazepine, vigabatrin, valproate, topiramate, clonazepam, and phenobarbital were ineffective. At age one year, she had generalized hypotonia, poor eye contact, smiled only out of context and did not respond to social interactions. She did not show any purposeful hand use and developed midline stereotypies such as hand wringing and mouthing, and tactile hypersensitivity such as hair brushing aversion. EEG demonstrated slow and epileptiform abnormalities prominent over the central regions and, during sleep, a pseudo-rhythmic pattern consisting of diffuse high voltage bursts of slow waves and spikes alternated with sequences of diffuse theta-delta activity. MRI, that was normal at the onset of seizures, revealed progressive cortical and subcortical atrophy. The association of hand stereotypies and infantile spasms suggested the diagnosis of early onset seizure variant of Rett syndrome. Blood samples were obtained after informed consent. DNA was extracted from circulating leukocytes and screened for mutations in the *CDKL5* gene by DHPLC (Transgenomics, San José, CA). The *CDKL5* coding sequence was analysed using primers and conditions as previously reported [16]. A PCR product with abnormal DHPLC profile was sequenced on both strands using PE Big Dye terminator cycle sequencing kit on an ABI Prism 310 genetic analyser (PE

Applied Byosystems, Foster City, CA), and revealed a *de novo* missense mutation: c.532C>T, p.R178W (Fig.3). At age 17 months, EEG demonstrated an epileptic encephalopathy and tonic seizures involving mainly the upper limbs corresponding to diffuse low voltage fast activity, followed by prolonged clusters of spasms with a time-locked diffuse slow wave EEG counterpart. In addition, massive myoclonic jerks of the upper limbs were observed during wake and sleep, with a diffuse brief discharge of poly-spikes and waves time-locked with the myoclonias. Head circumference dropped between the 50th and the 25th percentile and, despite partial seizure control with levetiracetam, clobazam and valproate, she showed virtually no developmental progress.

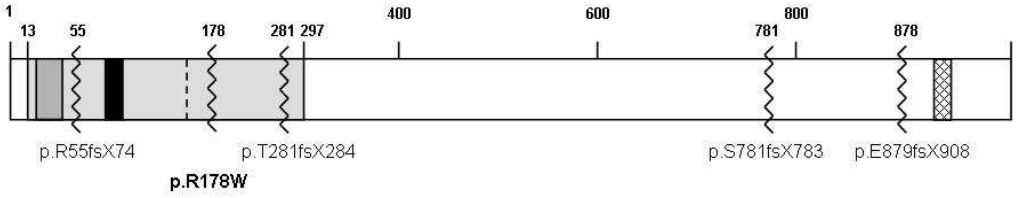

Figure 3. Schematic representation of the CDKL5 protein with mutation position. The catalytic domain (light grey) contains an ATP binding site (dark grey) and the serine-threonine protein kinase active site (bleck box). The conserved Thr-Xaa-Tyr motif is indicated with a sketch line. The signal peptidase I serine active site is represented by the reticulated box. The zigzag lines indicate the position of CDKL5 mutations. In bold type is represented the mutation described in the 5th case report with CDKL5 mutation. The other mutations are that described by Scala et al (p.R55fsX74 and p.E879fsX908) and Mari et al (p.T281fsX284 and p.S781fsX783) [16][17]. The numbers at the top refer to the amino acid position.

Discussion

In 1985 Hanefeld described the clinical picture of a young girl with early-onset infantile spasms and progressive encephalopathy characterized by dementia, stereotyped movements and acquired microcephaly [29]. Infantile spasms were the first symptoms of illness, occurring at the age of one year and were reported by the author as “atypical”. Indeed, she developed an EEG pattern of hypsarrhythmia only one year later. In 2004, mutations in *CDKL5* gene were found in female patients with infantile spasms and severe mental retardation [15]. After this original report, we and other published additional cases with *CDKL5* mutations [18] [16] [17] [19] [20] [21] [23] [22]. To date, 23 cases have been reported.

We report here another young female with *CDKL5* mutation. The causative relationship between the mutation and the phenotype is strengthened by the de novo origin and by the fact that the mutated amino acid lies within the kinase subdomain VIII involved in the substrate recognition [18]. It is conceivable that the change of the positively charged conserved arginine 178 for an uncharged tryptofane reduces substrate binding specificity.

The case reported here extends the description of the epileptic phenotype associated with *CDKL5* mutation. This is the first case of *CDKL5* mutation in which electroclinical features are documented since onset. In contrast with a previous study that classified this form of epilepsy among the early epileptic encephalopathy group [106] despite EEG data were lacking until several years of age, our observation demonstrates that early in the course, repeated EEGs do not display the typical epileptic encephalopathy pattern and lacked the interictal epileptiform abnormalities required for this definition [107]. An epileptic EEG pattern appears only later, along with the autistic features and sterotypies. Reviewing all the EEGs performed from the onset, we noticed a unique periodic pattern with low voltage background activity alternating with bursts of higher amplitude slow waves and no epileptiform abnormalities. Later in the course, while EEG maintains the same periodic pattern, the slow wave bursts appear enriched with spikes and spike waves either bilaterally or more prominently in one hemisphere.

We believe this data may be useful in the clinical practice in order to improve the precocious recognition of the early onset seizure variant of Rett syndrome, an age-specific, genetically-defined, X-linked epileptic syndrome. Based on our case as well as on the review of published cases, these children share the following characteristics: seizures beginning before 3 months, most often as infantile spasms and tonic seizures, intractability to conventional AEDs, severe psychomotor delay with autistic features and stereotypies, and progressive microcephaly. A greater awareness of this syndrome may lead more often to a diagnostic suspicion, which can be validated by the genetic test. Thanks to that, families will receive a correct diagnosis and an accurate recurrence risk assessment. We showed that ictal and interictal EEGs at onset may lack epileptiform activity but show a unique alternating pattern. Further observations will allow to better define this epileptic phenotype and recognize this rare condition as a specific entity.

Result 3.3

MECP2 deletions and genotype-phenotype correlation in Rett syndrome

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MECP2 deletions and genotype-phenotype correlation in Rett syndrome.

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Rett syndrome is a neurodevelopmental disorder that represents one of the most common genetic causes of mental retardation in girls. *MECP2* point mutations in exons 2-4 account for about 80% of classic Rett cases and for a lower percentage of variant patients. We investigated the genetic cause in 77 mutation-negative Rett patients (33 classic, 31 variant and 13 Rett-like cases) by searching missed *MECP2* defects. DHPLC analysis of exon 1 and MLPA analysis allowed us to identify the defect in 17 Rett patients: one exon 1 point mutation (c.47-57del) in a classic case and 16 *MECP2* large deletions (15/33 classic and 1/31 variant cases). One identical intragenic *MECP2* deletion, probably due to gonadal mosaicism, was found in two sisters with discordant phenotype: one classic and one "highly functioning" preserved speech variant. This result indicates that other epigenetic or genetic factors, beside *MECP2*, may contribute to phenotype modulation. Three out of 16 *MECP2* deletions extend to the adjacent centromeric *IRAK1* gene. A putative involvement of the hemizygosity of this gene in the ossification process is discussed. Finally, results reported here clearly indicate that *MECP2* large deletions are a common cause of classic Rett, and MLPA analysis is mandatory in *MECP2*-negative patients, especially in those more severely affected (p=0.044).

INTRODUCTION

Rett syndrome (RTT, OMIM#312750) has a large phenotypic variability [Percy, 2002] [Hagberg et al., 2002]. Beside the classic form, five distinct categories of atypical RTT have been delineated on the bases of clinical criteria: the infantile seizure onset variant, with seizure onset before regression; the congenital variant, lacking the normal perinatal period; the "forme fruste" with a milder and incomplete clinical course; the late regression variant, which is rare and still controversial; and the preserved speech variant (PSV), with some speech recovery [Hagberg et al., 2002; Hagberg, 1993]. In this last variant, a "highly functioning PSV" associated with acquisition of a more complex language function and a better hand use has been described by us [Zappella et al., 2003].

Mutations in exons 2-4 of *MECP2* account for approximately 80% of classic RTT, 50% of PSV and a lower percentage of other variants [De Bona et al., 2000; Miltenberger-Miltenyi et al., 2003; Renieri et al., 2003; Vacca et al., 2001; Yamashita et al., 2001; Zappella et al., 2003; Zappella et al., 2001]. Recently, mutations in another gene, *CDKL5* (OMIM #300203), have been found in RTT patients with the early onset seizures variant and in other atypical RTT cases [Evans et al., 2005a; Mari et al., 2005; Nectoux et al., 2006; Scala et al., 2005].

In order to search for missed *MECP2* defects explaining a proportion of mutation-negative RTT cases, different groups have investigated the presence of *MECP2* deletions, not detectable by PCR-based traditional techniques using either Southern blot, or Real Time quantitative PCR or multiplex ligation-dependent probe amplification (MLPA) [Archer et al., 2006; Ariani et al., 2004; Bourdon et al., 2001; Erlandson et al., 2003; Laccone et al., 2004; Schollen et al., 2003]. Moreover, recent studies have found a small subset of RTT patients with *MECP2* mutations in exon 1, previously considered non coding and therefore excluded from mutation analysis [Amir et al., 2005; Bartholdi et al., 2006; Mnatzakanian GN et al., 2004; Quenard et al., 2006; Saxena et al., 2006].

In this paper we investigated the spectrum and the frequencies of *MECP2* large deletions and exon 1 mutations in a group of 77 mutation-negative RTT patients, including 33 classic, 31 variants and 13 RTT-like cases in order to establish whether it is possible to delineate a genotype-phenotype correlation with

particular attention to deletions also involving *IRAK1*.

MATERIALS AND METHODS

Patients

We recruited 77 RTT patients from the Italian RTT database and biobank that have been screened by DHPLC in exons 2-4 and resulted negative (http://www.biobank.unisi.it) [Sampieri et al., 2006]. All cases contained in the bank have been clinically evaluated by the Medical Genetics Unit of Siena. Patients were classified in classic and variant RTT according to the international criteria [Hagberg et al., 2002]. Patients who show only 2 inclusion criteria among those required for Rett variant [Hagberg et al., 2002] and only 3 or 4 supportive manifestation are defined RTT-like cases. For each classic or variant RTT patient included in the present study a phenotypic score has been calculated using a severity score system modified from previously reported ones [Charman et al., 2005; Huppke et al., 2002; Kerr et al., 2001; Ronnett et al., 2003] (Table 1).

MECP2 quantitative analysis

We searched for *MECP2* large rearrangements by Multiplex Ligation-Dependent Probe Amplification (MLPA) using probe mix P015 (MRC Holland). The analysis was carried out as already described by Schouten [Schouten et al., 2002]. Briefly, 100 ng of genomic DNA was diluted with TE buffer to 5 µl, denatured at 98°C for 5 minutes and hybridized with SALSA Probe-mix at 60°C overnight. Ligase-65 mix was then added and ligation was performed at 54°C for 15 minutes. The ligase was successively inactivated by heat 98°C for 5 minutes. PCR reaction was performed in a 50 µl volume. Primers, dNTP and polymerase were added and amplification was carried out for 35 cycles (30 seconds at 95°C, 30 seconds at 60°C and 60 seconds at 72 °C). The amplification products were analyzed on an ABI model 310, using Genescan ROX 500 standards and Genescan software. Data from Genescan were copied to Excel files (Office for Windows) where the final results were calculated using Coffalyser software (MRC Holland). Dosage alterations were considered significant if sample values deviated more than 30% from the control.

Real Time quantitative PCR was used to confirm MLPA results and to analyze parents' DNA [Ariani et al., 2004].

MECP2 exon 1 analysis

Exon 1 mutations have been investigated by Denaturing High Performance Liquid Chromatography using the Transgenomic WAVE™ (Transgenomic, San Jose, CA, USA). We PCR amplified exon 1 using the following primers: *MECP2*_1F: 5'-GGAGAGAGGGCTGTGGTAAAAG-3' and *MECP2*_1R: 5'-CATCCGCCAGCCGTGTCGTCCG-3'. PCR products were denatured at 95°C, reannealed at 65°C for 10 minutes, and cooled to 4°C to generate heteroduplexes. The optimal column temperature for fragment analysis was calculated using the WaveMaker Software (Transgenomic, San Jose, CA, USA). DHPLC analysis was performed at two melting temperatures: 63.8°C and 68.8°C. Samples with abnormal DHPLC profiles were sequenced on an ABI PRISM 310 genetic analyzer (Applied Biosystems). Exon 1 PCR product of patient #86 was separated on 6% polyacrylamide gel to define exactly the deleted bases. Normal and mutant alleles were cut from the gel and sequenced individually.

Array-CGH

Array-based CGH analysis was performed using commercially available oligonucleotide microarrays containing about 43.000 60-mer probes (Human Genome CGH Microarray 44B Kit, Agilent Technologies, Santa Clara, Ca), as previously reported [Pescucci et al., 2007]. The average resolution is about 75 Kb.

X-inactivation

X-inactivation studies were performed using the assay as modified from Pegoraro et al. [Pegoraro et al., 1994]. Intensity of silver stained bands was measured using the Diversity Database program (Biorad) and the values were corrected for preferential allele amplification [Pegoraro et al., 1994]

Statistical analysis

The non-parametric Wilcoxon Mann-Whitney test with a significance level of 95% was used for comparing total severity score values of samples with and without *MECP2* deletions. The same test has been used to compare severity scores of patients with deletions involving exon 1 and 2 and those involving exon 3 and/or 4. Severity score for specific clinical features of patients with and without *MECP2* deletions were compared by Fisher exact test. Among *MECP2* deleted patients, the same tests ware used for comparing total and specific severity score values of patients with and without *IRAK1* deletion.

RESULTS

Among 77 mutation-negative RTT patients we identified one exon 1 point mutation and sixteen *MECP2* large deletions. In exon 1

we found a de novo deletion of 11 bp (c.47_57del) in one patient (#86) with a classic RTT phenotype (Tab. 1) (Fig. 1). The mutation creates a frameshift and a premature stop codon (p.Gly16GlufsX36).

MECP2 large deletions were found in fourteen sporadic cases with classic RTT and in two RTT sisters (#896 and #897) with discordant phenotype (Tab. 1 and 2). Statistical analysis revealed a significant difference between the total severity score of deleted patients (11.5+/-4) and that of non deleted patients (8.7+/-4.7) (p=0.044). Statistical analysis of severity scores for specific clinical features did not reveal significant differences except for "Speech". Among deleted patients, 1 had score 0 and 15 had score 1 or 2, while among non-deleted patients 19 had score 0 and 28 had score 1 or 2 (p=0.012).

The pedigree of the family with the discordant sisters is represented in Fig.2a. The younger sister is in the IV stage of classic RTT, while the elder sister fulfills the criteria for "highly functioning" PSV (Fig. 2) [Zappella et al., 2003]. MLPA showed that the deletions found in the two sisters involve the same amplicons (3, 4a, 4b and 4c) of *MECP2* (Tab. 2). Molecular investigation of the entire family by Real Time quantitative PCR revealed that the deletion is absent in parents' DNA (Fig. 2b). X-inactivation status was balanced in blood of both sisters (data not shown).

MLPA analysis showed that all *MECP2* large rearrangements identified in our series are partial deletions of the gene. Two deletions encompass exons 1 and 2, one deletion involves only exon 4 and thirteen both exons 3 and 4 (Tab. 2). Comparison of severity scores between patients with deletions involving exon 1 and 2 and those involving exon 3 and/or 4 did not reveal a statistical significant difference (p=0.98). Three deletions involve the adjacent *IRAK1* gene (Tab. 2). Array-CGH revealed that the proximal break-point is located between exon 3 and 3'UTR of IRAK1 in one patient (#951) and between 3'UTR of IRAK1 and CXorf12 in the other two cases (#100 and #1055) (Tab. 2). The distal breakpoint of all three cases is localized in *MECP2* intron 2 (Tab.2). The extension of the deletions in cases #100 and #1055 ranges from 20 to 75 Kb, while in case #951 from 14 to 46 kb.

Statistical analysis revealed that there is not a significant difference in the total severity scores between patients with and without *IRAK1* deletions (p=0.553). Similarly, significant difference is absent using severity score for specific clinical features between patients with and without *IRAK1* deletions.

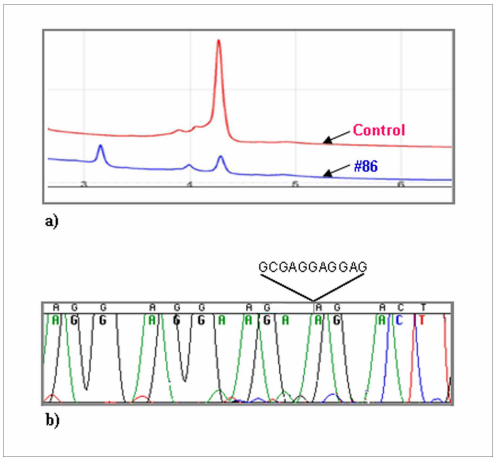

Figure 1. **Exon 1 molecular analysis of patient #86.** a) DHPLC profiles of RTT patient #86 and a control individual at 68.8°C. b) Sequence chromatogram of mutated exon 1 allele in patient #86. The 11 deleted bases are shown above the chromatogram.

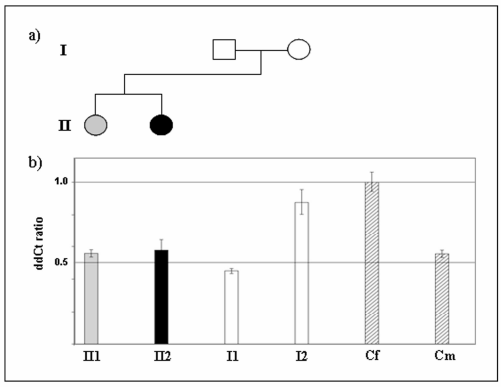

Figure 2. **RTT familial case.** a) Pedigree of the family. The elder sister (gray symbol) started to walk unsupported at the age of 2.5 and to say first words at the age of 2. Now, aged 32, she can walk unaided, she is able to speak in simple often echolalic phrases with good social interaction, she shows a normal head circumference, stereotypic hand movements and an exceptional recover of manual skills. She has never showed epileptic episodes. In the younger sister (black symbol), the ability to use her hands was overwhelmed at the age of 14 months by incessant hand stereotypes and generalized convulsions. Now, aged 26, she is not able to walk unsupported, she cannot speak, she has microcephaly, scoliosis, constipation, cold extremities, episodes of apnea and epilepsy barely controlled by therapy. b) qPCR results. *MECP2* ddCt ratios and standard deviations obtained for the two RTT sisters (II1 and II2), their parents (I1 and I2) and two healthy controls (Cf, female; Cm, male). The normal control female (Cf) and the mother (I1) show ddCt ratios of about 1, indicating a double copy of *MECP2*, while the male control (Cm), the father (I2) and the two sister (II1 and II2) show ddCt ratios of about 0.5, indicating a single copy of the gene.

Tab. 1: Phenotypic categories and severity scores of RTT patients.

Internal code	Phenotype	Age (years)	Head	Weight	Hand stereotypy	Voluntary hand use	Sitting	Walking	Speech	Epilepsy	Scoliosis	Intellectual disability	Total
#100	Classic	16	2	0	1	2	2	2	2	2	2	2	17
#263	Classic	6	2	2	2	2	0	0	2	1	0	2	13
#581	Classic	14	2	2	1	1	0	1	2	2	2[a]	2	15
#819	Classic	14	2	2	1	2	1	2	2	1	2[b]	2	17
#897[c]	Classic	28	2	2	0	1	1	1	2	2	1	2	14
#951	Classic	27	1	0	1	1	0	0	1	0	2[d]	2	8
#1004	Classic	5	1	0	1	1	0	0	2	0	0	2	7
#1055	Classic	8	2	2	2	1	0	2	1	1	1	2	14
#1063	Classic	14	1	0	1	1	0	0	1	2	1	2	9
#1239	Classic	4	2	1	2	2	0	0	1	0	0	2	10
#161	Classic	11	1	0	1	2	0	0	1	2	1	2	10
#166	Classic	17	1	2	2	2	0	2	1	0	2	1	13
#191	Classic	12	1	1	2	1	1	1	1	0	2	2	12
#380	Classic	19	1	2	1	2	0	0	2	1	2	2	13
#727	Classic	7	2	1	2	2	0	1	1	0	0	2	11
#86	Classic	21	2	2	1	1	0	1	1	2	0	2	12
#49	Classic	9	1	1	1	1	0	1	2	1	2	2	12
#91	Classic	13	2	2	1	2	0	2	2	2	2	2	17
#96	Classic	18	2	0	0	2	0	1	1	1	2	2	11
#97	Classic	6	1	1	2	2	1	2	2	0	0	1	12
#249	Classic	20	1	1	1	2	1	2	2	2	2	2	16
#406	Classic	3	0	1	2	1	1	2	2	0	0	1	10
#613	Classic	9	1	2	1	1	0	2	2	2	1	1	13

#744	Classic	13	2	2	2	1	0	2	2	2	2	2	17
#825	Classic	29	1	0	1	1	0	0	1	0	1	2	7
#968	Classic	5	2	1	1	1	0	2	2	1	0	2	12
#1161	Classic	5	2	2	1	1	0	0	1	1	0	2	10
#1377	Classic	2	2	1	1	1	1	0	1	0	0	2	9
#1374	Classic	8	0	0	1	1	2	2	2	2	0	2	12
#1403	Classic	18	1	2	2	2	0	0	2	0	1	2	12
#1311	Classic	17	1	1	2	2	0	1	2	1	1	2	13
#1314	Classic	26	1	0	1	2	1	1	0	1	2	2	11
#1383	Classic	16	1	2	1	1	1	2	2	1	1	2	14
#896[c]	PSV	32	0	0	1	0	0	0	0	0	0	0	1
#127	PSV	25	0	0	1	0	0	0	0	0	0	1	2
#129	PSV	14	1	1	1	1	0	0	1	2	0	1	8
#150	PSV	12	0	0	1	0	0	0	0	0	1	0	2
#152	PSV	7	1	0	1	1	0	0	0	1	0	0	4
#269	PSV	25	0	0	1	0	0	0	0	0	0	1	2
#270	PSV	19	0	0	1	0	0	0	0	0	1	0	2
#288	PSV	5	1	0	1	1	0	0	0	2	0	0	5
#299	PSV	12	0	0	1	1	0	0	0	0	0	0	2
#300	PSV	14	0	1	0	1	0	0	0	0	0	0	2
#313	PSV	20	0	0	1	1	0	0	0	0	1	2	5
#443	PSV	5	0	1	2	1	0	0	0	0	0	1	5
#458	PSV	17	1	0	1	1	0	0	0	1	0	1	5
#471	PSV	23	1	0	1	1	0	0	0	1	0	1	5
#493	PSV	12	1	0	1	1	0	0	0	1	0	2	6

#517	PSV	17	1	1	1	1	0	0	1	0	1	2	8
#523	PSV	19	1	0	1	1	0	0	0	0	0	0	3
#612	PSV	33	2	0	1	1	1	1	1	2	1	2	12
#662	PSV	13	1	0	1	1	0	0	0	0	0	0	2
#682	PSV	7	0	0	1	1	0	0	0	0	0	1	3
#921	PSV	10	0	0	2	1	0	0	0	0	0	0	3
#985	PSV	17	0	0	1	0	0	0	0	2	1	1	5
#207	Early seizure variant	20	2	0	0	2	0	0	2	2	0	2	10
#864	Early seizure variant	5	2	0	2	2	2	2	2	2	0	2	16
#962	Early seizure variant	2	2	1	1	2	0	2	2	1	1	2	14
#965	Early seizure variant	12	1	0	1	1	0	1	2	2	0	1	9
#1043	Early seizure variant	10	2	1	1	1	0	2	1	1	1	2	12
#1057	Early seizure variant	6	2	2	0	0	0	2	2	1	0	2	11
#156	Congenital	10	2	2	2	2	0	2	2	0	1	2	15
#731	Congenital	3	2	0	1	2	0	2	2	2	0	2	13
#107	Forme fruste	15	0	2	2	1	0	0	2	2	1	1	11

Severity score system modified from Kerr et al. 2001, Huppke P. et al. 2002, Ronnet G.V. et al. 2003, Charman T. et al. 2005. Head (postnatal microcephaly:2; deceleration of head growth:1; no deceleration of head growth:0), weight (below 3rd percentile:2; 3rd to 25th percentile:1; above 25th percentile:0), hand stereotypy (dominating or costant:2; mild or intermitten:1; none:0), volontary hand use (none:2; reduced or poor:1; good hand use:0), sitting (never learned to sit:2; loss of ability to sit:1; sitting unsupported at age of 5:0), walking (never learned to walk:2; loss of ability to walk:1; walking unsupported at age of 5:0), speech (never spoken:2; loss of ability to speak:1; more than 10 words at age of 5:0), epilepsy (barely or not controlled by therapy:2; controlled by therapy:1; no epilepsy at age of 5:0), scoliosis (severe:2; mild:1; absent:0), intellectual disability (apparently profound IQ<20:2; apparently severe IQ 20-40:1; IQ>40:0).

a: She underwent surgery for scoliosis at 17 years of age.

b: She underwent surgery for scoliosis at 13 years and 6 months of age.

c: Sisters

d: She underwent surgery for scoliosis at 13 years of age.

Note: in light grey are indicated patients with *MECP2* large deletions, in dark grey the patient with exon 1 mutation.

Tab.2: Results of quantitative analysis in deleted RTT patients.

Patients	Quantitative analysis results														
	L1CAM*	CXorf12**	IRAK1 3'UTR* *	IRAK1 Ex 3*	MECP2										SYBL1*
					Ex4d *	Ex 4c*	Ex4b *	Ex4a*	Ex 3*	Int2* *	Ex2**	Ex 2*	Ex1b *	Ex1a*	
#380	+	/	/	+	+	+	+	+	+	/	/	-	-	-	+
#727	+	/	/	+	+	+	+	+	+	/	/	-	-	-	+
#1004	+	/	/	+	+	+	-	-	-	/	/	+	+	+	+
#166	+	/	/	+	+	+	-	-	-	/	/	+	+	+	+
#161	+	/	/	+	+	+	-	-	-	/	/	+	+	+	+
#581[1]	+	/	/	+	+	-	-	-	-	/	/	+	+	+	+
#1239	+	/	/	+	+	-	-	-	-	/	/	+	+	+	+
#896[2]	+	/	/	+	+	-	-	-	-	/	/	+	+	+	+
#897[2]	+	/	/	+	+	-	-	-	-	/	/	+	+	+	+
#263	+	/	/	+	+	-	-	-	-	/	/	+	+	+	+
#1063	+	/	/	+	+	-	-	-	-	/	/	+	+	+	+
#819	+	/	/	+	-	-	-	-	-	/	/	+	+	+	+
#951	+	+	+	-	-	-	-	-	-	+	+	+	+	+	+
#1055	+	+	-	-	-	-	-	-	-	+	+	+	+	+	+
#100	+	+	-	-	-	-	-	-	-	+	+	+	+	+	+
#191	+	/	/	+	+	-	-	-	+	/	/	+	+	+	+

1: patient described in a previous paper by Ariani et al 2004. 2: Sisters. * : MLPA probes; * *: Array-CGH probes. Note 1: the grey area indicates the deleted region.Note 2: Array-CGH and MLPA probes are represented in the table respecting to their genomic position

DISCUSSION

In this study, we analyzed the presence of *MECP2* exon 1 mutations and gross rearrangements in a cohort of 77 mutation-negative RTT patients, including both classic, variant and Rett-like cases. In this work, respect to previous ones, the study population has a detailed clinical classification and there is homogeneity in data collection since all patients have been evaluated and followed-up by the same clinical centre, allowing a reliable genotype-phenotype correlation (Tab. 1). Using a combined approach including both exon 1 DHPLC analysis and MLPA analysis, we identified the genetic cause in 17 out of 77 (22%) RTT patients, negative to previous screenings. In particular, we found one exon 1 point mutation and sixteen *MECP2* large deletions.

Exon 1 DHPLC analysis identified only one mutation in a patient with classic phenotype, confirming that exon 1 mutation are not a common cause of RTT [Bartholdi et al., 2006; Evans et al., 2005b; Mnatzakanian GN et al., 2004; Quenard et al., 2006; Ravn et al., 2005; Saxena et al., 2006]. The deletion of 11 bp found in this study is a recurrent exon 1 mutation and it occurs in a region with an AAG repeat [Amir et al., 2005; Mnatzakanian GN et al., 2004; Quenard et al., 2006; Ravn et al., 2005; Saxena et al., 2006]. This mutation results in a frame-shift from amino acid in position 15 (in the MeCP2_e1 protein isoform), and the protein stops after 22 incorrect amino acids. Although, reading frame of the MeCP2_e2 isoform remains unaffected, Saxena et al. demonstrated that this mutation has a consequence also in MeCP2_e2 impairing its translation [Saxena et al., 2006]. The same authors detected the exon 1 deletion in a subject with a milder phenotype, suggesting that this mutation could predict a better prognosis. In contrast, we identified the deletion in a patient with a classic phenotype, with a severity score of 12/20 and who died at the age of 22 years (Tab. 1).

MLPA analysis allowed to identify sixteen large deletions in 15 out of 33 classic patients and 1 out of 22 PSV. No large deletions were found in the other atypical patients (6 early onset seizure variant, 2 congenital, 1 forme fruste and 13 Rett-like cases). Among classic cases, we found a slightly higher percentage of large deletions (45.4%) than previously reported (20 to 38%) [Ariani et al., 2004; Bourdon et al., 2001; Erlandson et al., 2003; Huppke et al., 2005; Laccone et al., 2004; Schollen et al., 2003]. These results clearly indicate that quantitative analysis is a fundamental step for the diagnosis of classic RTT cases. The low frequency of *MECP2* deletions in PSV (1/22) patients and the absence of such rearrangements in other variant and Rett-like patients suggest that they are rarely involved in atypical RTT. There is only another study which investigated the presence of *MECP2* large rearrangements in a large group of atypical RTT patients [Archer et al., 2006]. The authors detected large deletions in 4 out of 53 (7.5%) atypical cases, but they did not establish an association with a specific variant phenotype [Archer et al., 2006].

Using a severity score system, we have found a significant difference between our deleted and non-deleted patients ($p=0.044$). Patients with *MECP2* deletion have a higher (more severe) score (average total severity score of 11.5+/-4 versus 8.7+/-4.7). These data indicate that *MECP2* MLPA analysis is mandatory in severely affected cases. When we performed statistical analysis of severity scores for specific clinical features, we obtained a significant difference in the field of "Speech" ($p=0.012$). Almost all our patients (15/16) with *MECP2* deletions have never spoken or have lost the ability to speak. Comparison of "Speech" severity of our deleted patients with previously reported ones, confirms this finding, suggesting that quantitative analysis is fundamental in severely affected RTT cases with absent language [Archer et al., 2006] [Bartholdi et al., 2006].

We found a *MECP2* large deletion in only one patient classified as PSV (total severity score =1). This woman is the elder sister of a RTT patient with a classic phenotype. This familial case is very interesting, since despite the discordant phenotype, MLPA analysis revealed the presence of a *MECP2* large deletion involving exon 3 and partially exon 4 in both sisters. Although breakpoints have not been cloned, the two sisters probably harbor the same rearrangement. XCI assay on blood does not explain the discordant phenotype. The difference in disease severity could be due to more subtle epigenetic differences or to modifier genes. Moreover, quantitative analysis of the entire family revealed that the *MECP2* deletion is absent in parents, suggesting that one of them is a mosaic for the mutation in the gonadic tissue [Mari et al., 2004].

All *MECP2* large rearrangements identified in our study are partial deletions of the gene, frequently involving exons 3 and 4 (13/16) (Tab. 2). Statistical analysis of total severity scores revealed that there is not significant difference between patients with C-

terminal deletions and patients with deletions involving exons 1 and 2 (p=0.98). Intron 2 of *MECP2* is involved in almost all deletions. This region contains a series of repetitive elements, mostly Alu repeats, that may represent recombinogenic factors [Laccone et al., 2004]. In addition, seven deletions have the proximal breakpoint located between MLPA probes 4c and 4d (Tab.2). By UCSC Genome Browser, we identified one repetitive region (g. 20624-20529; Locus: AF030876) that probably induces the rearrangements. The already known *MECP2* deleted prone region (g.22243-22093; Locus: AF030876), located in exon 4, is involved in three rearrangements of our study (#161, #166 and #1004) (Tab. 2) [Laccone et al., 2004]. Among the three cases with deletions involving the adjacent *IRAK1* gene, the distal breakpoint is located between the 3'UTR of *IRAK1* and *CXorf12* in two patients, while in the third case it is located inside *IRAK1,* between exon 3 and the 3'UTR region (Tab. 2).

We wondered whether *IRAK1* deletion may contribute to some specific clinical features. *IRAK1* encodes the interleukin-1 receptor-associated kinase 1, a proinflammatory cytokine that functions in the generation of systemic and local response to infections [Cao et al., 1996]. These properties make *IRAK1* a good candidate for altered infections susceptibility. However, this is difficult to establish in RTT patients because their long term immobilization often leads to pulmonitis ab ingestis. To better evaluate a possible effect on infections susceptibility, specific information about infancy is necessary. We ask parents about the occurrence of infections in their daughters during infancy and we ruled out the possibility of recurrent significant episodes of infections.

A previous study by Archer et al. found a significant increased frequency of additional congenital anomalies in patients with large deletions compared with cases with any other *MECP2* mutation [Archer et al., 2006]. The authors proposed that the extension of the *MECP2* deletions into *IRAK1* and potentially other genes in the immediate vicinity may be associated to the presence of such anomalies [Archer et al., 2006]. However , the other studies did not describe major congenital anomalies in RTT cases with deletions involving *IRAK1*, as in our cohort of patients [Li et al., 2007; Ravn et al., 2005].

Among the three RTT patients of our series with *IRAK1* deletion, two have a slightly discordance between bone and chronological age. One out of three has precocious ossification of the ulnar half of the distal growth cartilage of radius leading to an asymmetric growth which in turn causes a curvature of radius (Madelung deformity). The pathogenic mechanisms of Madelung deformity is thought to reside in a difference of the ossification rate in the two moieties of the distal growth cartilage of radius. Given that IRAK1 regulates NFkB activation and that NFkB is critical for osteoclasts differentiation, deletion of *IRAK1* may contribute to the impairment of ossification in these patients [Cooke et al., 2001; Iotsova et al., 1997; Lee et al., 2003]. A specific haplotype in *IRAK1* has been found associated with radial bone mineral density, reinforcing the involvement of this gene in bone homeostasis [Ishida et al., 2003].

In conclusion, this study confirms that *MECP2* large deletions are a common cause of classic RTT, underlining the importance of quantitative studies in a complete diagnostic strategy. The introduction of quantitative studies allowed us to obtain an overall *MECP2* mutation detection rate of about 90% in patients with classic RTT (www.biobank.unisi.it), suggesting that it is unlikely that other genes are involved. The remaining fraction of mutation-negative classic cases could be due to mutations in regulatory regions of the gene. On the contrary, the low fraction of *MECP2* deletions in RTT variants may suggest genetic heterogeneity.

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3.4 Modifier genes in Rett syndrome regions

MECP2 mutations are found in about 90% of classic RTT patients and in 20-40% of variant cases [11], [12] [13] [14]. A number of studies aimed to establish a genotype/phenotype correlation have yielded discordant results [36] [37] [38] [39] [40]. Whereas some investigators found no significant correlation between mutation type and clinical features, we and others have found a partial correlation [41] [37] [14] [38] [12] [40] [13] [39] [24]. In particular, we have preliminary evidence that missense, early and late truncating mutations are found in classic patients, whereas only missense and late truncating mutations are identified in PSV cases [24]. The discrepancies among the different studies can be due to variation in the degree of accuracy in phenotype definition. Alternatively, the influence of XCI status or modifier genes can contribute in modulating the phenotype. In support of the XCI hypothesis, skewed XCI (presumably favouring inactivation of the mutant allele) has been observed in healthy carrier mothers of RTT patients and partially skewed XCI in less severe RTT phenotypes [13] [39] [42] [37] [43]. However, such a role was not confirmed in other studies showing random XCI in healthy carrier females and highly skewed X inactivation in classic RTT patients [13] [44] [45]. Furthermore, it has been demonstrated that XCI may vary remarkably between tissues [46] [47]. Thus, the extrapolations of results based on sampling peripheral tissues, such as lymphocytes, to other tissues, such as brain, may be misleading. Studies performed on RTT brain tissues suggest that balanced XCI patterns are prevalent [48] [49]. However, XCI has been investigated in a limited number of regions in few RTT brain samples and no definitive conclusions can be drawn. Taken together, these studies leave unresolved the issue of genotype-phenotype correlations and suggest that additional modifier genetic factors can have a role in determining this variability. It is in fact possible that differences in expression/activity levels of genes regulated by *MECP2*, belonging to its signalling pathway or more generally contributing to the regulation of the same processes, might ameliorate/worsen the effects of *MECP2* mutations. Such differences might be due to the effect of polymorphisms inside the genes influencing mRNA stability or protein activity (in the case of functional polymorphisms) or to segmental genomic variations (deletions/duplications) which might result in alterations of gene dosage.

3.4.1 *CDKL5* as modifier gene of RTT

Our group and others have shown that mutations in the *CDKL5* (cyclin-dependent kinase-like 5) gene are found in patients with a phenotype overlapping with that of RTT [15] [18] [16] [17] [102] [20]. This phenotype in most cases is in accordance with that described by Hanefeld as the infantile seizure onset variant of RTT [29]. We also demonstrated that *Mecp2* and *Cdkl5* have an overlapping temporal and spatial expression profile during neuronal maturation and synaptogenesis, that the two proteins interact both in vivo and in vitro and that CDKL5 is a kinase able to phosphorylate itself and to mediate MeCP2 phosphorylation [17]. Given these results, we hypothesized that differences in activity levels of *CDKL5* might contribute to the phenotypic heterogeneity observed in *MECP2* mutated RTT patients. In particular, since *CDKL5* mutations are associated with the infantile seizures onset variant of RTT, it is possible that functional polymorphisms in this gene may contribute to the onset and severity of seizures in RTT girls.

Methods

Patients' materials and phenotype definition

Patients' material has been selected from the cohort of RTT patients available at the Italian Rett database (http://www.biobank.unisi.it) [94]. Among the total of 227 probands, the bank includes 128 classic RTT patients and 63 RTT variants, classified according to the international diagnostic criteria [Hagberg, 2002 #443). Among the remaining 36 patients, 18 have been classified as RTT-like, i.e., cases who do not completely fulfil the international clinical criteria for RTT, and 18 as not determinable (ND), when the very young age of the patient does not allow a definitive clinical classification. The Italian Rett database contains freely available information about the specific *MECP2* or *CDKL5* mutation identified in each patient included in the collection. *MECP2* mutations have been identified in 118 out of 128 classic cases (90%) and in 28 out of 63 variant cases (44%), in 17 out of 18 ND cases, and in 5 out of 18 RTT-like patients.

For grading the severity of epilepsy in *MECP2* mutated patients, we have used the following scoring system modified from previous ones [Kerr, 2001 #523] [39] [108] [109] (Tab.1).

Tab. 1 Scoring for different clinical features

Head	2	postnatal microcephaly
	1	deceleration of head growth
	0	no deceleration of head growth
Weight	2	Below 3rd percentile
	1	3rd to 25th percentile
	0	Above 25th percentile
Hand stereotypy	2	Dominating or constant
	1	Mild or intermittent
	0	None
Voluntary hand use	2	None
	1	Reduced or poor
	0	Good hand use
Sitting	2	never learned to sit
	1	loss of ability to sit
	0	sitting unsupported at age of 5 years
Walking	2	never learned to walk
	1	loss of ability to walk
	0	walking unsupported at age of 5 years
Speech	2	never spoken
	1	loss of ability to speech
	0	more than 10 words at age of 5 years
Epilepsy	2	Barely or not controlled by therapy
	1	Controlled by therapy
	0	No epilepsy at age of 5 years

Scoliosis	2	Severe
	1	Mild
	0	Absent
Intellectual disability (Wechlsler scale)	2	Apparent profound IQ<20
	1	Apparent severe IQ: 20-40
	0	IQ>40

To date, it has been possible to evaluate the severity of epilepsy in 125 *MECP2* mutated RTT patients. Among them 59 have Score=0, 39 have Score=1 and 27 have Score=2.

Genotyping of polymorphism p.Q791P.

We investigate the frequency of p.Q791P (rs35478150) in 125 *MECP2*-mutated patients (http://www.biobank.unisi.it). The polymorphism has been characterized using transgenomic WAVE denaturing high performance liquid chromatography (DHPLC). We established allele frequencies, extending the analysis to 200 normal individuals of Caucasian origin, since all our patients came from this background.

Statistical analysis

The Hardy-Weinberg distribution has been defined in a control population of 200 individuals. Allele/genotype association of the polymorphism has been evaluated using a Fisher exact test between RTT patients and the control population. Statistical significance of correlations between the polymorphism and epilepsy severity score (Tab. 1) has been assessed with Fisher exact test [110].

Results

Genotype frequencies in *MECP2* mutated RTT patients were: 90% Q:Q, 9% Q:P and 1% P:P. These frequencies did not differ from frequencies observed in the control population. Moreover, genotype frequencies were in Hardy-Weinberg equilibrium. The genotypic and allelic distributions of the p.Q791P polymorphism were not significantly different between the different RTT groups in regard of the epilepsy severity score ($p>0.05$). However, we observed that the genotype Q:P was higher (23%) in RTT patients with epilepsy "barely or not controlled by therapy" (Score=2) compared with patients without epilepsy at the age of 5 years (Score=0) (7%) or with patients with epilepsy controlled by therapy (Score=1) (6%) ($p=0.1$) (Tab.2).

Table 2: Epilepsy scores and genotypes of CDKL5 polimorphism (p.Q791P) in *MECP2*-mutated RTT patients.

	Q:Q	*Q:P*	*P:P*	*Total*
0	55	4	0	59
1	36	2	1	39
2	22	5	0	27
Total	113	11	1	**125**

3.4.2 Chromosome 16p duplication

With the aim to understand the genetic differences between the classic RTT syndrome and the milder phenotype described in preserved speech variant (PSV), we searched for differences in segmental genomic variations in two RTT sisters (patients #896 and 897) with the same *MECP2* mutation but discordant phenotype.

. The classic RTT patient (#897) is 26 years old and she is in the IV stage of the disease (Fig. 4b, right). The child had a normal psychomotor development in the first year of life. Her progress then ceased and autistic features became evident. At 14 months of age, generalized convulsions appeared. The ability to use her hands was overwhelmed by incessant hand stereotypes. Presently, she is not able to walk unsupported and she cannot speak. Epilepsy is still present and barely controlled by therapy. She has microcephaly, scoliosis, constipation, cold extremities and episodes of apnea. The elder sister(#896), now aged 32 years, is still in the III stage and fulfils the criteria for "highly functioning" PSV [34] (Fig. 4b, left). She showed a psychomotor delay since the first months of life. She could walk unsupported at the age of 2.5 years and she could say her first words at the age of 2 years. Presently, she can walk unaided even if on a broad basis and she has a good social interaction with a friendly behaviour. She is able to speak in simple phrases and answer appropriately talking about her daily-life activities, even if often in an echolalic manner. She has never showed epileptic episodes. Now she shows a normal head circumference, stereotypic hand movements and an exceptional recover of manual skills, being able to make simple drawing and to write her name and address.

Both sisters present the same *MECP2* deletion involving part of exon 4 (Results 3.3). Molecular investigation of the entire family by Real Time quantitative PCR revealed that the deletion is absent in parents' DNA (Results 3.3). X-inactivation status was balanced in blood of both sisters (Results 3.3).

We performed whole genome array-CGH analysis on both girls in order to investigate whether segmental genomic variations could explain the different phenotype observed in the two sisters.

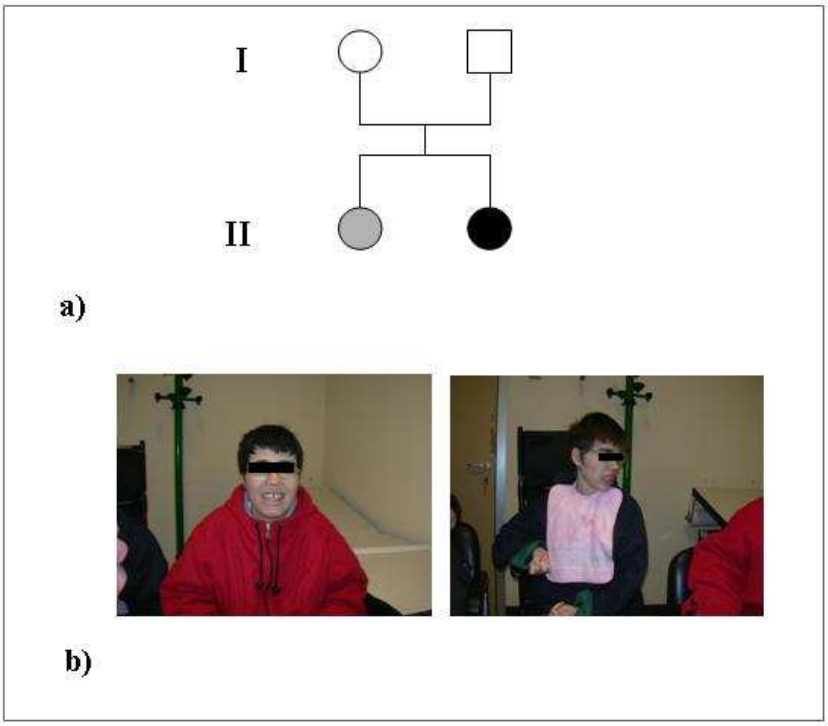

Figure4. RTT familial case. a) Pedigree of the family. White symbols represent asymptomatic subjects, the black symbol stands for classic RTT, while the gray symbol stands for "highly functioning PSV". b) The two RTT sisters. On the left, the elder sister classified as "highly functioning PSV" (#896); on the right, the younger sister with a classic RTT phenotype (#897).

Methods

Oligo array-CGH experiments using Agilent platform with 15 kb resolution (Agilent Human Genome CGH Microarray Kit 105A) have been used [111] [112] (Fig.5). Array-CGH results have been validated by Real Time quantitative PCR (Applied Biosystems) in all members of the family (#896, #897, #898, #899) in order to determine the parental origin of the identified rearrangement (Fig.7). With the aim to verify if the variation is already reported as copy-number polymorphism (CNP), we consulted the BioXRT database, (http://projects.tcag.ca/bioxrt/). Moreover, we determined gene content of the rearranged regions utilizing the published working draft sequence of the human genome (http://genome.ucsc.edu) (Fig.6).

Results

Whole genome array-CGH analysis revealed a duplication of about 370 Kb on chromosome 16p11.2 in the classic RTT patient (#897) inherited from the healthy father (#898) and absent in the mother (#899) and PSV sister (#896) (Fig.5 and 7). Regarding the proximal breakpoint, the last normal oligonucleotide is located in 28,623 Mb and the first duplicated in 28,539 Mb. Concerning the distal breakpoint, it is located between 28,949 and 28,952 Mb (last oligonucleotide duplicated and first normal, respectively). Consulting BioXRT database, we found that the region is subjected to copy number variations (CNV) (http://projects.tcag.ca/bioxrt/). The duplicated segment includes 10 known genes (Fig.6).

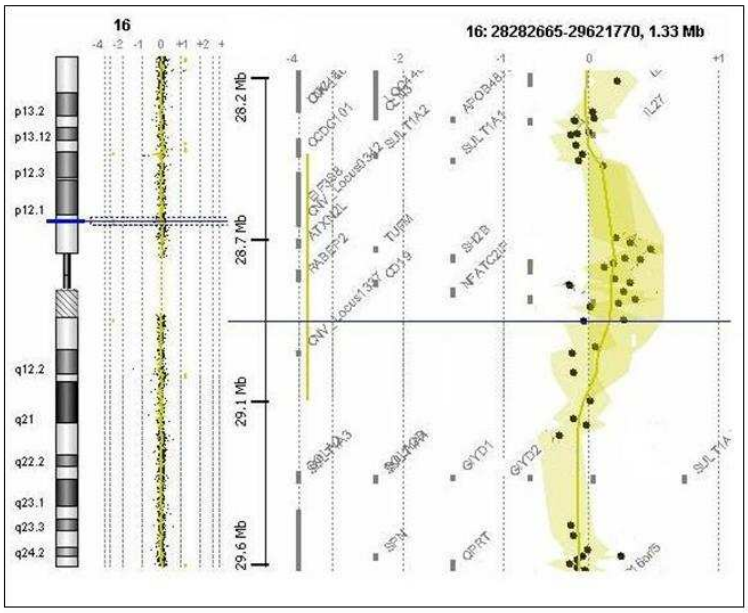

Figure 5. Array-CGH results. Chromosome 16 array-CGH ratio profile using DNA from the patient and reference DNA from a normal female. The log2 ratio of the chromosome 16 probes are plotted as a function of chromosomal position. Oligo with a value of zero represent equal fluorescence intensity ratio between sample and reference. Each dot represent a single probe (oligo) spotted on the array. Copy number gain shift the ratio on the right

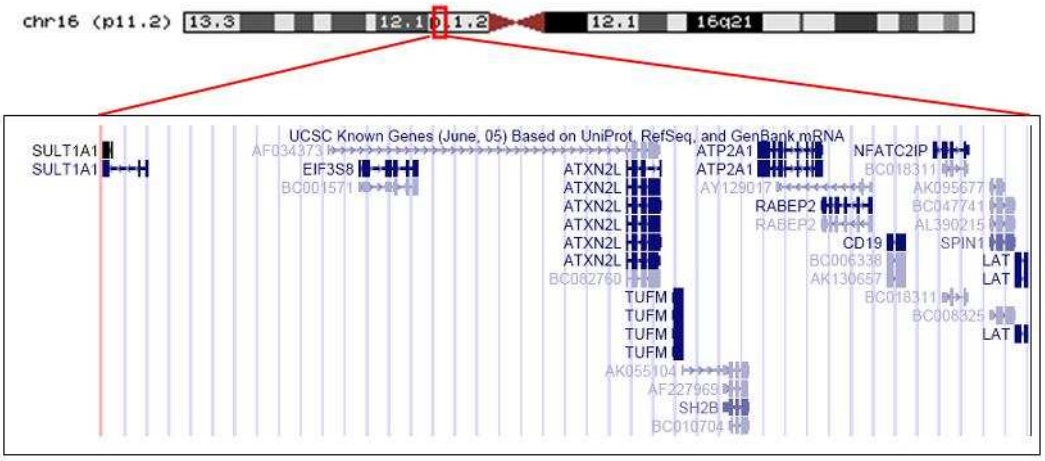

Figure 6. Chromosome 16 ideogram and gene content of the duplicated 16p11.2 region (UCSC Genome Browser;http://genome.ucsc.edu).

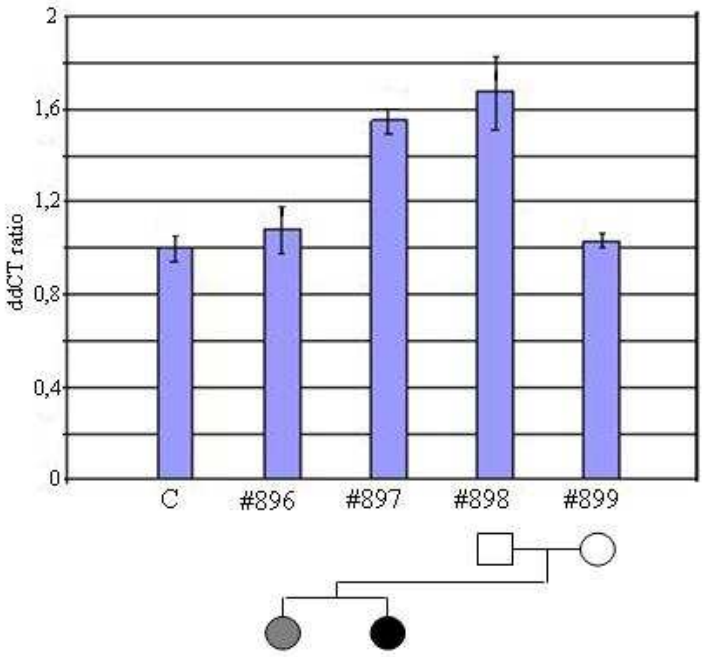

Figure 7. Real-Time quantitative PCR validation experiment. The Real-Time probe covers part of the REBEP2 gene, located in the middle of the rearrangement. RABEP2 ddCT ratio and standard deviation of a control individual (C), PSV patient (#896), her cassic RTT sister (#897), the father (#898) and mother (#899) are indicated. The control individual, PSV patient and her mother show a ddCT ratio of about 1, while the classic RTT girl and her father show ddCT ratio of about 1.5, indicating a double copy of the gene.

3.4.3 Chromosome 15q rearrangements

In 2004, by markers analysis, we found three RTT patients with both a 15q11-q13 rearrangement and a *MECP2* mutation [113]. In particular, we found maternally inherited 15q11-q13 deletions in two classic RTT girls and one paternally inherited duplication in a PSV patient. Neither of these rearrangements was found in 200 healthy controls. All three patients carry the same *MECP2* mutation, p.R133C [113]. The 15q11-q13 region is a very interesting region since maternally duplications on 15q11-q13 have been found in a fraction of autistic patients [114] [115] [116]. In 2005 studies of post-mortem human brain, both RTT and autism samples, exhibited expression defects in two 15q11-13 genes, *UBE3A* and *GABRB3*, suggesting that overlapping pathways are deregulated in these disorders [117]. Homologous association of 15q11-13 *GABRB3* alleles occurs in mature neurons within brain, and it is deficient in RTT and autism. Very recently, it has been demonstrated that 15q11-13 *GABAa* receptor gene are biallelically expressed in control brain samples whereas they are subjected to epigenic dysregulation in Autism and RTT [118]. Given these data, we performed array-CGH analysis in the three RTT patients in order to determine the extension of the rearrangements and to define a minimal common region for the duplication and the deletions.

Methods

We characterized the extension of the 15q11-13 rearrangements found in RTT patients #10, #203 and #307 by array CGH analysis with 15 kb resolution (Agilent Human Genome CGH Microarray Kit 105A) [111] [112] (Fig.8).

Results

The array-CGH analysis showed the presence of a 15q11 complex rearrangement in patients #10. Regarding the proximal breakpoint, the last normal oligonucleotide is located in 21,856 Mb and the first deleted in 21,982 Mb. The first and the last duplicated oligonucleotides are located in 22,077 and 22,346 Mb, respectively. Concerning the distal breakpoint, the first normal oligonucleotide is located in 22,374 Mb (Fig. 8a).

In patient #203, array-CGH results revealed the presence of a deletion of about 188 Kb. The proximal breakpoint is located between 21,856 and 21,982 Mb, while the distal breakpoint between 22,077 and 22,138 Mb (Fig. 8b).

In patient #307, array-CGH analysis showed the presence of a duplication of about 311 Kb. The proximal breakpoint is located between 22,021 and 22,077 Mb, while the distal breakpoint between 22,346 and 22,374 Mb (Fig. 8c).

The published working draft sequence of the human genome (http://genome.ucsc.edu) did not report any known gene in the rearranged regions. The rearranged regions include the CNV-Locus 1327 corresponding to a predicted gene.

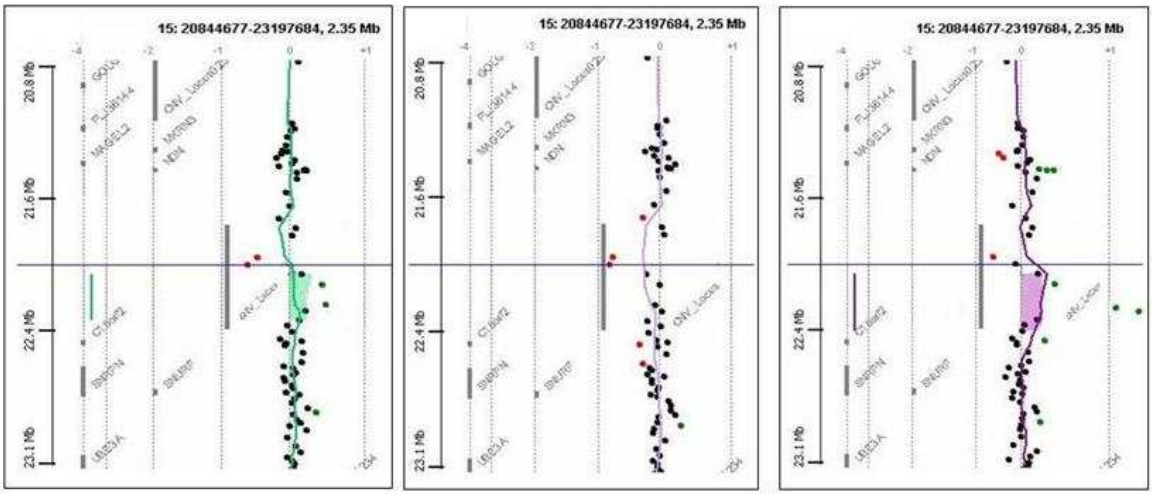

Figure 8. Molecular data of patients. Three different chromosome 15 array-CGH ratio profile. For each experiment have been used DNA from the patient and reference DNA from a normal female. In each panel are visualized the log2 ratio of the chromosome 15 probes plotted as a function of chromosomal position. Oligo with a value of zero represent equal fluorescence intensity ratio between sample and reference. Each dot represent a single probe (oligo) spotted on the array. Copy number gain or loss shift the ratio on the right or on the left respectively. a) RTT patient #10; b) RTT patient #203; c) RTT patient #307.

3.4.4 Discussion

MECP2 mutations are associated with a broad spectrum of clinical phenotypes in RTT girls, including the infantile seizures onset variant and the preserved speech variant [27] [6] [28] [29]. Moreover, even among individuals belonging to the same RTT category (classic or variant), a high level of variability in type and age of onset, severity of impairment and profile of clinical course has been described. To date, two different genetic factors have been implicated in influencing the phenotype in RTT: X-chromosome inactivation (XCI) and the type and position of *MECP2* mutations. However, these factors do not give the full explanation of RTT phenotypic heterogeneity, suggesting the involvement of additional genetic modifiers. This study is the first one that faces the complex issue of the identification of these genetic factors.

We have first studied the role of one candidate modifier gene, *CDKL5*, in phenotype modulation of *MECP2* mutated RTT patients. In particular, since *CDKL5* mutations are associated with the infantile seizure onset variant of RTT, we investigated whether the non-conservative polymorphism p.Q791P may contribute to determine the severity of epilepsy in RTT girls. Although the effect is not significant due to the small size of the population, we observed that the genotype Q:P was higher in RTT patients with untreatable seizures compared with patients without precocious epilepsy or with patients with epilepsy controlled by therapy. Additional patients are needed to confirm our preliminary observations.

Segmental genomic variations (deletions/duplications), resulting in alterations of gene dosage, may also contain candidate modifier genes of RTT. We have started to search such variations by array CGH analysis in two sisters with the same *MECP2* mutation but discordant RTT phenotype (classic and PSV). This analysis revealed a duplication of about 370 Kb on chromosome 16p11.2 in the classic RTT patient inherited from the healthy father and absent in the mother and PSV sister. The duplicated segment includes 10 known genes. Among them, *ATP2A1* (ATPase, Ca(2+)-Transporting, fast-twitch 1) encodes for a Ca(2+)-transporting ATPase found in different membranes that lower cytoplasmic Ca(2+) concentration. Loss of function of *ATP2A1* causes Brody myophaty, characterized by increasing impairment of muscular relaxation during exercise [119] [120]. Another duplicated gene, *ATXN2L* encoding for ataxin type 2 related protein, seems quite interesting. Its function is

presently unknown; however, it is a member of the spinocerebellar ataxia (SCAs) family, which is associated with the SCA group of neurodegenerative disorders. Given these data, it can be hypothesized that potential alterations of the expression of *ATP2A1* and/or *ATXN2L* might contribute to modulate the neuro-muscular RTT phenotype. Another interesting gene as candidate modifier of RTT is *SULT1A1* (Sulfotranferase 1A1). This gene is expressed in many human tissues, including fetal brain and encodes for an enzyme catalyzing the sulfation of neurotransmitters, steroid hormones, acetaminophen, and p-nitrophenol [121] [122]. Numerous studies have demonstrated that *SULT1A1* level of activity is influenced by common genetic polymorphisms (functional SNPs) and gene copy number differences [123] [124] [125] [126]. Hebbring et al. underlined the pharmacological implications of these common variants that may help explain individual differences in drug toxicity and/or efficacy in the clinical setting [126].

In 2004, by marker analysis, we found three RTT patients with both a 15q11-q13 rearrangement and a *MECP2* mutation [113]. In order to verify whether these variations could contain genes that can modulate phenotypic outcome in RTT patients, we decided to characterize the extension of the rearrangements using array CGH. This analysis revealed that the rearranged regions do not contain any known gene. We are now investigating if these regions contains MeCP2 binding sites that can regulate the expression of genes flanking or more distal to the rearrangements. Downstream the rearranged regions there are very interesting candidate modifier genes such as UBE3A and GABR, that are involved in Prader-Willi syndrome, Angelman syndrome and Autism [117].

In conclusion, all these results address future studies aimed to investigate the effects of multiple genetic factors that can determine the complex and fascinating phenotypic heterogeneity of RTT syndrome. These studies, if successful, will have a broad impact not only on the elucidation of pathogenic mechanisms underlying RTT, but also on the design of future therapeutic strategies.

4. Future perspectives

We will continue to investigate the role of candidate modifier genes in RTT. To this aim, we are collecting detailed clinical data in order to define for each patient of the biobank all the phenotypic scores indicated in Tab 1. These severity scores will be correlated with the genotype of the polymorphism p.Q791P in *CDKL5* and other candidate modifier genes. In particular, we are planning to analyze MeCP2 target genes (such as *BDNF*), genes involved in cognitive impairment (such as *APOE*) and genes that interact with *MECP2* and participate in silencing functions (*Sin 3A*, the six subunits of histone deacetylase complex). Investigation of the single and combined effect in multivariate analysis, will allow to weight the contribution of these modifier genes. Further investigation by array-CGH in RTT patients with discordant phenotype, will allow to detect possible additional rearranged regions (http://www.biobank.unisi.it). Gene content of these regions will be studied to select new candidate modifiers of RTT.

We are also collaborating with Prof. Janine LaSalle (Medical Microbiology and Immunology, and Rowe Program in Human Genetic, University of California, Davis, USA) who has performed a large scale mapping of neuronal MeCP2 binding site in order to verify if the rearrangements characterized in Results 3.4 contains these regulatory elements. This collaboration could reveal additional interesting genes contributing to RTT phenotype modulation.

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