University of Siena

Ph.D. in Medical Genetics

MOLECULAR DEFECT IN MECP2-NEGATIVE RETT PATIENTS

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Thesis suitable for the title of "Doctor Europeus"

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To the Ph.D. Thesis reviewers

Siena May 28, 2006

Please receive the Ph.D. thesis of Francesca Ariani.

She started the Ph.D. course in Medical Genetics in October 2002, after a degree in Biological Sciences. During these years, she carried out several lines of research which include Rett syndrome, X-linked mental retardation and glaucoma (Ariani F. et al. *Graefes Arch Clin Exp Ophthalmol.* 2006 Jan 27; 1-6).

She chose to discuss the final Thesis on Rett syndrome since she recognized this field more stimulating and fruitful. Within the field of Rett syndrome, she presents in this thesis only the work related to the identification of the molecular defect in MECP2-negative patients. However, she contributed to three additional papers (*Clin Genet* 2003 Dec;64(6):497-501; *Eur J Hum Genet* 2004 Aug;12(8):682-5; *Clin Genet* 2005 Mar;67(3):258-60) and helped me in writing the review: Rett syndrome: the compelx nature of a monogenic disease (*J Mol Med* 2003 Jun;81(6):346-54).

Starting from this year, in Italy, it is possible to get the title of "Doctor Europeus". This title can be conferred by the University of Siena, which is one of the Italian pioneer Universities in this field, when the following criteria are fulfilled:

- the authorization to the final PhD dissertation is accorded in the light of the reports on the Thesis compiled by at least two professors belonging to two superior education institutions of two member states of the European Community different from that in which the doctorate is held;
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- the PhD thesis must have been prepared partially following a research stay of at least a three-month period in one member state of the European Community different from that in which the doctorate is held.

I ask you to be the external reviewer of this thesis. Please send me back your general opinion together with specific comment whenever necessary.

Sincerely

Prof. Alessandra Renieri

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Acknowledgements

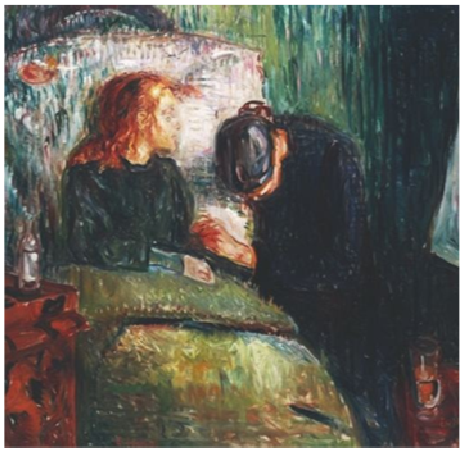
During these years, I have shifted my research interests from the "biomorphic forms" (W. Kandinsky "Sky Blue", 1940) of the university studies to patients and their families (E. Munch "The Sick Child", 1907). It has been a very exciting experience and I would like to thank prof. Alessandra Renieri, director of the Medical Genetics Unit of Siena, for this opportunity. I'm also very grateful to her for all she taught me and for the supervision of my work. Finally, I thank her for giving me the opportunity to know a lot of Italian and foreign researchers working in this field and to exchange ideas with them.

I would like also to thank prof. Orsetta Zuffardi and her group for the collaboration in performing the array-CGH experiment described at page 32.

A special thank to all my colleagues…They are all females and even if someone think that sometimes we are affected from the "double X syndrome"…I'm very happy to work with them!

Finally I would like to thank my family and Paolo for their support and patience….

W. Kandinsky "Sky Blue", 1940



E. Munch "The Sick Child", 1907

1. Introduction

1. Introduction

Rett syndrome (RTT, OMIM#312750) is a progressive neurodevelopmental disorder affecting almost exclusively girls. The syndrome was originally described by Andreas Rett, an Austrian paediatrician, in 1966, but it only became known worldwide in 1983, when Hagberg and colleagues reported 35 girls with strikingly similar clinical features of progressive autism, loss of purposeful hand use, ataxia and acquired microcephaly (1) (2). RTT is now recognised as a distinct clinical entity with an estimated prevalence of 1 in 10.000 to 20.000 female births and represents a leading cause of mental retardation and autistic behaviour in girls (3). In addition to the classic form, a number of RTT variants have been described and the preserved speech variant (PSV) is probably the most common (4-9). The early observation that the syndrome occurred exclusively in females suggested an X-linked dominant inheritance with possible male lethality. Since RTT familial cases are extremely rare, it took years of research to find data supporting this genetic model. In 1999, it was firstly demonstrated that RTT is caused by mutations in the *MECP2* gene, located in Xq28 (10). *MECP2* mutations account for approximately 70-80% of cases with classic RTT and for a lower percentage of variant patients. While much has been learnt about RTT since its first description, several questions have still to be answered and the pathogenic mechanisms underlying the disorder are still not well understood. To fully explain RTT phenotype will require the identification of the genetic defect in cases without an apparent *MECP2* mutation and the characterization of MeCP2 target genes, particularly those active in the central nervous system.

1.1 Clinical features of RTT

The established diagnostic criteria for RTT have been recently revised in order to clarify previous ambiguities in interpretation of clinical features (11). These criteria for classic RTT include a period of normal development, during which girls tend to reach motor, language and social milestones at the expected rate and age. Their neurological development is then arrested and begins to regress according to a predictable course that comprises four stages. During **Stage I** (6-18 months), patients cease to acquire new skills; they show decelerating head growth and autistic features. In **Stage II** (1-4 years), RTT girls lose the ability to speak and the purposeful use of the hands. During this stage, patients show the classic 'hand-washing' stereotypic activity, irregular breathing patterns, truncal and gait ataxia/apraxia; about half of them also develops seizures. 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 stereotypic hand activities persist. Other somatic and neurologic handicaps, such as severe scoliosis, reduced somatic growth and epilepsy, become evident. During **Stage IV** (5-15 years and older), seizures become less frequent, but somatic and neurologic deterioration continues, resulting in spastic quadriparesis (Fig. 1a). In addition to the classic form, five distinct categories of atypical RTT have been delineated on the bases of clinical criteria (12). These variants have some, but not all diagnostic features of RTT and can be milder or more severe. They include: i) the infantile seizure onset variant, with seizure onset before regression; ii) "forme fruste" with a milder and incomplete clinical course; iii) the congenital variant, lacking the normal perinatal period; iv) the late regression variant, which is rare and still controversial; v) the preserved speech variant (PSV), in which girls recover the ability to speak in single words or third person phrases and display an improvement of purposeful hand movements at Stage 3 of disease progression (Fig. 1b). Furthermore, in our italian cohort of patients, it has been described a “highly functioning PSV” associated with acquisition of more complex language function including use of first person phrases (9). In this variant, girls acquire a better control of their hands and they are able to draw figures and write simple words (Fig. 1c and 1d). The degree of mental retardation in these girls is also milder than in PSV and their I.Q. can be as high as 50.

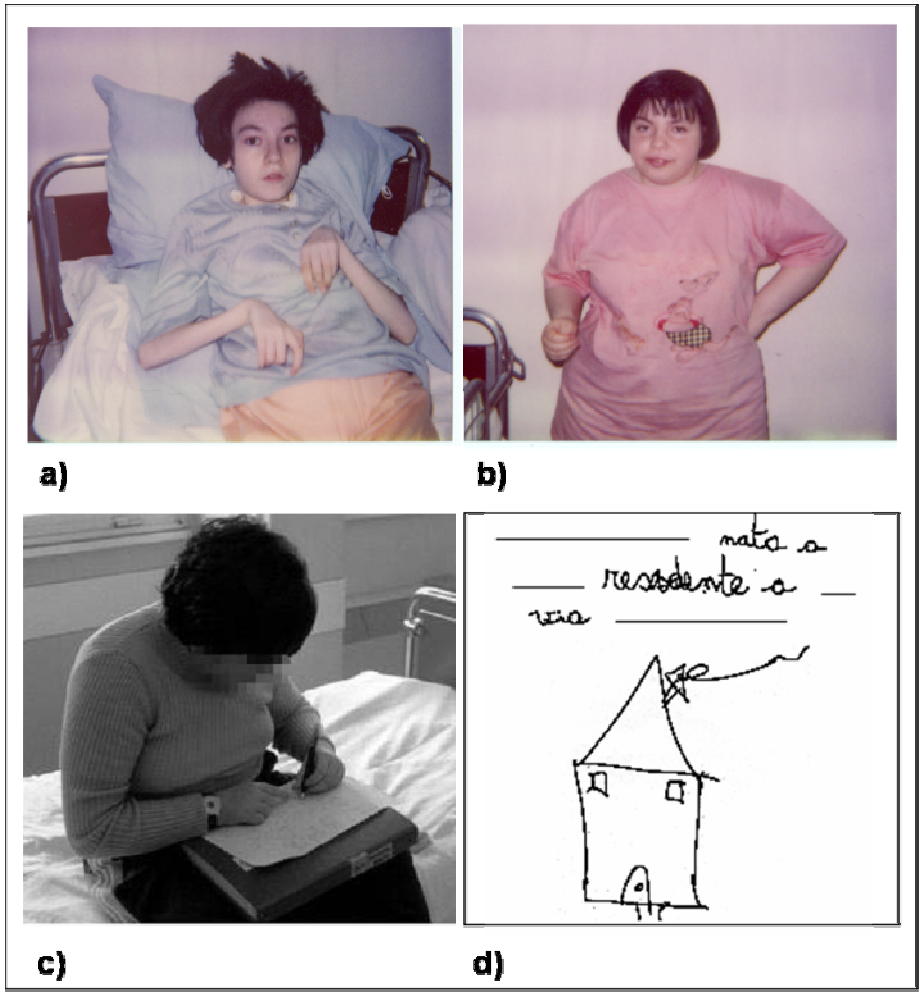


Fig. 1. RTT phenotypes. a) A classic RTT patient at the age of 12 years. b) A preserved speech variant (PSV) patient at the age of 21 years. c) A "highly functioning PSV" patient, at the age of 13 years, while she is writing the letters of her name. d) Drawing by a "highly functioning PSV" patient, indicating the recover of manual skills. The girl is also able to write her name and address.
(a and b reproduced with permission from (8); c reproduced with permission from (9)).

1.2 Genetics of RTT

MECP2 mutations in RTT and RTT-like phenotypes

Mutations in *MECP2* account for approximately 70-80% of females with classic RTT, 50% of PSV cases and a lower percentage of other variants (5-9) (13) (14). Following the first report of *MECP2* involvement in RTT, several mutation databases have been developed (http://homepages.ed.ac.uk/skirmis/; http://mecp2.chw.edu.au/; http://www.biobank.unisi.it). There are eight common mutations which are located at CpG dinucleotides and account for 65% of all described mutations in *MECP2*.

Studies aimed to establish a genotype/phenotype correlation have yielded conflicting results (8) (15-21). The results discrepancy may arise from differences in study design such as assessment scale of phenotype severity and classification of *MECP2* mutations. In addition, biological factors such as the pattern of X-inactivation, the effects of modifier genes and genetic background may account for the differences. There is some agreement that missense mutations have a milder effect than nonsense mutations and that early truncating mutations (located in the MBD or TRD), by causing complete loss of MeCP2 function, are preferentially associated with a more severe phenotype than late truncating mutations (located in the C-terminal domain) (14). Furthermore, the common Arg270X mutation seems to be associated with increased mortality (22).

Although initially thought to be a disorder exclusively affecting females, males with a RTT-like phenotype have been described. These cases include males with a 47,XXY kayotype, males who are a mosaic for severe *MECP2* mutations and males who may have milder *MECP2* mutations (23-25).

MECP2 mutations in non-RTT phenotype

A large degree of phenotypic variability has been observed in individuals with *MECP2* mutations. In 2000, we described a family in which a *MECP2* mutation segregated in male patients with recessive X-linked mental retardation (XLMR) and spasticity (26). Other studies identified *MECP2* mutations in males with nonspecific mental retardation, males with severe neonatal encephalopathy, males with language disorder and schizophrenia, males with PPM-X syndrome, Angelman syndrome and infantile autism (15) (27-36).

Concerning the type of mutations, *MECP2* early truncating mutations have been found associated with severe encephalopathy in male siblings of RTT females, while late truncating and missense mutations in MBD and TRD have been identified in nonsyndromic X-linked recessive mental retardation cases. In addition, missense mutations either in the C-terminal domain or in the connecting peptide between MBD and TRD, which are never described in RTT, have been found in XLMR patients. Successively, some of these *MECP2* missense changes turned out to be non pathogenic variants (37-39).

Finally, recent data show that duplications spanning the *MECP2* locus have been found in several patients with severe mental retardation and progressive spasticity and in members of three families with non specific mental retardation (40) (41).

Effect of X-chromosome inactivation

The *MECP2* gene is located at q28 on the X chromosome and it has been demonstrated to be subjected to X-inactivation in mice and humans (42) (43). Consequently, the pattern of X chromosome inactivation (XCI) has been postulated to explain the phenotypic heterogeneity associated with *MECP2* mutations. In support of this 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 (8) (18) (30) (44) (45). 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 (8) (26) (46). Furthermore, it has been demonstrated that XCI may vary remarkably between tissues (47) (48). 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 (49) (50). However, XCI has been investigated in a limited number of regions in few RTT brain samples. Consequently, no definitive conclusions can be drawn.

The *MECP2* gene and its function

MECP2 is a four-exons gene located in Xq28. It encodes two separate isoforms depending on the use of alternative splice variants: a protein of 486 amino acids (MeCP2_e2; encompassing part of exon 2 but not exon 1) and the recently identified protein of 498 amino acids (MeCP2_e1; encompassing exon 1 but not exon 2) (Fig. 2) (51) (52). The MeCP2_e1 isoform is more abundant in brain, while the MeCP2_e2 isoform predominates in other tissues such as fibroblast and lymphoblast cells. MeCP2 has two main functional domains: the Methyl-CpG-Binding Domain (MBD), shared by exon 3 and 4, which binds exclusively to symmetrically methylated CpGs and the Transcription Repression Domain (TRD), located in exon 4, which is able to recruit co-repressor complexes (Sin3A/HDACI or Ski/N-CoR/HDACII) that mediate repression through deacetylation of core histones (Fig. 2) (53-55). MeCP2 also has HDAC independent silencing activity (56). In addition to this role as transcriptional repressor, it has been recently demonstrated that MeCP2, interacting with the RNA-binding protein Y box-binding protein 1 (YB1), is also implicated in regulating RNA splicing (57).

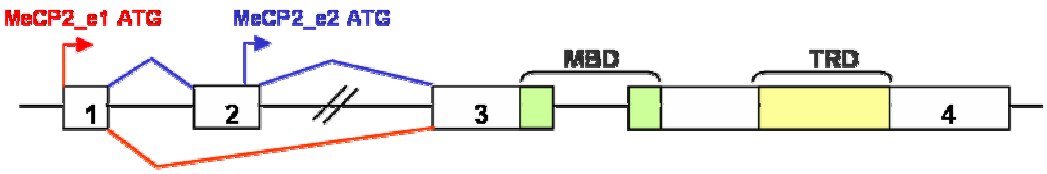


Fig. 2. Structure of the *MECP2* gene and mRNA. Alternative splicing for the MeCP2_e2 isoform is shown in blue and for the MeCP2_e1 isoform in red. The region encoding the MBD (Methyl-CpG Binding Domain) is in green and the TRD (Transcription Repression Domain) in yellow.

Mouse models of RTT

In order to clarify the mechanisms of RTT pathogenesis, a variety of mouse models have been produced. Two different groups generated mice with fully deleted *Mecp2* sequences (58) (59). *Mecp2* heterozygous female mice are viable, fertile and appear normal even in early adulthood. However, at about 6 months of age, they begin to show neurological symptoms reminiscent of RTT. Interestingly, the delay before symptoms onset overlaps in human and heterozygous mouse, despite their profoundly different rates of development. These results suggest that stability of brain function rather than development may be compromised by the absence of MeCP2. 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. Deletion of *Mecp2* restricted to neuronal lineages results in the same phenotype, showing that the absence of normal protein function in neurons is sufficient to cause the disease (59). Furthermore, *Mecp2* deletion in post-mitotic neurons lead to a delayed RTT-like phenotype, suggesting that the disease is caused by the absence of a continuous function of the protein in mature neurons (58) (60) (61). In addition, mice expressing a truncated MeCP2 protein that retains partial function display a milder phenotype than that shown by the null mice (62). Brain tissues from these mice contained hyper-acetylated H3 histone, indicating that transcriptional alterations may underlie the phenotype. Since the phenotype in male mice is milder than in male humans, in whom *MECP2* mutations are considered embryonic lethal, the possibility of functional redundancy of other methylated-DNA binding proteins such as MBD2 (Methyl Binding Domain 2) has been raised. MBD2, which usually functions as a transcriptional activator in the presence of wild type MeCP2, acts as a silencer and associates with the MeCP1 histone deacetylase complex in MeCP2-deficient cells, possibly replacing some silencing function normally dependent on MeCP2 (63). However, in *Mecp2* null mice, concomitant loss of *Mbd2* does not worsen the phenotype, suggesting that Mbd2 alone does not compensate for the loss of MeCP2 (59). Recently, mouse models demonstrated that also slightly increased levels of MeCP2 have a deleterious effect on brain function (64). In particular, twofold over-

expression of *Mecp2* in wild type mice resulted in the development of progressive neurological deterioration with seizures, motor dysfunction, and reduced survival.

MeCP2 targets

The involvement of MeCP2 in methylation-specific transcriptional repression suggests that RTT may result from inappropriate expression of a large number of genes. However, experiments of gene expression profiling in neuronal and non-neuronal tissues from Mecp2 knockout mice and/or from RTT patients have identified only small and unreproducible differences. This could be due to functional redundancy and other members of the MBD protein family may compensate for loss of MeCP2 function. Alternatively, expression changes may be limited to very restricted brain areas or cell types and thus they might be lost by these studies. Finally, the microarray technology may not be sufficiently sensitive to detect subtle expression changes of a limited number of genes.

Recently, a candidate gene approach has been used to identify the brain derived neurotrophic factor (BDNF) gene as a MeCP2 target in mammals (65) (66). BDNF is a neurotrophin essential for survival, growth and maintenance of neurons during development (67). In addition, BDNF has important functions for neuronal plasticity, learning and memory (68-70). MeCP2 was found to bind the promoter of *Bdnf*, thereby repressing its expression in resting neuronal cultures. Following membrane depolarization, phosphorylated MeCP2 dissociates from the promoter and *Bdnf* expression is induced. 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 (Fig. 3). However, more recently, it has been demonstrated that BDNF protein level are reduced in *Mecp2* knockout mice (71). 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.

One class of genes that have been intensively studied as MeCP2 targets are the imprinted genes. In 2005, convincing evidence emerged that MeCP2 is important for imprinting at the *Dlx5* (distal-less homeobox 5) locus (72) (73). 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 (Fig. 3). 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. Since DLX5 regulates GABA neurotransmission and osteogenesis, loss of imprinting at the *DLX5* locus might account for epilepsy, osteoporosis and somatic hypoevolutism observed in RTT (Fig. 3).

Phenotypic overlap between RTT and Angelman syndrome (AS) led to investigate expression levels of another imprinted gene, *UBE3A* (ubiquitin protein ligase E3A). AS is caused by repression of the maternally expressed copy of *UBE3A* (74). Regulation of *UBE3A* expression involves the *UBE3A* antisense transcripts that have been suggested to inhibit transcription from the paternal allele (75) (76). It has been demonstrated that *UBE3A* mRNA and protein levels are decreased in brains of MeCP2 deficient mice and RTT patients (Fig. 3) (77) (78). This correlates with a biallelic production of *UBE3A* antisense RNA and changes in chromatin structure (78). The authors concluded that MeCP2 deficiency causes epigenetic aberrations at the PWS/AS imprinting center that result in loss of imprinting of the *UBE3A* antisense gene, increase in *UBE3A* antisense RNA level and consequently decrease in UBE3A production (Fig. 3).

Other genes that have been subsequently found to be regulated by MeCP2 in brain tissues are the glucocorticoid inducible genes, *Sgk1* and *Fkbp5* (79). Nuber et al observed increased levels of *Sgk1* and *Fkbp5* mRNAs in *Mecp2*-null mice (Fig. 3). Given the known deleterious effect of glucocorticoid exposure on brain development, the authors hypothesized that disruption of MeCP2-dependent regulation of stress responsive genes contribute to RTT symptoms (Fig. 3).

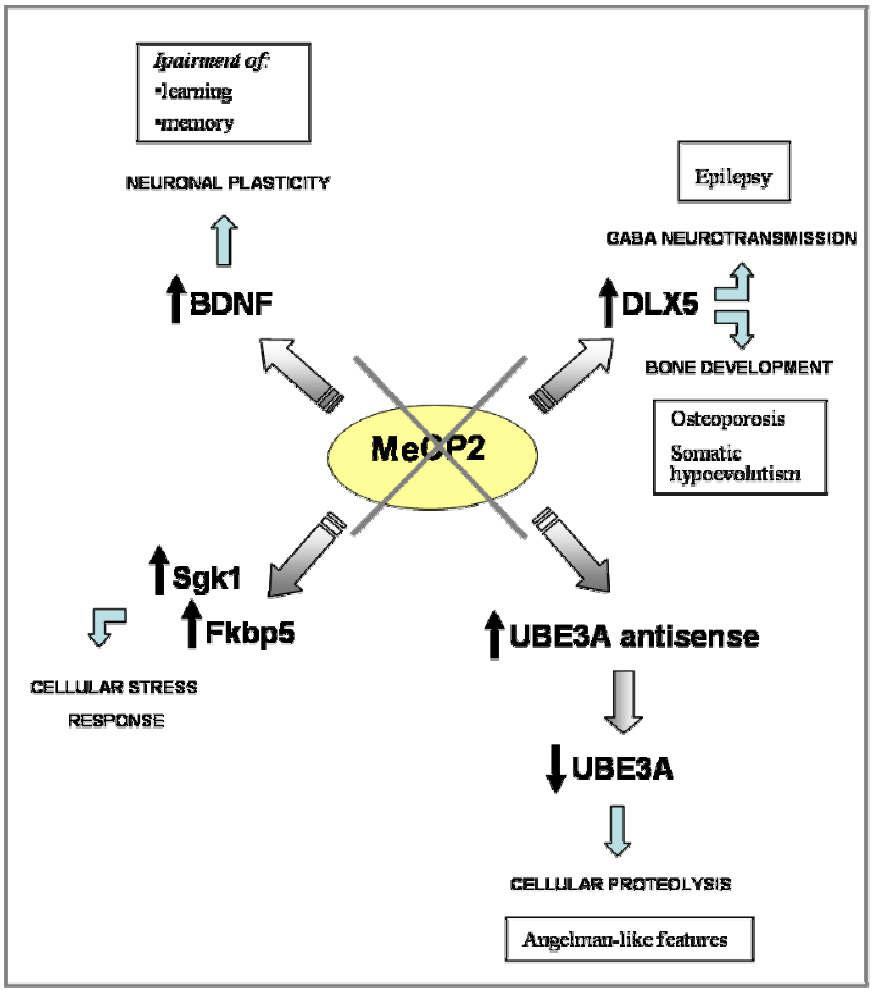


Fig. 3. MeCP2 targets. Loss of MeCP2 leads to altered expression of specific genes: *BDNF*, *DLX5*, *Sgk1*, *Fkbp5, UBE3A* antisense and, consequently, *UBE3A*. Expression changes of *BDNF* may be responsible for impairment of learning and memory. Over-expression of *DLX5* may account for epilepsy, osteoporosis and somatic hypoevolutism of RTT. Over-expression of the glucocorticoid inducible genes *Sgk1* and *Fkbp5* may lead to altered cellular stress response, but actually it is not possible to predict the phenotypic consequences. Finally, the over-expression of antisense *UBE3A* results in under-expression of *UBE3A*, the gene responsible for the Angelman syndrome, possibly explaining the Angelman-like features present in patients with RTT. (Figure adapted from (80)).

2. Rationale, aim and outline of the study

2. Rationale, aim and outline of the study

RTT is a devastating neurodevelopmental disorder that represents one of the most common genetic causes of mental retardation in girls. *MECP2* mutations account for about 70-80% of classic RTT cases and for a lower percentage of variant patients.

This study has been focused on the identification of the genetic defect in RTT patients without an apparent *MECP2* mutation. We tried to achieve this goal through the combination of several strategies including the set up and use of a clinically and molecularly well-defined patients database, the improvement of traditional methods employed to analyze the *MECP2* gene and the identification of new genes involved in RTT.

At the beginning of this study, the availability of a large number of RTT samples has been essential. A DNA and cell lines bank of RTT patients has been present in the Medical Genetics Unit of Siena since 1998. Given that this large collection of patients represents a very important tool for the scientific community, we inserted bank samples in an on-line catalogue available upon request to all researchers working on RTT **(Result 3.1)**.

We then start to investigate the molecular cause in our cohort of mutation-negative RTT patients by searching missed *MECP2* defects **(Result 3.2).**

Mutation-negative RTT cases could be due to the limited sensitivity of the methodology used for *MECP2* mutation analysis. In particular, the traditional techniques employed for *MECP2* analysis (DGGE, SSCP, DHPLC, direct sequencing) were prone to miss large rearrangements. We therefore decided to develop a fast and sensitive assay to detect such rearrangements by Real Time quantitative PCR and we employed this method to analyze our cohort of patients. *MECP2* rearrangements were then characterized by MLPA or array-CGH.

In 2004, two studies described a new isoform of MeCP2 and showed that it is the predominant form in the human brain. The new isoform has a longer N-terminus, transcribed from exon 1 of *MECP2*. Exon 1 was previously considered non coding and was therefore not included in *MECP2* mutation screening. Given these data, we analyzed our RTT patients for mutations in exon 1**.**

To explain the proportion of mutation-negative RTT cases, we also hypothesized the existence of another RTT locus **(Result 3.3)**. We observed two girls with clinical features typical of the infantile seizure onset variant of RTT. In these patients, *MECP2* point mutations and gross rearrangements were excluded by DHPLC and Real Time qPCR. The infantile seizure onset variant of RTT 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. *ARX* and *CDKL5* genes have been associated with ISSX. Based on the phenotypic similarities between the two syndromes, we analyzed both *ARX* and *CDKL5* for mutations in the two RTT variant patients. The identification of *CDKL5* deletions in the two girls led us to extend the analysis to the other *MECP2*-negative patients of our collection. We found *CDKL5* mutations in two other girls with the infantile seizure onset variant, strengthening the correlation between *CDKL5* and RTT. 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 firstly decided to investigate the expression patterns of *Mecp2* and *Cdkl5* in embryonic and postnatal mouse brains. Successively, we performed a series of experiments aimed to investigate whether MeCP2 and CDKL5 directly interact in vitro and in vivo. Finally, we analyzed the functional activity of CDKL5.

This study has been very interesting not only because it allowed us to define the molecular diagnosis in a fraction of "unsolved" RTT cases, but also because it established the molecular bases for future studies aimed to understand the mechanisms of RTT pathogenesis.

3. Results

Result 3.1

On-line RTT database

Unpublished data

A bank of RTT DNA and cell lines is active in the Medical Genetics Unit of Siena since 1998. During the PhD, I have been involved in the creation of an on-line database containing all RTT patients collected in the bank. The database is now available and represents a precious resource for all researchers working on RTT. The general home page of the web site is available at http://www.biobank.unisi.it. This introductory page gives access to three independent databases: 1-X-Linked Mental Retardation; 2-Rett syndrome; 3-Other (containing biological samples from patients affected by other genetic disorders).

Fig. 4. General home page of the bank. Here, users can find links to the three distinct databases: 1-XLMR; 2- RTT; 3- Other. Moreover, this page gives access to general information useful for site navigation and use.

Methods

The RTT database is maintained and updated on the server of the University of Siena. The database has been created using the Access software (Microsoft Office; http://www.microsoft.com) for data management. The web-site is written in VBScript and takes advantage of a Microsoft IIS (Internet Information Server) server with ASP (Active Server Pages) technology.

How to use the database

By accessing the RTT section, users can see a list of all patients available with additional specific information. At present, the site contains 606 samples (208 probands and 398 relatives). Of the 208 probands, 149 have a known mutation while for the others 59 mutation screening failed to identify any pathogenic change.

The database is organized on two levels: a "public" level freely available to the general public and an "administrator" level accessible only to bank administrators through the use of a password. This level contains personal data of patients and relatives and detailed clinical information.

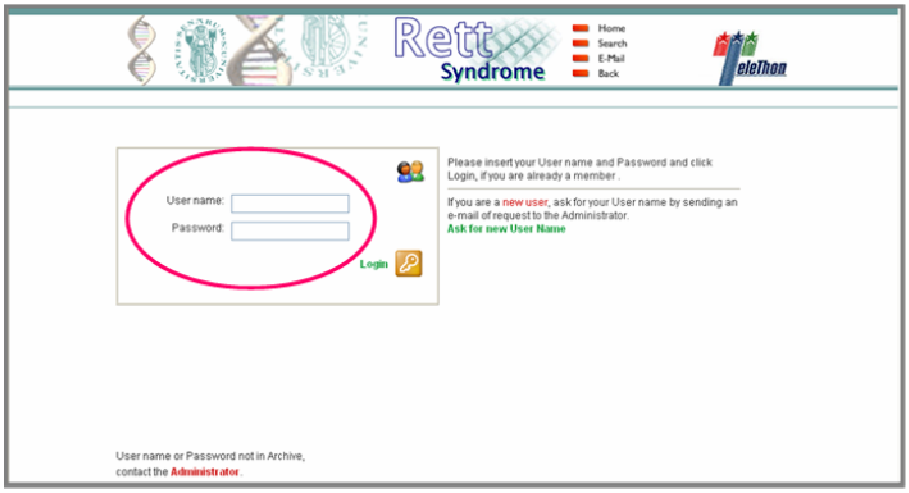


Fig. 5. Login page. A username and a password guarantee a regulated access to information. Only administrators can insert new records, modify information and access to patients personal data and clinical details.

In both cases, the main page consists of a table of all available samples with the following information: **a) CODE**: a progressive number which identifies RTT families; **b) INTERNAL CODE**: the personal codes assigned to each family member; **c) PHENOTYPE**: classic RTT, PSV, high functioning PSV, early seizures variant, "forme fruste", congenital variant, RTT-like, or ND (< 4 years) when the age of the patient does not allow an accurate clinical classification; **d) GENE NAME**: the name of the mutated gene ("Unknown" if the mutation has not been found); **e) MUTATION TYPE**: missense, early truncating, late truncating, gene deletion and gene duplication; **f) NUCLEOTIDE CHANGE**; **g) AMINOACID CHANGE**; **h) ADDITIONAL INFO**: a page in which external users can only visualize information about the X-inactivation status and mutations inheritance, while bank curators can visualize also personal data of patients and relatives**; i) BIOLOGICAL SAMPLE AVAILABLE:** lymphoblastoid cell lines, leukocytes in DMSO medium, plasma, DNA, fibroblasts; **l) REFERENCE:** PubMed references of articles including the specific patient.

The website is interactive, with a user-friendly graphical interface. Researchers can rapidly verify if there are the patients they need by exploiting the **"Search by"** option available directly on the main page. This option allows users to select patients by: i- **Mutated gene**; ii- **Mutation type**; iii- **Nucleotide change**; iv- **Aminoacid change**; v- **Phenotype**; vi- **Survival**. Users can choose to search by one single option or to combine two or more options (Fig. 6a and 6b). As a result of the search, they will visualize a table with all RTT patients fulfilling the requested features (Fig. 6c).

Finally, on the main page, users can find links to three other pages: i) **List of mutations**, a table with all mutations identified in bank patients; ii) **Graph of mutations**, a dynamic graph showing the position of the pathogenic mutations and their relative frequency; iii) **Rare variants**, a table with all non-pathogenic rare variants identified in bank patients.

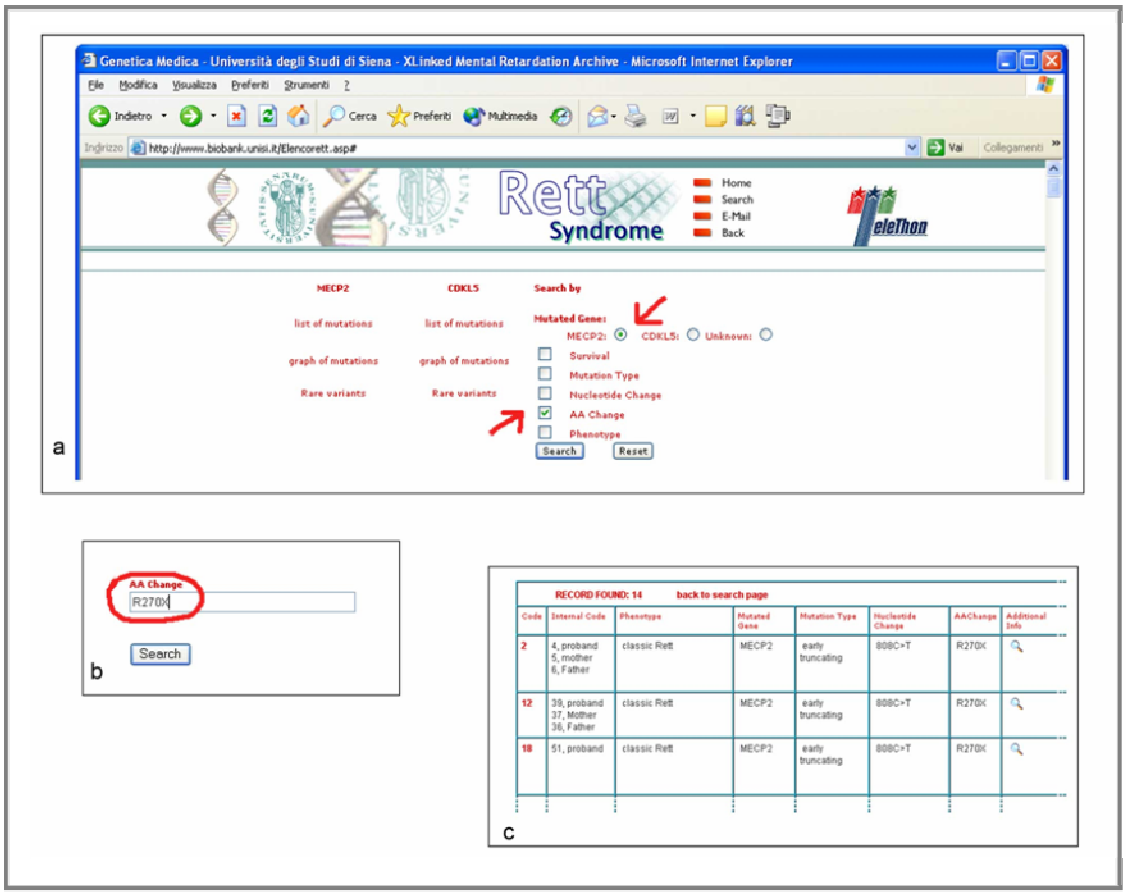


Fig. 6. Search of specific RTT samples in the database. a) Users can search RTT patients with a specific aminoacid change through the "Search by" option available on the main page of the website. b) The "Search" option gives access to a page in which users can indicate the specific aminoacid change, for example R270X. c) As a result, users visualize a table containing all the fields present in the general table and listing all bank patients with R270X.

How to improve the database

Given the complexity of RTT phenotype, we are actually working on the improvement of the structure and information content of the web site. For example, we want to increase the amount of information related to each RTT patient creating a new schedule containing an accurate list of clinical features. Moreover these clinical data will be associated to a phenotypic severity score (Table 1, modified by (81) (82)). This amelioration will make possible a better definition of the clinical phenotype of each patient and a more accurate genotype-phenotype correlation.

Furthermore, we plan to create an additional schedule (named "Additional anomalies") in which there will be the possibility to insert clinical features not generally associated with RTT phenotype or less frequently evaluated in these patients. For example, our bank contains some RTT patients with particular features such as eyes anomalies (microphthalmia and optic nerve coloboma) or bone anomalies (ectrodactyly). This improvement will better define the phenotype of each patient and will make the bank a precious resource for all researchers studying these very rare patients.

We also plan to add a new column named "Inheritance" to the main page table. Usually RTT is a sporadic disease but some exceptions to this rule do exist. In fact, three RTT cases of the bank are familial. In these familial cases, affected members show variations in age onset, severity of impairment and clinical course. These rare cases are very precious to study possible modifier genes of RTT phenotype.

In addition, our bank includes patients with particular chromosomal rearrangements and without any point mutation identified. For example, the bank contains a patient with RTT-like features and an interstitial deletion of chromosome 2 (2q34) and another patient with atypical RTT and an interstitial duplication of chromosome 11 (11p23.3) (83) (84). The bank, making these rare samples available to the scientific community, represents an important resource to identify the specific gene/s responsible for the phenotype. Consequently, we plan to allow users to immediately visualize this information on the main page.

Table 1

Scoring for different clinical features

Head	2	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
Epilepsy	2	First seizure in the first year of life
	1	First seizure after the first year of life
	0	Epilepsy at age of 5
Hand stereotypy	2	Dominating or constant
	1	Mild or intermittent
	0	None
Voluntary hand use	2	None
	1	Reduced or poor
	0	Hand use normal
Walking	2	Never learned to walk
	1	Loss of ability to walk
	0	Walking unsupported at age of 5
Speech	2	Never spoken
	1	Loss of ability to speech
	0	More than 10 words at age of 5
Sitting	2	Never learned to sit
	1	Loss of ability to sit
	0	Sitting unsupported at age of 5 years
Scoliosis	2	Severe
	1	Mild
	0	Absent
Intellectual disability	2	Apparent profound IQ<20
	1	Apparent severe IQ: 20-40
	0	IQ>40

Result 3.2

Identification of missed *MECP2* defects

Real-time quantitative PCR as a routine method for screening large rearrangements in Rett syndrome: Report of one case of *MECP2* deletion and one case of *MECP2* duplication

Ariani F, Mari F, Pescucci C, Longo I, Bruttini M, Meloni I,
Hayek G, Rocchi R, Zappella M, Renieri A.

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HUMAN MUTATION 24:172–177 (2004)

METHODS

Real-Time Quantitative PCR as a Routine Method For Screening Large Rearrangements in Rett Syndrome: Report of One Case of MECP2 Deletion and One Case of MECP2 Duplication

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Mutations in the X-linked methyl-CpG-binding protein 2 gene (MECP2) are found in 70–80% of cases of classical Rett syndrome (RTT) and in about 50% of cases of preserved speech variant (PSV). This high percentage of MECP2 mutations, especially in classical RTT cases, suggests that another major RTT locus is unlikely. Missed mutations may be due to the limited sensitivity of the methodology used for mutation scanning and/or the presence of intronic mutations. In a double-copy gene, such as MECP2 in females, current methodologies (e.g., DGGE, SSCP, DHPLC, direct sequencing) are prone to miss gross rearrangements. Three previous reports during 2001–2003 have shown the presence of large deletions in a fraction of MECP2-negative classical RTT patients. We developed a reliable, single tube, quantitative PCR assay for rapid determination of MECP2 gene dosage. This method involves a multiplex reaction using a FAM labeled TaqMan probe with a TAMRA quencher derived from MECP2 exon 4 and two primers derived from the same exon and RNAaseP as an internal reference. The copy number of the MECP2 gene was determined by the comparative threshold cycle method (ddCt). Each sample was run in quadruplicate. We validated this assay through the analysis of 30 healthy controls (15 female and 15 male) and we then applied this method to eight classical RTT and six PSV patients, all negative for MECP2 mutations. We identified gross rearrangements in two patients: a deletion in a classical RTT patient and a duplication in a PSV patient. Our results confirm that a fraction of MECP2-negative RTT cases have MECP2 gross rearrangements and we propose real-time quantitative PCR as a simple and reliable method for routine screening of MECP2 in addition to DHPLC analysis. Hum Mutat 24:172–177, 2004. © 2004 Wiley-Liss, Inc.

KEY WORDS: quantitative PCR; Rett syndrome; RTT; MECP2; PSV

DATABASES:
MECP2 – OMIM: 300005; 312750 (RTT); GenBank: XM_048395.1, AF030876 http://mecp2.chw.edu.au (RettBASE)

INTRODUCTION

Rett syndrome (RTT; MIM# 312750) is a progressive neurodevelopmental disorder seen almost exclusively in girls, with an estimated prevalence of approximately 1 in 10,000–15,000 females. The classical form of the disease was originally described by Andreas Rett in 1966. RTT girls are born healthy, and appear to develop normally until 6 to 18 months of age. Their neurological development then regresses, with the loss of skills already gained, such as speech and purposeful hand movements. These girls develop microcephaly, seizures, ataxia, autistic features, intermittent hyperventilation, and stereotypic hand movements. Some patients who do not manifest all the typical features of RTT are considered to have a variant form of the disease. Among RTT variants, preserved speech variant (PSV) is probably the most common [Zappella, 1994; Zappella et al., 1998; De Bona et al., 2000]. PSV patients recover some degree of speech (in single words and in some cases using third person phrases) and show improvement in purposeful hand movements.

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RTT is caused by mutations in the gene encoding methyl CpG binding protein 2 (MECP2; MIM# 300005) related to the Xq28 locus [Amir et al., 1999]. MECP2 mutations have been identified in 70 to 80% of patients with classical RTT [Huppke et al., 2000; Bienvenu et al., 2000; Cheadle et al., 2000; De Bona et al., 2000; Laccone et al., 2001; Nielsen et al., 2001; Miltenberger-Miltenyi and Laccone, 2003] and in about 50% of cases with PSV [De Bona et al., 2000; Zappella et al., 2001]. The routine diagnosis of RTT is mainly performed by using techniques like DGGE, SSCP, DHPLC, or direct sequencing of the coding exons and immediate flanking intronic regions of MECP2. However, these methods do not allow the detection of major rearrangements in the gene. So far, three groups have reported the use of Southern blotting to screen for gross rearrangements in MECP2 [Bourdon et al., 2001b; Schollen et al., 2002; Yaron et al., 2002]. Another study reported FISH analysis to detect MECP2 rearrangements [Bourdon et al., 2001a]. In the present study, we used, for the first time, real-time quantitative PCR for detecting major rearrangement in MECP2. This method involves standard PCR in conjunction with a fluorescent TaqMan method and an ABI Prism sequence detector (Applied Biosystems, www.appliedbiosystems.com).

MATERIALS AND METHODS

Patients

We investigated eight cases of classical RTT and six cases of PSV, all negative for the MECP2 mutation [De Bona et al., 2000; Vacca et al., 2001]. All the girls with classical RTT were diagnosed according to the international criteria based on the Rett Syndrome Diagnostic Criteria Work Group [Trevathan and Moser, 1988]. The cases of PSV fulfilled the criteria given by Hagberg and Skjedal for RTT variants [Hagberg and Skjeldal, 1994]. Blood samples were obtained from patients and available parents after informed consent.

DNA Extraction

DNA was extracted from peripheral blood using a QIAamp DNA Blood Kit (Qiagen, www.qiagen.com). DNA extracted by phenol/chloroform was used in some experiments. The use of the QIAamp DNA Blood Kit is recommended for obtaining a high-quality DNA template, which is very important for the reliability of the experiment [Aarskog and Vedeler, 2000]. We used the OD260/280 method on a photometer to determine the appropriate DNA concentration [Sambrook et al., 1989]. DNA was diluted in HPLC pure water to a concentration of ~50 ng/µl and stored at 4°C.

Primers/Probes and TaqMan Reaction

The TaqMan probe and primers were designed using the Primer Express software (Applied Biosystems), following the criteria indicated in the program. We designed the MECP2 probe to be complementary to a segment located in the middle of exon 4, in a region corresponding to the first part of the TRD domain of the protein. MECP2 exon 4 forward primer: 5′-TCA GAG GGT GTG CAG GTG AA-3′; MECP2 exon 4 reverse primer: 5′-TTG AAA AGG CAT CTT GAC AAG GA-3′; MECP2 exon 4 TaqMan probe: 5′-AGG GTC CTG GAG AAA AGT CCT GGG AAG-3′. The MECP2 probe contained a fluorophore 5′FAM as reporter and a 3′TAMRA as quencher. The size of the amplicon was 72 bp. We used an RNAseP kit as an internal reference (Applied Biosystems). This kit contains 20× RNAseP Mix with a VIC-labeled probe and specific primers for the RNAseP gene. 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 of each plate were prepared from a single PCR Mastermix consisting of: 2× TaqMan Universal PCR Master Mix, 900 nM MECP2 forward primer, 900 nM MECP2 reverse primer, 200 nM MECP2 probe, 20× RNAseP Mix, and HPLC pure water. A total of 100 ng of DNA (5 µl) 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 sec 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. Two known control samples (diploid samples) were analyzed on each reaction tray for calibration, as required in the comparative threshold cycle (ddCt) method. In addition, each plate run was monitored with two control male samples (haploid samples). No-template control (background) was also included in each assay.

Data Analysis

Data evaluation was carried out using the ABI Prism sequence detection system and Microsoft Excel (www.microsoft.com). Each sample was run in quadruplicate for the quantification of the MECP2 gene as compared to the internal RNAseP control gene. The threshold cycle number (Ct) was determined for all PCR reactions. The threshold was automatically set at 10 standard deviations (SDs) above the mean baseline emission, representing the background level, and was calculated from cycles 1–20. In most cases, the threshold was manually adjusted within the logarithmic curve, above the background level and below the plateau phase. Data analysis was performed only for samples with at least three amplifications (triplicates). A comparative Ct method, as previously described by Livak [1997], was used to calculate the relative gene number. To use this method, some assumptions must be verified. In particular, the efficiencies of the target and endogenous control amplifications must be approximately equal. We therefore performed a validation experiment to determine the efficiencies of MECP2 and RNAseP amplifications. By diluting a standard sample in steps and measuring the Ct value at each dilution, we constructed two calibration curves from which the PCR efficiencies could be determined (Fig. 1A). We prepared four 10-fold dilutions of total DNA and each amount of the standard sample was amplified in triplicate. The amplification of MECP2 and RNAseP was performed in the same tube (multiplex). We obtained an amplification efficiency close to 100% (~99%) for the two genes, signifying that both reactions proceeded with very high efficiencies. In addition, the validation experiment showed an absolute value of the slope of log input vs. dCt very close to 0 (0.009), demonstrating that the efficiencies of the two systems are approximately equal (Fig. 1B).

Using the comparative Ct method, the starting copy number of the unknown samples was determined in comparison with the known copy number of the calibrator sample, using the following formula: ddCt = [dCt RNAseP (calibrator sample) – dCt MECP2 (calibrator sample)] – [dCt RNAseP (unknown sample) – dCt MECP2 (unknown sample)]. The relative gene copy number was calculated by the expression $2^{-(ddCt \pm s)}$, where s represents the SD of the difference calculated from the Ct SD of both MECP2 and RNAseP. Using this calculation, a ddCt ratio of about 1 for a diploid sample and about 0.5 for an haploid sample is expected.

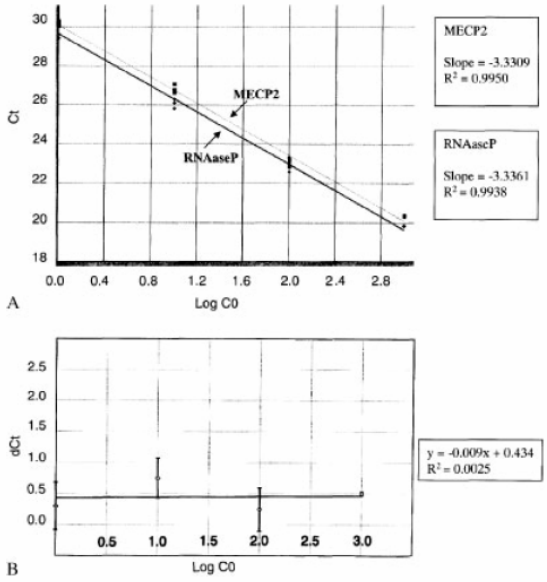

FIGURE 1. **Validation experiment for assessing the efficiencies of the target (MECP2) and the reference amplification (RNAaseP). A:** Standard curves plotting log starting copy number (C_0) vs. Ct. Each symbol represents the amplification of standard DNA (1, 10, 100, and 1000 ng, from left to right) for MECP2 (■) and RNAaseP (◆). Each amount of standard DNA was amplified in triplicate for both reactions. PCR efficiencies are obtained from the slopes of the fitted lines as: $E = 10^{-(\text{slope})^{-1}} - 1$. **B:** Plot of log input amount vs. dCt. The dCt value is determined by subtracting the average RNAaseP Ct value from the average MECP2 Ct value for each step of standard DNA dilution.

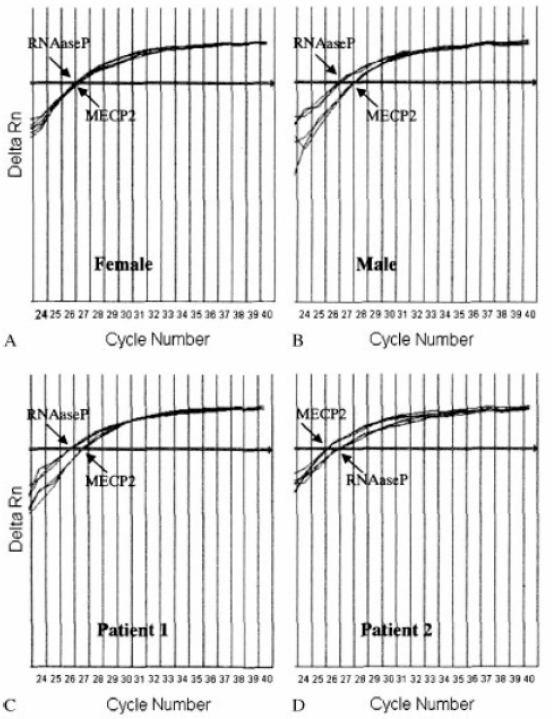

FIGURE 2. **PCR amplification plot of MECP2 and RNAaseP in multiplex single tube assay. A:** Healthy control female with a double copy of MECP2 (ddCt ratio 1.00). **B:** Healthy control male with a single copy of MECP2 (ddCt ratio 0.47). **C:** RTT female (Patient 1) with MECP2 deletion (ddCt ratio 0.55). **D:** PSV female (Patient 2) with MECP2 duplication (ddCt ratio 1.59). The dRn value represents the quantity of fluorescent probe degraded and fits an exponential function generating an amplification plot for each well. dRn is plotted on the y-axis and the cycle number is on the x-axis. After sufficient hybridization, the probe is cleaved by 5′ nuclease activity of the Taq DNA polymerase, the intensity of the reporter (FAM for the MECP2 probe and VIC for the RNAaseP probe) fluorescent emission increases above background and crosses the threshold. The point at which the amplification plot crosses the threshold is defined as Ct, and Ct is reported as the cycle number at this point. In the normal control female, the Ct values of MECP2 and RNAaseP quadruplicates were almost identical, whereas in the healthy control male and in the RTT female Patient 1, the decrease in the starting copy number of the MECP2 gene results in a shift of the amplification plot to the right. In PSV female Patient 2, the Ct value of MECP2 is shifted to the left when compared with RNAaseP. The decrease in the Ct value when estimated by the comparative Ct method gave a ddCt ratio of 1.59.

RESULTS

Assay Development and Data Analysis

We performed separate and multiplex preruns, varying the concentrations of MECP2 primers and probe, and we assessed the conditions for the highest intensity and specificity of reporter fluorescent signal. Each sample was run in quadruplicate. The quadruplicate runs of MECP2 and RNAaseP showed an almost complete overlap in the exponential and plateau phases of parallel amplification plots. The high quality of the amplification plots was due to the accuracy of pipetting of PCR reagents and to the DNA extraction method. DNA extracted by the QIAamp Blood Kit resulted in amplification plots of higher quality, with low Ct SD values (mean 0.11; range 0.05–0.20). DNA extracted using the phenol/chloroform method produced amplification plots with higher Ct SD values (mean 0.18; range 0.13–0.31) (data not shown).

In a healthy control female, the amplification plots of MECP2 and RNAaseP quadruplicates crossed the threshold at approximately the same point (Ct) (Fig. 2A). In a healthy control male, the Ct value of MECP2 showed an increase of approximately one cycle when compared with RNAaseP (Fig. 2B). We validated the method through the analysis of 15 male (MECP2 single copy) and 15 female (MECP2 double copy) healthy controls. Using the ddCt method, we quantified the MECP2 copy number. The values of the MECP2 ratio ($2^{-(\text{ddCt}\pm s)}$) obtained from the 15 male and 15 female healthy controls showed no overlap between the two groups (Fig. 3; Table 1).

The first column of Table 1 shows the range of variation of ddCt ratio values obtained from male and female controls ($2^{-(\text{ddCt})}$). The second and the third column show the lower and the upper ranges of the ddCt ratio ($2^{-(\text{ddCt}\pm s)}$). From these results, we set the following threshold values for the ddCt ratios: a value

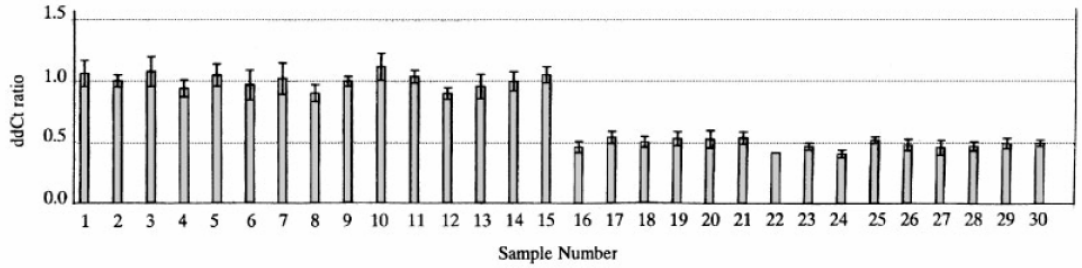

FIGURE 3. **MECP2 ddCt ratios and SD of 15 healthy females and 15 healthy males. The female samples (1–15) show ddCt ratios of about 1, indicating a double copy of MECP2, while the male samples (16–30) show ddCt ratios of about 0.5, indicating a single copy of the gene. The two groups are clearly distinguishable and show no overlap in ddCt ratios.**

TABLE 1. Copy Number (ddCt Ratio) of MECP2 Gene

Samples	ddCT ratio range	ddCT ratio lower range	ddCT ratio upper range	95% CI
Male controls	0.41–0.54	0.38	0.59	0.41–0.57
Female controls	0.90–1.12	0.84	1.23	0.89–1.12

under 0.59 indicates a single copy gene, while a value between 0.84 and 1.23 indicates a double copy gene. These results showed that the method is sensitive enough to detect a 50% difference in DNA template, as shown by the ddCt ratio values. The fourth column shows the 95% confidence interval (CI).

Analysis of RTT Patients Negative for MECP2 Mutations

We selected eight patients with classical RTT and six with PSV in which a SSCP screening on MECP2 failed to detect any mutation [De Bona et al., 2000; Vacca et al., 2001]. We rescreened these patients by DHPLC, a more sensitive technique [Buyse et al., 2000], without any additional results. However, all these approaches do not allow for the detection of gross rearrangements in MECP2. For this reason, we decided to analyze this group of patients by quantitative real-time PCR. We identified large rearrangements in two patients (Fig. 2C, and D). In one classic RTT female (Patient 1), the Ct value of MECP2 showed an increase when compared to RNAaseP. The increase in Ct value, when calculated by the comparative Ct method, resulted in a ddCt ratio of 0.55. This result was consistent with an MECP2 deletion.

In a second RTT female (Patient 2), the MECP2 Ct showed a decrease compared to the known normal female sample. The decrease in Ct value, when estimated by the comparative Ct method, gave a ddCt ratio of 1.59. This result was consistent with a MECP2 duplication. A similar result was not found in 100 normal chromosomes.

A brief description of the clinical phenotype of Patients 1 and 2 follows:

Patient 1 (#581), a girl with typical RTT syndrome, is presently 22 years old. The first symptoms of this disorder started at the age of 15 months. She then underwent the subsequent stages of the disease: stage IV with a progressive motor decay was evident at 12 years of age. At 14 years of age, she was no longer ambulatory and showed a severe scoliosis. She suffers generalized convulsions that are difficult to treat.

Patient 2 (#612) is an example of PSV variant; she is presently 34 years old. She was born by dystocic delivery, of third-cousin consanguineous parents. The disorder started between the ages of 1 and 2 years. She was able to walk alone, speak in short sentences, and attend school until 9 years of age, when her language and motor activities began to progressively decline. At this point, she entered puberty and treatment for seizures was initiated. One year later, she began to suffer drug-resistant generalized tonic-clonic and myoclonic-astatic seizures. These seizures persist today, with a high frequency. Electroencephalogram (EEG) showed diffuse, slow, background activities, with spikes and sharp waves in the frontal area. Diffuse cerebral and cerebellar atrophy was found at MRI. Presently, she is hypotonic, has a hypomimic face, and is not ambulatory. She cannot speak and is unable to use her hands for precise movements. She has always presented occasional hand-washing movements and repetitive truncal rocking.

DISCUSSION

So far, Southern blotting has been used by three different groups to detect the presence of large MECP2 rearrangements in RTT patients negative for point mutations. One group identified two large MECP2 deletions out of 10 mutation-negative classical RTT patients, the second identified one out of three, and the third identified three out of three [Bourdon et al., 2001b; Yaron et al., 2002; Schollen et al., 2002]. In another study, MECP2 gross rearrangements were investigated by FISH analysis and no deletions were found in a group of 25 classical RTT females [Bourdon et al., 2001a].

In this study, we used, for the first time, real-time quantitative PCR for detecting major rearrangements in

MECP2. We developed a multiplex assay that is sensitive enough to detect small differences in MECP2 gene dose (50%). Applying this method to 15 male and 15 female normal controls, the ddCt ratio values of the two groups were clearly distinguishable and showed no overlap, indicating high sensitivity and specificity of this assay. Real-time quantitative PCR, when compared to the Southern blot technique, is faster, requires a minimal template, and involves no radioisotopes. The sample throughput of the method is high, since 96 separate reactions (24 separate individuals) can be analyzed in 2 hr. Although real-time quantitative PCR requires specific equipment, the method has proven to be precise and reproducible [Heid et al., 1996; Lie and Petropoulos, 1998; Wilke et al., 2000; Aarskog and Vedeler, 2000].

Using this method, we analyzed eight cases of classical RTT and six cases of PSV, all MECP2 mutation-negative. We identified a deletion in one out of eight classical RTT patients and a duplication in one out of six PSV patients. Previous studies identified deletions in 6 out of 41 mutation-negative classical RTT cases [Bourdon et al., 2001a, 2001b; Schollen et al., 2002; Yaron et al., 2002].

No large rearrangements were previously found in RTT variants (12 tested patients) [Schollen et al., 2002; Yaron et al., 2002] and no specific data were reported for PSV variants. To our knowledge, this is the first report of a large MECP2 duplication in a PSV patient.

Duplications associated with RTT phenotype were never reported. We ruled out the possibility of this rearrangement being a polymorphism, since it was not identified in 100 chromosomes. Using this method, the size of the duplication can not be established. Consequently, we can not distinguish between a duplication of the entire gene and a partial duplication involving the TRD domain, where the MECP2 probe binds to its complementary sequence. The presence of a partial duplication could lead to a disruption of the protein from the TRD domain downstream. On the contrary, the presence of an entire duplication of the gene could suggest that MECP2 is dosage-sensitive. A detailed characterization of the duplication may lead to better define genotype–phenotype correlation.

With the use of this method, there is still a percentage of RTT patients without mutations. This could be due to the presence of a rearrangement at 5′ or at 3′ of the gene that is not detectable with this method or to intronic mutations. To increase the sensitivity of this assay, it will be interesting to test in parallel other parts of MECP2 with primers and probes located in the other exons of the gene.

In conclusion, our results confirm the importance of searching for genomic rearrangements in RTT patients. We demonstrated that a fraction of MECP2-negative patients have large deletions or duplications. We developed a new, fast, and sensitive method to detect such rearrangements and we propose to introduce this technique in the routine screening of MECP2, as a complementary tool to mutation scanning methods. Therefore, the DNA of patients with a clinical suspicion of RTT syndrome or variants should undergo real-time PCR in addition to DHPLC analysis. This association should increase the sensitivity of mutation identification.

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Screening of *MECP2* large rearrangements by Real-time quantitative PCR

Unpublished data

Once developed a Real Time quantitative PCR assay to screen for large rearrangements in *MECP2*, we decided to employ the method to analyze all mutation-negative RTT patients contained in our bank.

Methods

See Ref. (85).

Results

We identified eight new *MECP2* large deletions involving exon 4. In particular, the deletions were found in six sporadic cases with classic RTT and in one familial case with two RTT sisters with strikingly different phenotypes. The pedigree of the family is represented in Fig. 7a. The younger sister is in the IV stage of classic RTT, while the older sister fulfills the criteria for the "highly functioning" PSV (Fig. 7b) (9). Molecular investigation of the entire family by Real Time qPCR revealed a *MECP2* deletion in both sisters, absent in parents (Fig. 7c). A detailed clinical description of both sisters is given below.

II1 (#896). She is the elder sister, now aged 32. The mother had a normal pregnancy and delivery and the child was born pre-term at the 8th month of gestation. 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. She has a good social interaction with a friendly behavior. She is able to speak in simple phrases, 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 exceptionally recover of manual skills (Fig. 7b, left).

II2 (#897). She is the younger sister, now aged 26. The mother had a normal pregnancy and delivery. 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, stipsis, cold extremities and episodes of apnea. She fulfills the criteria for classic RTT (Fig. 7b, right).

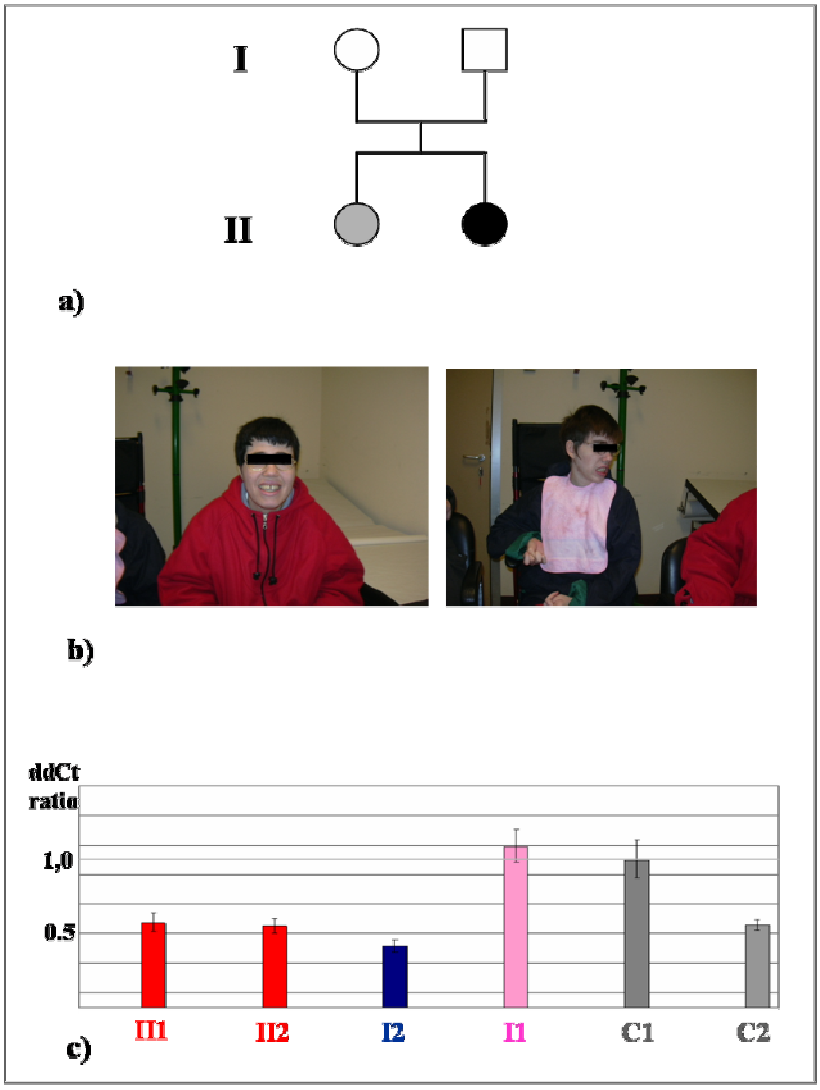


Fig. 7. RTT familial case. a) Pedigree of the family. White symbols represent asymptomatic subjects, the black symbol stands for classic RTT, while the gray symbol stands for "highly functioning PSV". b) The two RTT sisters. On the left, the elder sister classified as "highly functioning PSV"; on the right, the younger sister with a classic RTT phenotype. c) Real Time quantitative PCR results. *MECP2* ddCt ratios and standard deviations of the RTT sisters (II1 and II2), their parents (I1 and I2) and two healthy controls (C1, female; C2, male). The normal control female (C1) and the mother (I1) show ddCt ratios of about 1, indicating a double copy of *MECP2*, while the male control (C2), the father (I2) and the two sisters (II1 and II2) show ddCt ratios of about 0.5, indicating a single copy of the gene.

Characterization of *MECP2* deletions

Unpublished data

We decided to characterize the nine *MECP2* deletions identified by Real Time qPCR. To this aim, we carried out quantitative analysis by Multiple Ligation-dependent Probe Amplification (MLPA). This is a recently developed technique that allows multiplex detection of copy number changes of genomic DNA sequences.

Ordinary multiplex PCR requires one pair of primers for every fragment to be amplified. Given that the efficiency of different primer pairs is not equal, this technique cannot easily be used for relative quantification of target sequences. MLPA reactions are more robust as all fragments are amplified with the use of only one pair of PCR primers. MLPA probes consist of two parts: one synthetic oligonucleotide and one phage M13 derived single stranded DNA fragment (Fig. 8a). Each part contains one of the two sequences recognized by the universal PCR primers. In addition, the M13 derived fragment contains a non hybridizing stuffer sequence of variable length. The two parts of the MLPA probes are then hybridized to each target sequence, enzymatically ligated and products are amplified using the universal PCR primers (Fig. 8b, 8c and 8d). The stuffer sequence makes amplification products different in length and allows to identify them by size separation. In the analysis, the signal strengths from the probes are represented by corresponding peak areas (Fig. 8e). Peak areas are then compared with those of a control individual.

For MLPA analysis of *MECP2,* we used a specific probe mix developed by MRC Holland. This mix contains probes for exons 1, 2, 3, and 4 of *MECP2* and three additional probes for the flanking genes *IRAK1*, *L1CAM* and *SYBL1*.

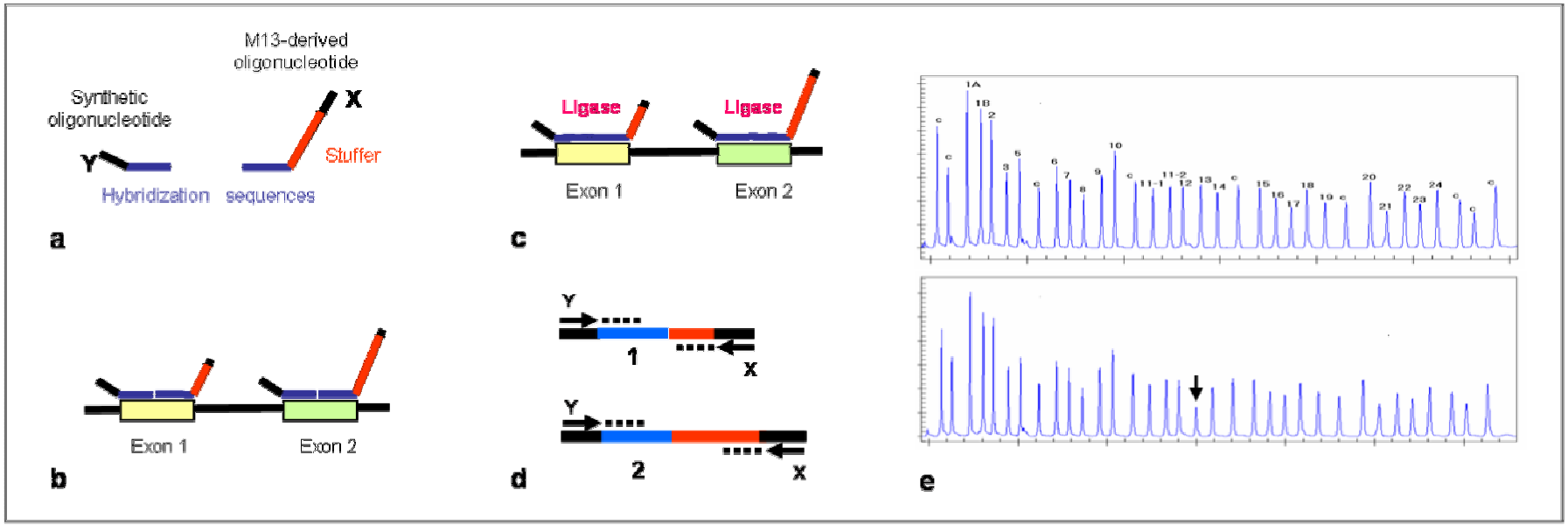


Fig. 8. Principle of MLPA. a) MLPA probes. The synthetic oligonucleotide contains the sequence recognized by the universal primer Y (in dark), while the M13 derived fragment contains the specific sequence for universal primer X (in dark) and the stuffer sequence (in red). Target sequences are indicated in blue. b) Hybridization of MLPA probes. Genomic DNA is denatured and the two parts of each MLPA probe are hybridized to the target sequences. c) Ligation reaction. Only perfectly matched probes are ligated by a thermostable ligase. d) PCR reaction. All the ligated probe products are amplified by PCR using only one primer pair (X and Y). The amplification product of each probe has a unique length and can be identified by size separation. e) Example of fragment analysis by capillary electophoresis. Analysis of a control DNA (upper panel) and a test DNA (lower panel). Compared to a control reaction, the relative peak area of each amplification product reflects the relative copy number of the target sequence of that probe in the analyzed sample. Here, the analysis of test DNA shows a decrease in peak area of exon 13 compared to control, indicating the presence of a deletion.

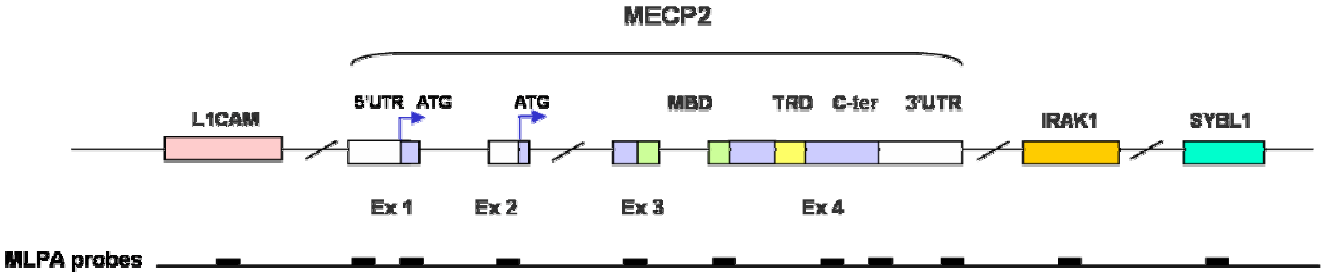
Methods

We analyzed *MECP2* by MLPA using probe mix P015 (MRC Holland). The analysis was carried out as already described by Schouten (86). 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 (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.

Results

MLPA analysis showed that all rearrangements are partial deletions of the *MECP2* gene (Fig. 9). In fact, all deletions involve exon 3 and 4, but not exon 2 of *MECP2*. In addition, in all samples, the *L1CAM* gene is not involved in the rearrangement. Among the four deletions involving the 3' amplicon of exon 4 (4d), two extend to involve the *IRAK1* gene and only one both *IRAK1* and *SYBL* genes (Fig. 9). In the familial case, MLPA results showed that the deletion in the two RTT sisters (#896 and #897) involves exon 3 and partially exon 4 (amplicons 4a, 4b and 4c), suggesting that they probably harbor the same rearrangement.

a



b

c

		MECP2									
Samples	L1CAM	1a	1b	2	3	4a	4b	4c	4d	IRAK1	SYBL1
#581	1,02	0,93	0,78	0,80	0,40	0,33	0,43	0,45	1,07	0,85	0,97
#100	0,97	0,87	0,70	0,80	0,32	0,35	0,62	0,49	0,59	0,54	0,89
#263	1,05	1,06	0,91	0,86	0,44	0,40	0,35	0,54	1,19	0,97	0,82
#919	0,72	1,03	1,02	0,82	0,47	0,26	0,56	0,45	0,56	0,94	0,94
#1055	1,02	0,96	1,02	0,80	0,51	0,35	0,51	0,43	0,47	0,46	0,86
#951	0,97	1,02	0,73	0,90	0,46	0,28	0,59	0,55	0,69	0,55	0,62
#1004	0,97	1,05	0,97	0,88	0,37	0,38	0,43	0,96	1,10	1,06	0,93
#896	0,93	1,01	1,06	0,93	0,38	0,46	0,38	0,46	1,07	1,08	0,77
#897	0,92	1,02	0,91	0,91	0,38	0,39	0,59	0,55	1,1	0,89	0,90

Fig. 9 Characterization of MECP2 deletions. a) MECP2 and flanking genes (not to scale). The four exons of MECP2 are shown. The coding sequences of MECP2 are in blue. The Methyl-CpG-Binding Domain (MBD) is in light green and the Transcription Repression Domain (TRD) in yellow. b) Position of MLPA probes. c) Results of the MLPA analysis for each probe. Samples codes are shown on the left. Values indicating a half gene dosage are in red.

Characterization of the *MECP2* duplication

Unpublished data

By Real Time quantitative PCR, we identified a PSV patient with an additional copy of *MECP2* (85). Quantitative analysis of parents DNA revealed that they have a normal *MECP2* gene dosage. In addition, we ruled out the possibility that this rearrangement may be a polymorphism since it was not identified in 100 chromosomes. To characterize the rearrangement, we performed an array-CGH experiment.

Methods

Genomic DNA of a normal female control was obtained from Promega. Genomic DNA of the patient was isolated from an EDTA peripheral blood sample by using a QIAamp DNA Blood Kit according to the manufacturer protocol. We used the OD260/280 method on a photometer to determine the appropriate DNA concentration.

Ten micrograms of genomic DNA both from the patient (test sample) and the control (reference sample) were sonicated. Test and reference DNA samples were subsequently purify using dedicated columns (DNA Clean and Concentrator, Zymo Research) and the appropriate DNA concentrations were determine by a DyNA Quant™ 200 Fluorometer (GE Healthcare).

Array based CGH analysis was performed using commercially available oligonucleotide microarrays containing about 43.000 60-mer probes with an estimated average resolution of approximately 75 kb (Agilent Technologies). DNA labelling was executed according to the Agilent protocol (Oligonucleotide Array-Based CGH for Genomic DNA Analysis 2.0v). Labelled samples were subsequently purified using CyScribe GFX Purification kit (Amersham) according to the manufacturer protocol. Test and reference DNA were pooled and mixed with 50 µg of Human Cot I DNA (Invitrogen), 50 µl of Blocking buffer (Agilent Technologies) and 250 µl of Hybridization buffer (Agilent Technologies). Before hybridization to the array, the mix was denatured at 95° C for 7 minutes and then pre-associated at 37°C for 30 minutes. Probes were applied to the slide using an Agilent microarray hybridization station. Hybridization was carried out for 40 hrs at 65° in a rotating oven (20 rpm). The array was disassembled and washed according to the manufacturer protocol. The slides were scanned using an Agilent G2565BA DNA microarray scanner. Image analysis was performed using the CGH Analytics software v. 3.1 with default settings.

Results

Array-CGH unexpectedly showed the presence of an additional X chromosome in the PSV patient. Clinical features of females with the 47,XXX karyotype (also called triple X syndrome) are not medically significant. Consequently, the altered *MECP2* gene dosage probably is not involved in the PSV phenotype.

Screening of mutations in exon 1

Unpublished data

In 2004, two different studies have identified an alternatively splice transcript of *MECP2* consisting of exons 1, 3 and 4 leading to a new protein isoform (MeCP2_e1), which represents the predominant form in the brain (Fig. 2) (51) (52). Exon 1 was previously considered non coding and was therefore excluded from *MECP2* mutation analysis. Mnatzkanian et al. examined 19 mutation-negative RTT patients and identified two girls with distinct deletions in exon 1(52).

Given these results, we decided to analyze exon 1 in our collection of *MECP2*-negative RTT patients.

Methods

We PCR amplified exon 1 of *MECP2* using the following primers: *MECP2*_1F: 5'-GGAGAGAGGGCTGTGGTAAAAG-3' and *MECP2*_1R: 5'-CATCCGCCAGCCGTGTCGTCCG-3'. Mutation analysis was performed by Denaturing High Performance Liquid Chromatography using the Transgenomic WAVE^{TM} (Transgenomic, San Jose, CA, USA). 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.

Results

In one case, a girl with classic RTT (#86), we found a deletion of 11 bp, c.47_57del (Fig. 10). The mutation creates a frameshft and a premature stop codon (p.Gly16GlufsX36). The mutation was not found in parents. A clinical description of the patient is given below.

Proband (#86)

The patient died at 22 years. She was examined in our Unit when she was 20. Pregnancy and delivery were normal. Her development was normal until 19 months when she showed motor regression. At 5 years of age generalized convulsions, stereotypic hands activities and hyperventilation were noted. At the time of the examination her head circumference was 52 cm (<3° cnt), weight was 36 Kg (<5° cnt) and height was 146 cm (<5° cnt). She was able to walk with support and to stand alone only for few moments.

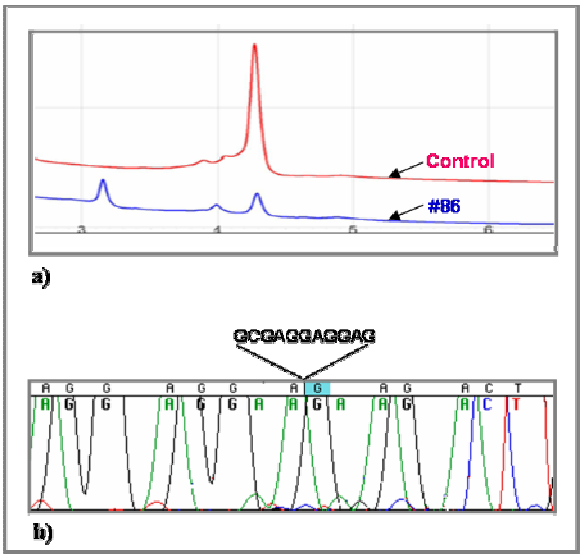


Fig. 10. Exon 1 molecular analysis of patient #86. a) DHPLC profiles of RTT patient (in blue) and a control individual (in red) at 68.8°C. The control shows a unique peak (homoduplex molecule), while sample #86 shows additional peaks (homoduplex plus heteroduplex molecules). b) Sequence chromatogram of deleted exon 1 allele in patient #86. The 11 bp deleted bases are shown above the chromatogram.

Result 3.3

Identification of a new RTT gene

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

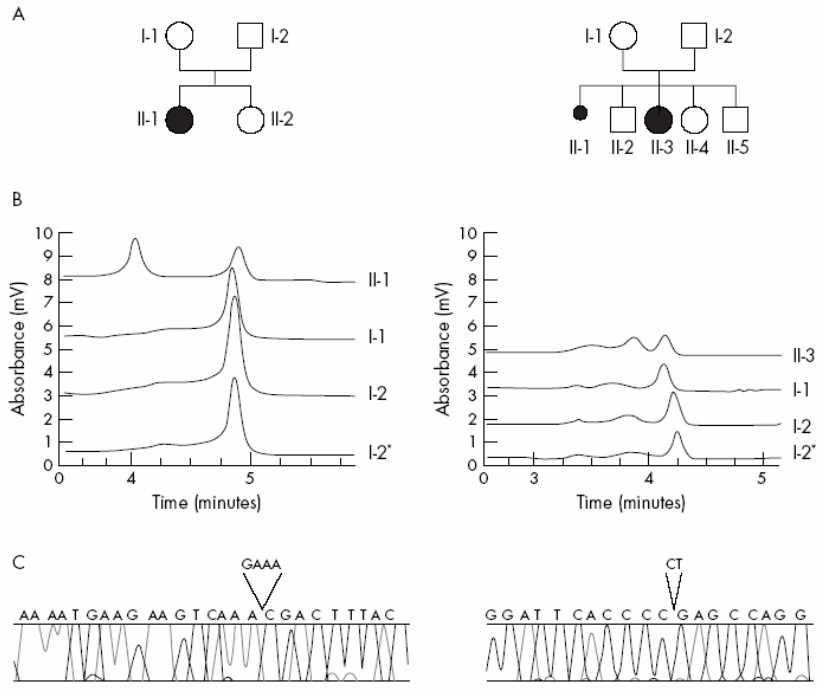

Figure 1 Pedigree (A), DHPLC analysis (B), and DNA sequencing (C). Left side, case 1, right side, case 2. (B) An asterisk indicates that the DNA of the father is 1:1 mixed with a control male. (C) The chromatograms represent the mutated sequences separated from normal alleles on 6% polyacrylamide gel.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

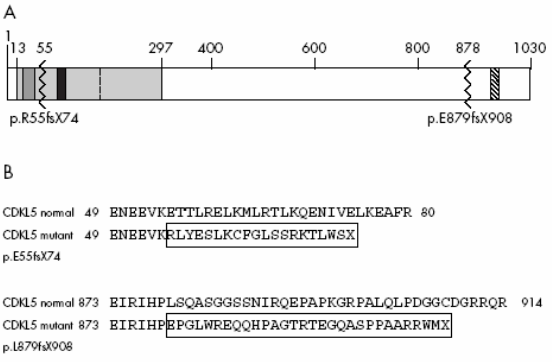

Figure 2 CDKL5 protein with mutation positions (A) and alignment between the normal and the mutated amino acid sequences (B). (A) The catalytic domain (light grey) contains an ATP binding site (dark grey) and the serine–threonine protein kinase active site (dark box). The conserved Thr-Xaa-Tyr motif 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

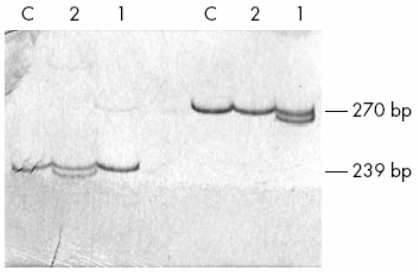

Figure 3 RT-PCR analysis of patients (1 and 2) and a control individual (C). The polyacrylamide gel shows RT-PCR products. RT-PCR products spanning exons 17–18 (left) and exons 4–6 (right). In both cases, the mutant allele is evident as a lower band. Size is indicated on the right.

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.[25] 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.[23 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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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

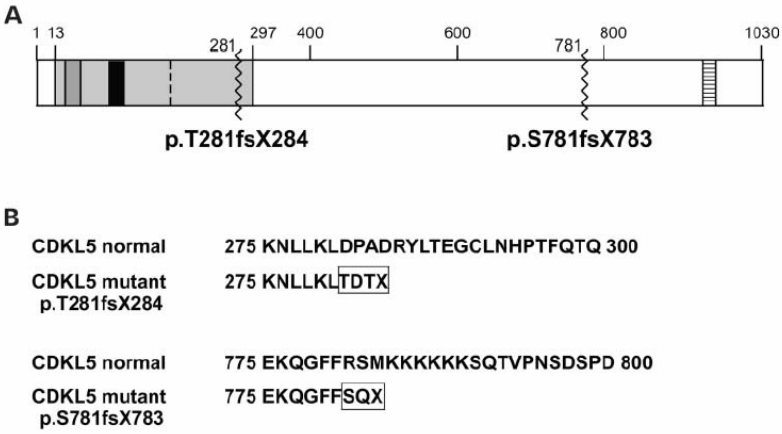

Figure 1. Schematic representation of the CDKL5 protein with mutation position and alignment between the normal and the mutated amino acid sequences. (**A**) The predicted human CDKL5 protein and the positions of the new identified mutations are represented. The catalytic domain (light gray box) contains 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

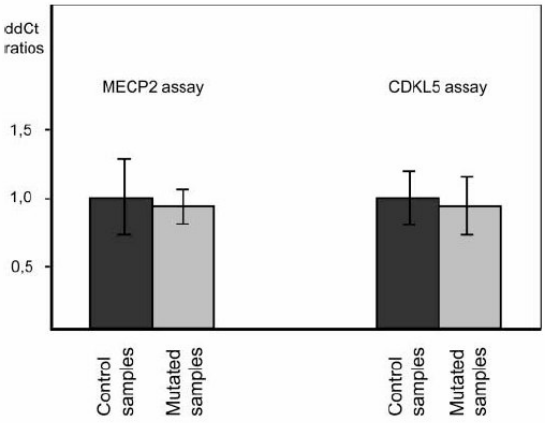

Figure 2. Real-time qPCR analysis. On the left, *MECP2* assay. Mean values of *MECP2* ddCt ratios and standard deviations (SD) of five control samples (dark gray column) and three *MECP2* mutated samples (light gray column). On the right, *CDKL5* assay. Mean values of *CDKL5* ddCt ratios and SD of five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

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

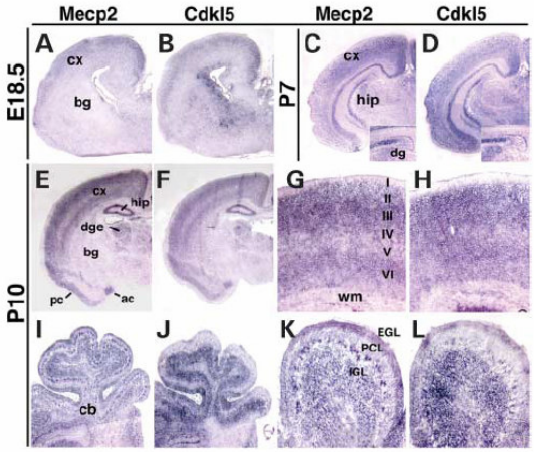

Figure 3. Mecp2 and Cdkl5 expression patterns during neural differentiation. Mecp2 (**A**) and Cdkl5 (**B**) expressions are first detected in E18.5 brains in the neurons migrated into the cerebral cortex (cx). (**C** and **D**) Sections of posterior forebrain hybridized for Mecp2 and Cdkl5, respectively. 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.

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 Mecp*2* 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

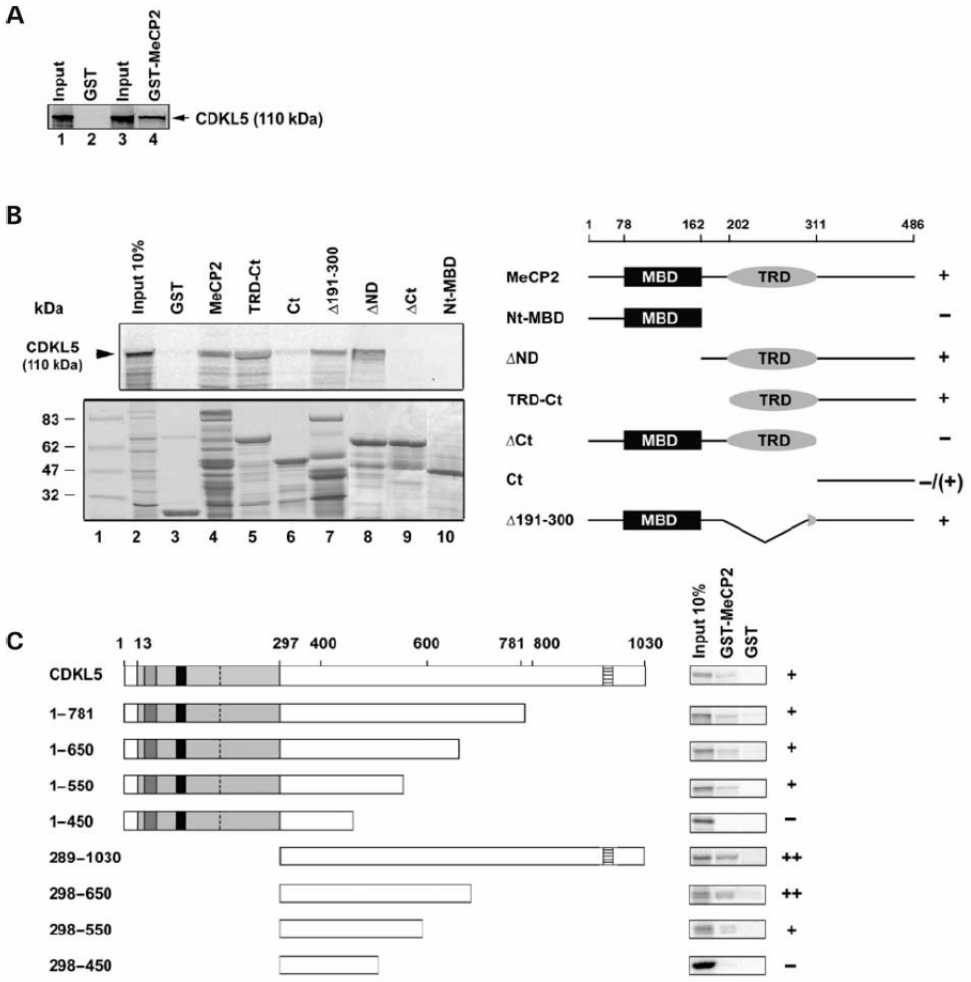

Figure 4. CDKL5 and MeCP2 interact *in vitro*. (**A**) GST pull-down assay in which *in vitro* translated [^{35}S]methionine labeled CDKL5 was incubated with immobilized recombinant GST and GST–MeCP2. Retained proteins were separated on an 8% SDS–PAGE and CDKL5, indicated to the right, were visualized 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

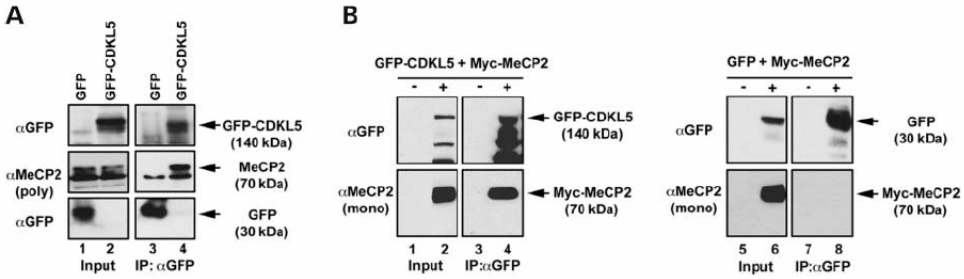

Figure 5. CDKL5 and MeCP2 interact also *in vivo*. (**A**) Coimmunoprecipitation of overexpressed GFP–CDKL5 and endogenous MeCP2. The left panels show a western blot on 10% of the GFP (lane 1) and GFP–CDKL5 (lane 2) human 293T cell extracts used for the immunoprecipitation. To the right, the figure shows 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

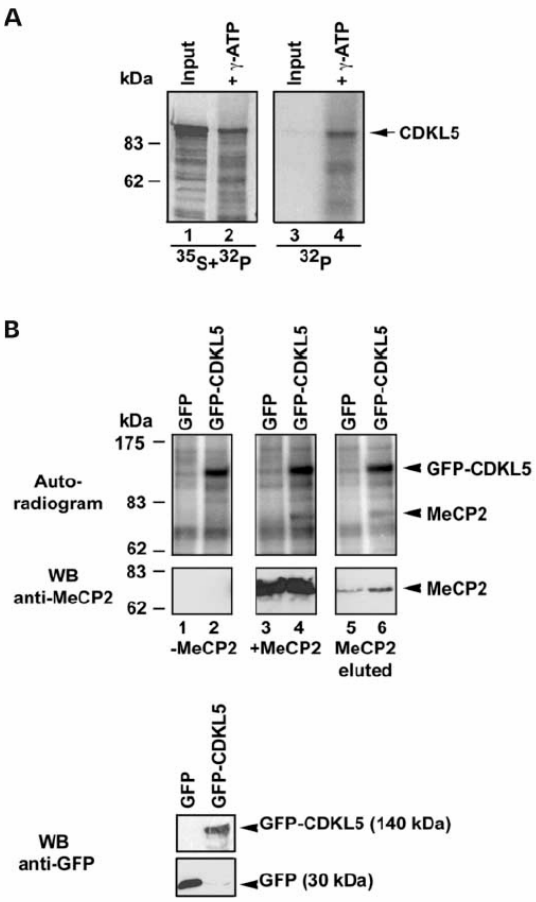

Figure 6. CDKL5 possesses a kinase activity and mediates MeCP2 phosphorylation. (**A**) Autophosphorylation assay in which CDKL5 translated *in vitro*, in the presence of [^{35}S]methionine, was incubated with [γ-^{32}P]ATP for 30 min at 30°C. Proteins were fractionated on an 8% SDS–PAGE, the gel dried 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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4. Discussion

4. Discussion

The aim of this work was to identify the genetic defect in mutation-negative RTT patients. We used a combined strategy based on both the improvement of traditional techniques employed for *MECP2* analysis and the search of other genes involved in RTT. By this approach, we identified the molecular cause in 14/74 (19%) mutation-negative RTT patients. In particular, we identified the genetic defect in 9/25 (36%) classic and in 5/49 (10%) atypical *MECP2*-negative RTT patients.

Among classic RTT cases, we found a high percentage of *MECP2* large deletions (32%). Combining the results obtained by other quantitative studies performed in classic RTT patients, the percentage of mutation-negative cases with *MECP2* large deletions is similar (87-93). These results clearly indicate that large deletions of *MECP2* are an important cause of classic RTT and confirm the importance of quantitative studies in a complete diagnostic strategy.

Subsequent analysis by MLPA showed that all *MECP2* deletions identified involve exon 3 and 4, but not exon 2, suggesting that one breakpoint is located in intron 2. These results are in agreement with a previous study conducted by Laccone et al. (90). They characterized a series of *MECP2* large deletions and found that the majority of proximal breakpoints were located in intron 2. Examination of the *MECP2* genomic sequence revealed that intron 2 contains a series of repetitive elements (mostly Alu repeats) and the authors suggested that these elements represent potent factors contributing to genomic rearrangements. Furthermore, they found that the distal breakpoint of *MECP2* deletions frequently occurs in a specific region of exon 4 (DPR, deleted prone region, from c.1057 to c.1207 of the coding sequence; Locus: XM_048395 counting from the first translated ATG). Sequence analysis revealed the presence of a perfect χ sequence in the DPR, suggesting that it may contribute to generate the rearrangements. In contrast, in our group of RTT patients, MLPA analysis showed that the DPR may represent a recombinogenic element in only one case (#1004). These results suggest that other mechanisms are involved in our RTT cases.

Among classic RTT cases, we also found a mutation in the recently identified exon 1 of *MECP2* in one out 25 (4%) mutation-negative patients. No exon 1 mutations were found in atypical RTT cases of our bank. The other groups who identified pathogenic mutations in exon 1 reported discordant data about their overall prevalence in classic or atypical RTT (52) (92) (94-96). Amir et al. reported the more comprehensive study,

analyzing a large group of mutation-negative RTT patients (95). They found that mutations in exon 1 account for 3% of classic cases and 4% of atypical RTT. All these results suggest that mutations in exon 1 are not a common cause of RTT. However, given the identification of exon 1 mutations in both classic and atypical RTT cases, analysis of this exon should be included in a complete molecular test for the syndrome.

The deletion of 11 bp found in patient #86 had been previously reported by Mnatzakanian (52). The same deletion was successively identified by other three groups (92) (95) (96). The high frequency of this mutation could be due to the fact that it occurs in a region with an AAG repeat which could be prone to recurrent de novo deletions. This mutation results in a frame-shift and generates a missense protein sequence after aminoacid 15, which stops after amino acid 36 in the MeCP2_e1 protein isoform. In contrast, the reading frame of the MeCP2_e2 isoform is unaffected, as the deletion is positioned 103 nucleotides upstream of the e2 protein translation start site. Recently, Saxena et al. investigated the effect of this frequent deletion on both RNA transcription and protein translation (96). They found that, although RNA expression for both protein isoforms was detected from the mutant allele, evaluation of MeCP2 protein in uncultured patients lymphocytes by immunocytochemistry revealed that MeCP2 protein production was restricted. These data demonstrated that translation but not transcription of the MeCP2_e2 isoform is ablated by the deletion, suggesting that nucleotides within the deleted sequence in the 5'-UTR of the e2 trancript are essential for protein translation.

Comparison between clinical features of all reported RTT cases with the same 11 bp deletion in exon 1, does not suggest a clear genotype-phenotype correlation. In fact, we identified the deletion in a girl with classic phenotype but the other studies found the mutation in both classic and atypical RTT cases (52) (92) (95) (96). Saxena et al. hypothesized that the exon 1 deletion may be milder in its clinical consequences but this is in contrast with our results. In fact, patient #86 was severely affected and died at 22 years. The difference observed in the severity of the disease between patients bearing the same mutation could be due to different pattern of X-inactivation or modifying factors.

Among atypical RTT patients, we found only one *MECP2* large deletion in a patient classified as "highly functioning" PSV. This patient represents a very interesting familial case. In fact, this girl is the elder sister of a RTT patient with a classic phenotype. Quantitative analysis by Real Time PCR revealed the presence of a *MECP2* large deletion involving exon 4 in both sisters. Quantitative analysis by MLPA showed that the deletion involves exon 3 and partially exon 4 in both patients. Although breakpoints have not been

cloned, the two girls probably harbour the same rearrangement. In addition, quantitative analysis of the entire family revealed that the *MECP2* deletion is absent in parents. To explain the origin of the deletion in the two sisters, two alternative hypotheses can be formulated. The first hypothesis is that one of the two parents can be a mosaic for the mutation in the gonadic tissue. The alternative hypothesis is that the mother bears a cryptic chromosomal rearrangement in Xq28, possibly an inversion. During meiosis, the inversion may produce gametes with chromosomal imbalances.

At present, the frequency of *MECP2* large deletions in RTT variant cases is not well defined. There is only another study which investigated the presence of such rearrangements in a large group of atypical RTT patients (93). The authors detected *MECP2* large deletions in 4 out of 53 (7,5%) mutation-negative patients with atypical RTT, but the lack of an accurate clinical classification does not allow to define the frequency of such rearrangements in PSV cases and other variants.

The story of the *MECP2* duplication is very instructive. On one side, it teaches that the diagnosis of RTT variants is quite difficult on a clinical basis. On the other side, it teaches that standard karyotype is a fundamental step in a diagnosis setting, even when the clinical hint has a specific direction.

Presently, we do not know whether the patient is a phenocopy of PSV bearing a point mutation in a yet unknown gene or the triple dosage of the X chromosome in combination with other factors, such as the status of X-inactivation, induces an over-expression of the *MECP2* gene. Actually, we are performing Real Time quantitative experiments on RNA extracted from the lymphoblastoid cell line of the PSV patient to define whether the RNA levels are altered respect to other triple X healthy individuals. All these results will be published together with the characterization of the *MECP2* deletions, possibly in the same journal of the original publication, in the aim to correct the information for the scientific community.

Among atypical RTT cases, we also identified mutations in another gene, *CDKL5* (OMIM#300203; also known as *STK9*), demonstrating the genetic heterogeneity of the syndrome. In particular, we found *CDKL5* mutations in 4/49 (8%) atypical patients. Interestingly, all four *CDKL5* mutations were identified in patients with a specific RTT phenotype, largely overlapping the infantile seizure onset variant. Consequently, in our experience, *CDKL5* mutations seem to be associated with this specific RTT variant. In particular, we detected *CDKL5* mutations in 40% (4/10) of infantile seizure onset variant

cases of our collection. However, the analysis of a larger number of patients is necessary to establish the exact frequency of *CDKL5* mutations in RTT.

Chromosomal rearrangements at the *CDKL5* locus have been previously associated with other neurological phenotypes. In two unrelated female patients with West syndrome a *de novo* balanced X-autosome translocation was shown to disrupt the *CDKL5* gene (97). In a male patient with X-linked retinoschisis (XLRS) and seizures, which are not typically associated with XLRS, a large deletion involving at least the last codon of *CDKL5* and the flanking *RS1* gene was identified (98). From these studies, it appears that the phenotype associated with *CDKL5* gross rearrangements is variable and this phenotypic heterogeneity may be at least in part explained by alterations in contiguous genes. Other recent reports have identified *CDKL5* point mutations in patients with slightly different neurological phenotypes (99-101). Since these patients are seen by different clinicians, part of this variability may be due to different clinical sensitivity. Moreover, *CDKL5* mutations may cause both the infantile seizure onset variant of RTT and a less defined phenotype ranging from autism to mental retardation. However, in all cases, a seizure disorder appears to be a prominent and integral manifestation of the disease.

CDKL5 point mutations reported so far vary from substitutions in the N-terminal catalytic domain to frameshift mutations in the N- or in the C-terminal portion of the protein (99-103). Mutations located in the conserved kinase domain probably influence the catalytic activity of the protein. A recent study demonstrated that removal of the C-terminal domain increases *CDKL5* expression, enhances autophosphorylation activity and causes perinuclear localization, indicating that mutations affecting the C-terminus can modify these functions (104).

The observation that mutations in *MECP2* and *CDKL5* cause similar phenotypes suggests that these genes may be involved in the same molecular pathway. To verify this hypothesis, we compared the expression patterns of *Mecp2* and *Cdkl5* in embryonic and postnatal mouse brains and we demonstrated that the two genes have an overlapping temporal and spatial expression profile during neuronal maturation and synaptogenesis. We then demonstrated that the two proteins physically interact and that CDKL5 is indeed a kinase able to phosphorylate itself and to mediate MeCP2 phosphorylation.

In a recent study, Lin et al confirmed the interaction between MeCP2 and CDKL5, but their results did not suggest that MeCP2 is a direct substrate of CDKL5 (104). Consequently, the authors hypothesized that MeCP2 may act to recruit CDKL5 to a DNA binding complex that contains a functional substrate of the kinase.

In conclusion, all these results support the hypothesis that the functions of *MECP2* and *CDKL5* are tightly linked and contribute to explain the involvement of *CDKL5* in RTT. Additional studies are necessary to define this new molecular pathway, other components of which could be responsible for the remaining percentage of mutation-negative RTT cases.

5. Future perspectives

Based on the results obtained in the last few years, which allowed us to have a clearer picture of the molecular defects in typical and atypical RTT cases, we decided to go ahead and to investigate the mechanisms leading from a mutation to disease pathogenesis. To this aim, we plan to investigate the consequences of *MECP2* and *CDKL5* mutations in RTT lymphoblastoid cell lines.

Recently, we demonstrated that the two genes have an overlapping temporal and spatial expression profile during neuronal maturation and synaptogenesis (103). In addition, we demonstrated that the two proteins physically interact and that CDKL5 is able to mediate MeCP2 phosphorylation (103). These results suggest that the two proteins might belong to the same molecular pathway and that CDKL5 might be involved in MeCP2 regulation.

Considering these data and the similar phenotypes caused by mutations in *MECP2* and *CDKL5*, we hypothesized that at least some of the genes differentially expressed in the absence of MeCP2 or CDKL5 might be the same. These common genes probably represent important factors in disease pathogenesis of RTT.

To verify this hypothesis, we will perform microarray gene expression profiling on RNA isolated from lymphoblastoid cell lines of RTT patients with *MECP2* or *CDKL5* mutations and we will compare their expression profiles with those of controls. The genes differentially expressed in *MECP2* or *CDKL5* mutated patients will be then compared to determine whether it is possible to define a common “molecular signature” for RTT.

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- 2000-2001: worked as student in the Research Project: "Molecular and cytogenetic analysis of Silver-Russell syndrome", under the supervision of prof. Maria Luisa Giovannucci Uzielli, Medical Genetics, Meyer Pediatric Hospital of Florence, Italy.

- 2002-today: worked as Ph.D student in the Research Project: "Identification of the molecular defect in MECP2-negative Rett patients" under the supervision of prof. Alessandra Renieri, Medical Genetics, Department of Molecular Biology, University of Siena, Italy.

List of publications

Articles:

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