Clinical genetics of Rett syndrome and mental retardation

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INTRODUCTION

Aims of the thesis

During the course of my PhD program I was mainly involved in genetic counseling. I followed patients with different phenotypes, the majority of which were pediatric and adult patients with Rett syndrome (RTT), RTT variants and mental retardation. A large part of these cases were admitted to Child Neuropsychiatry of the General Hospital of Siena, others to Neurology and Pediatrics.

Given the huge number of Rett patients afferent to our Medical Genetics Unit I gained experience in RTT both from a clinical, as well as a molecular point of view. I am now able to consider a possible diagnosis of RTT or of a RTT variant and to suggest an appropriate molecular analysis. The RTT story arose at the beginning of my PhD program, towards the end of 2001. MECP2, the gene responsible of RTT had already been known since 1999. During genetic counselling I had the opportunity to personally seeing almost 100 RTT families. Some of them were met only once and others were followed for years in order to reach a definitive molecular diagnosis or to provide the parents with pre-conceptual counselling and eventually with prenatal diagnosis. My thesis will focus on the wide clinical spectrum of RTT mostly from a clinical genetics point of view.

We became familiar with the preserved speech variant of RTT (PSV) and for the first time we described a duplication of the MECP2 gene in a PSV. An extensive clinical description of this patient will be part of my thesis.

The possibility to see patients directly made it possible for us to analyze new genes in MECP2 negative RTT patients and RTT-like phenotypes. With this strategy we were able to demonstrate that the CDKL5 gene is involved in RTT and it would appear responsible for a specific phenotype: the early seizure variant of RTT.

By adopting the clinical approach many hints for research became evident. As will be extensively described in the body of the thesis, RTT is mostly caused by “de novo” mutations in the MECP2 gene and thus the recurrence risk for the following pregnancy of a couple with a RTT girl is considered very low. The recurrence risk is not null since very few cases of germ-line mosaicism have been described
(recurrence of the disease in sibs with an apparently de novo mutation). I witnessed the first case of germ-line mosaicism in RTT identified by prenatal diagnosis. The couple with the positive prenatal test decided to interrupt the pregnancy and to devolve fetal tissues of the affected fetus for research purposes to our Institute. Thanks to the availability of these tissues we are designing a panel of experiments aimed at the evaluation of the consequences of MECP2 absence on brain development at this stage.

Since the RTT phenotype is almost exclusively seen in female patients, it has been thought for a long time that MECP2 mutations could be lethal in males. In the near past we have described a phatogenic MECP2 mutation in two related male patients with severe mental retardation and progressive spasticity. This report demonstrated that, in males, MECP2, can be responsible for severe mental retardation associated with neurological disorders. In a section of this thesis I will describe a family with two brothers with microcephaly and mental retardation and some female relatives with isolated microcephaly. Probandes were tested for MECP2 mutations and resulted negative. A possible X-linked semi-dominant inheritance was hypothesized and a linkage study on the X chromosome was performed.

Different considerations have to be made for mentally retarded patients for whom a specific condition has been excluded. Attempts to give specific guidelines for diagnostic investigations aimed at detecting the etiological diagnosis in patients with mental retardation have been made in recent years. However, there is not yet a worldwide consensus especially for cytogenetic and molecular investigations. As already mentioned, I had the opportunity of seeing many sporadic or familial cases with mental retardation, either syndromic or not. In the last part of this thesis I will report on a male patient with severe mental retardation and mild dysmorphic features in whom an interstitial deletion of the short arm of chromosome 3 was identified. I will discuss the phenotype and review the literature of the very few cases with deletions overlapping with that of our patient.

Given the enormous and precious number of patients we currently visit during genetic counselling we decided to create a bank for both RTT and XLMR patients. Biological samples (lymphoblastoid cell line and DNA) of each patient are stored and are available for researchers after intent statement. The bank also contains clinical and genetic information of each patient. Selected data are also accessible to
the general public without a password. RTT bank includes only patients afferent to our Institute, while XLMR bank contains patients seen in clinical centers, including ours, spread all over Italy. A detailed description of RTT and XLMR databases will be reported in this thesis.
Rett syndrome

Rett syndrome (RTT, OMIM#312750) is a progressive neurodevelopmental disorder almost exclusively affecting females. It was first described by the Austrian pediatrician, Andreas Rett, who casually observed, in the waiting room of his surgery, two girls having similar stereotypic hand movements. Afterwards Andreas Rett, reviewing patients he had observed in the past, found other cases with similar stereotypic hand movements and clinical history and published an article in 1966 where he described all the observed patients (Rett 1966). However, the article by Andreas Rett was ignored for years. In 1983 a collaborative study on 35 patients of a group of child neurologists, such as Hagberg, Aicardi, Dias e Ramos, awakened the interest on RTT and made the syndrome known worldwide (Hagberg et al. 1983).

RTT is the second most frequent cause of mental retardation in females. It has an incidence of approximately 1/10,000-15,000 female births. RTT patients show a well-defined clinical course and peculiar characteristics. RTT is an X-linked dominant condition mostly caused by “de novo” mutations in the MECP2 gene, coding for a transcriptional silencer (Amir et al. 1999).

An analytic clinical analysis of a huge number of RTT patients allowed Hagberg and Witt-Engerström to define a characteristic clinical course of the syndrome, which can be divided into 4 stages (Hagberg and Witt-Engerstrom 1986). Affected girls usually have a normal birth and neonatal course followed by apparent normal psychomotor development during the first 6-18 months of life. Analysis of retrospective data shows that the majority of these children have mild hypotonia and they are described as very placid infants. Afterwards patients show a short period of developmental arrest (stage 1, stagnation stage) followed by a rapid regression in language and motor skills between 1-3 years (stage 2, rapid destructive/regression stage). The hallmark of the disease is the loss of purpose hand use and its replacement with repetitive, usually mid-line, stereotyped hand movements (Fig. 1). During stage 2 the syndrome becomes more recognizable. The girls show a deterioration of mental functioning, they lose verbal language if already acquired and autistic features appear. Additional characteristics include
Figure 1. Particular of the hands of RTT girls, showing the typical stereotypic hand movements: hand twist and wringing (a-b), hand washing (c), conjunction of hands in the mid-line which are brought frequently to the mouth (hand-mouthing activities) (d), hand clapping (e), fingers movement with the hand in a mid-line position (f) and rolling pills movements of the fingers (g-h).

bruxism, episodic apnea and/or hyperpnea, seizures or EEG anomalies, gait ataxia and apraxia, and deceleration of head growth, which can result in acquired microcephaly. The following stage is called pseudostationary stage and is apparent between 2 and 10 years of age. During this stage (stage 3) there is an amelioration of autistic features and the growth failure and scoliosis become more evident. Stiposis and cold extremities can be also present. The fourth and last stage is characterized by a late motor deterioration. It is present usually after 10 years of age and girls can show a spastic quadripareisis and they can necessitate of an artificial feeding (Fig. 2).
Figure 2. A 12 year-old patient with classic RTT in stage 4. Note the spastic paraparesis and the thin habitus. Reproduced with permission from Zappella et al 2001 (Zappella et al. 2001).

Making a clinical diagnosis of RTT is not a simple task, especially in very little girls (stage 2) or when the affected girls are already in the fourth stage. Besides specific signs or symptoms, the peculiar clinical course of the syndrome helps in the diagnosis. Therefore, the young girl should be followed over the years. Clinical experience, a high degree of “sensitivity” to developmental history and systematic follow up, sometimes for years, will provide the best hints. For the older patients, accounts by mothers, who are in the majority of cases astute observers, can result illuminating.

At a distance from the first original reports (Rett 1966; Hagberg and Witt-Engerstrom 1986) it has become clear that the RTT phenotype is much more variable than originally believed. Besides the classic form several RTT variants have been described. The phenotypic spectrum of RTT varies from the most severe cases, including the congenital form and the early seizure variant or Hanefeld variant, to the milder forms, comprising the “forme fruste”, the preserved speech variant (PSV) and the late regression variant (table 1). Together with the clinical variation in RTT phenotypes it also has to be considered that there is overlap between the variants of RTT, and that the borderlines between these are far from clear. For instance, Hagberg and Gillberg stated that the three girls who were described by Zappella in 1992 as having preserved speech variant could also be defined as “forme fruste” (Hagberg 1993, Zappella, 1992). Furthermore they assert that some patients described as having the early seizure variant were not widely discrepant from cases
described as congenital RTT (Hagberg 1993). This wide clinical spectrum led clinicians to establish in 1988 and 1994 strict clinical criteria for diagnosis of classic RTT cases and variant of RTT, respectively (Trevathan and Moser 1988; Hagberg and Skjeldal 1994).

In 1999, the MECP2 gene was found to be responsible for RTT (Amir et al. 1999). Until today MECP2 mutations have been found both in classic RTT patients and in a minority of RTT variants cases. Is RTT synonymous with MECP2 mutations? This question could be appropriate for many other conditions. Once the molecular cause of a disease has been found we become aware that the clinical spectrum is larger than previously believed. Classic RTT occurs with and without MECP2 mutations. In fact, only 80% of classic RTT cases harbors mutations in the MECP2 gene. Thus the diagnosis of classic cases remains clinical. There is the possibility that we are missing mutations in the MECP2 negative patients given the not absolute sensibility of the available techniques. However, MECP2 mutations are less frequently found in RTT variant patients. This fact can be better explained by genetic heterogeneity. Recently, mutations in the CDKL5 gene have been found in patients with the early seizure variant of RTT, demonstrating that other genes than MECP2 can be responsible of a RTT-like phenotype (article 3; Tao et al. 2004; Weaving et al. 2004). Is it possible that these two genes, given the overlapping phenotypes they cause, can be part of the same molecular pathway? Attempts to find a connection between the two proteins are ongoing. Probably other genes associated with RTT-like phenotypes will be rapidly discovered.

Table 1. Rett syndrome and variants.

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<td>Classic Rett</td>
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Mental retardation

Mental retardation (MR) is the most frequent cause of serious handicap in humans, and an important health-care problem throughout the world. It is characterized by a significantly sub-average intellectual functioning in conjunction with significant limitations in adaptive functioning, occurring before age 18 (Association 1994). Intellectual functioning is commonly determined by performance in a series of standardized tests that allow the definition of the “Intelligence Quotient” (IQ) of an individual. Assuming a population mean IQ of 100, MR can be divided into four categories, based on IQ measurements: MILD with an IQ between 50 and 70; MODERATE, with an IQ between 35 and 50; SEVERE, with an IQ between 20 and 35 and PROFOUND with an IQ below 20.

It is calculated that moderate to profound MR has a prevalence of 0.3-0.5% in the population, and that this prevalence increases to about 1-1.5% when mild MR is included in the estimate (Chelly and Mandel 2001). The causes underlying the majority of MR are still unknown; about 25-40% of severe MR and most of mild MR cases remain unexplained (Chelly and Mandel 2001). In the portion of cases with known etiology, environmental causes, including very premature birth, head trauma, brain infections, fetal alcohol syndrome or exposure to toxic agents, are estimated to underlie 10-30% of moderate to profound MR and about 15% of mild MR (Knight et al. 1999). An additional 30-40% of moderate to profound MR and 15% of mild MR are due to chromosomal or single-gene alterations (Knight et al. 1999). In patients with chromosomal rearrangements MR is usually present in association with a specific pattern of physical or neurological abnormalities. In these cases, the term “syndromic MR” is used. These include well-known and relatively common disorders such as Down syndrome (trisomy 21), Prader-Willi and Angelman syndromes (deletion of 15q11.3 region), William syndrome (deletion in 7q11.2) or Wolf-Hirschorn syndrome (deletion of distal 4p region). In addition, subtle subtelomeric rearrangements missed by standard cytogenetic analysis have been estimated to account for about 7% of children with moderate to profound MR and 0.5% of children with mild MR (Knight et al. 1999). Our present knowledge of the monogenic causes of MR is still limited, although great progress has been
made in recent years, especially in the field of X-linked disorders. Monogenic MR disorders include both syndromic MR and non-syndromic forms, in which MR is the only obvious symptom. An excess of males between mentally retarded patients has long been noticed and explained with the presence of mutations in genes on the X chromosome. It is calculated that XLMR may account for about 20-25% of mentally retarded males (Turner 1996). Up to now, 20 genes have been found involved in “non-specific” MR (MRX), where MR is the only consistent feature: RSK2, ARX, IL1RAPL1, TM4SF2, PQBP1, ZNF41, FTSJ1, JARID1C, OPHN1, DLG3, XNP, AC54, PAK3, AGTR2, ARHGEF6, FMR1, FMR2, MECP2, SLC6A8, GD11 (Fig.3) (Jensen et al. 2005; Renieri et al. 2005). The reported mutation frequency of each gene is low (0.01-1%) although large cohort of patients have been screened only for few genes. Mutations in some of these genes (RSK2, MECP2, ATRX, ARX, FMR1) have been also identified in syndromic XLMR (MRXS) (Renieri et al. 2005).

**Figure 3.** Known MRX genes. The position of all known MRX genes on the X chromosome is reported. Genes indicated on the left are those involved both in MRX and MRXS; their associated syndromes are indicated between brackets. Genes indicated on the right are those involved in MRX. Modified from Renieri et al. 2005 (Renieri et al. 2005).
RESULTS

Cohort of Rett patients

From 2001 I have personally seen almost 100 RTT patients out of the 137 patients afferent to the Medical Genetics of Siena for whom a MECP2 mutation was proven. The majority of these patients were admitted to Neuropsychiatry of Siena and in part to Pediatrics and Neurology Units of Siena. Among the 137 cases with a molecular diagnosis of RTT, 93 were classic RTT, 19 preserved speech variants, 6 forme fruste, 4 early seizure variants. In 15 patients an exact classification as classic form or variants could not be defined since they were younger than four years. Among the patients for whom a MECP2 mutation could not be found there were some classic RTT and suspected PSV, 7 early onset seizure variants of RTT and some patients with a RTT-like phenotype that is patients who do not fulfill the criteria for classic RTT or variants (Trevathan and Moser 1988; Hagberg and Skjeldal 1994).

Until recently, RTT was considered a monogenic disorder due to mutations in a single gene named MECP2 (Amir et al. 1999). However, about 20-30% of classic RTT cases and an higher percentage of patients with variants of RTT do not have MECP2 mutations, raising the question of which is the molecular defect in these patients. In some forms, such as the early seizure variant, MECP2 mutations have never been described. Recently, mutations in the CDKL5 gene have been found in some cases with this variant of RTT (Tao et al. 2004; Weaving et al. 2004). However, CDKL5 does not seem the disease gene for all MECP2 negative patients since analysis of this gene in a cohort of classic RTT patients and patients with variants of RTT did not reveal any mutation.

In order to reveal the molecular cause of those cases who lack a molecular diagnosis it is important to collect a great number of patients. The aim of our RTT bank is to make such a large panel of patients available to researchers working on RTT. In addition, it is known that patients with the same MECP2 mutation can have different phenotypes, suggesting that other factors contribute to the phenotypic outcome. The availability of a large number of MECP2 or
CDKL5 mutated patients with different phenotypes will bring to the identification of possible modifier genes.

The general home-page of the bank is available at http://www.bank.unisi.it. This is an introductory page where users can find general information useful for site navigation and use.

**Figure 4.** Home-page of the bank. Here you can find the link for the three different databases: RTT, XLMR and “other”.

In particular, users can find the following sections: 1- **Bank organization**, describing the general organization of the bank and its sections. 2- **Guidelines**, containing a description of the procedures users have to follow for storing their samples in the bank and for requesting samples stored in the bank. 3- **Services**, containing a list of all services available at the bank. 4- **Contact information**, for contacting bank administrators. 5- **Forms**, containing the forms that users have to fill in order to take advantage of bank services. Moreover, this introductory page gives access to the three distinct databases which compose the bank: 1- XLMR; 2- RTT; 3- Other. The first two databases are funded through a Telethon grant. The “other” database will contain a list of biological samples from patients affected by other genetic disorders, different from RTT and other forms of XLMR. By clicking on one of the three links users can have access to the specific databases.
By accessing the RTT section of the bank users can see a list of all patients available with additional specific information (see below). At present the site contains 187 probands and their relatives. Of the 187 probands, 137 have a known mutation while for the others 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 an username and password and containing personal data of patients and relatives and detailed clinical information when available. In both cases, the main page of the site consists in a table containing a list of all samples available in the bank (fig. 5).

![Figure 5](image-url)

**Figure 5.** Page publicly available which contains a list of all samples available in the bank. In the upper part there are links for the list of mutation page, for the page where the graphs of mutations are located and the link for the rare variants. On the upper right part of the page there are the “search” tools.

The following information is available for each sample: a) **CODE:** This is a progressive number which identifies the single families, usually consisting of the affected girl and the parents and other relatives when available. b) **INTERNAL CODE:** This column of the table contains the personal codes which are assigned to each individual (proband, parents, other relatives) when it comes to our attention. These codes allow single individuals to be identified without using the personal data. Together with the personal code, the relationship within the family (proband, father, mother, etc) is visible in this column, so that users can
immediately know for which individuals biological samples are available. c) PHENOTYPE: This column reports the phenotype of the proband, if classic RTT or RTT variants (PSV and high functioning PSV, forme fruste variant, congenital variant, Rett-like phenotypes which include patients with a suggestive phenotype but which can not be classified in one of the specific phenotypes reported above) In addition, for very young patients for which an accurate clinical evaluation is not possible the phenotype is indicated as “ND (age less than 4 years)”. d) GENE NAME. This column reports the name of the gene mutated in each patient. In those patients for which the causative mutation has not been identified the gene name is designed as “Unknown”. e) MUTATION TYPE. Pathogenic mutations are classified in five general categories: i- Missense mutations in which a single aminoacid has been substituted with a different one; ii- Early truncating mutations which interrupt the protein eliminating part or all of the MBD and/or TRD domain; iii- Late truncating mutations which interrupt the protein in the C-terminal portion after the TRD domain; iv- Gene deletion in which the entire MECP2 gene has been lost on one of the two X chromosomes; v- Gene duplication in which a duplication including the entire MECP2 gene is present. f) NUCLEOTIDE CHANGE. The change at nucleotide level is reported according to the standard nomenclature (den Dunnen and Antonarakis 2001). g) AMINOACID CHANGE, which indicates the change at protein level according to the standard nomenclature (den Dunnen and Antonarakis 2001). h) ADDITIONAL INFO. This column contains an icon which links to another page containing additional information about the family. In this page bank administrators can visualize all available information about the family, including personal data of patients and relatives. On the contrary, external users can visualize only information about the X-inactivation status of the patient and the inheritance mode (“de novo” mutation, apparently sporadic, carrier mother, mosaicism in one parent). i) AVAILABLE BIOLOGICAL SAMPLE. In this column users can find a list of the biological samples available for each family. On the general table only the kind of available biological sample is reported (Lymphoblastoid cell line, Leukocytes in DMSO medium, Plasma, DNA, fibroblasts). In order to know for which family members a specific biological sample (for example DNA) is available users must simply pass with the cursor on
the sample type and a list of all family members for which that sample is available will appear. 1) REFERENCE. This column contains a link which allows to view the PubMed reference of papers in which the specific patient was published.

In addition to simply look through the list of available samples users can rapidly verify if there are the patients they need by exploiting the “Search by” option available directly on the site main page. This option allows users to select patients by: i- Mutated gene (MECP2 or CDKL5); ii- mutation type; iii- Nucleotide change; iv- Aminoacid change; v- Phenotype. Users can choose to search by one single options or to combine two or more options; for example they can search all patients with missense mutations or they can search all patients with missense mutations in the MECP2 gene and PSV phenotype. This extremely flexible search option allow users an accurate selection of patients so that they can immediately evaluate if the kind of samples they need are present in the bank and choose which samples to request before contacting bank administrators.

Finally, on the main page of the bank, users can find links to three additional pages: a) List of mutations. This page contains a table which lists all mutations identified in the patients of the database. In the table the following information are available: i- Nucleotide change; ii- Aminoacid change; iii- Frequency, expressed as the number of patients bearing the specific mutation; iv- Phenotype, reporting all different phenotypes found associated to that specific mutation inside the database; v- Reference, which allow to view the PubMed reference of papers in which the specific patients were published. The table is automatically updated every time a new mutated patient is inserted in the database. b) Graph of mutations (fig. 6). c) Non pathogenic rare variants. This page contains a table listing all non pathogenic rare variants identified in the patients of the bank. The table includes the following information: i- ID, that is a progressive number which identifies the single variant; ii- Nucleotide change; iii- Aminoacid change; iv- Number of patients, that is the number of individuals (patients and relatives) in which the variant has been identified; v- Internal Code, that is the internal code of all the individuals in which the variant is present; v- Reference, which
allows to view the PubMed references of papers in which the specific variant has been reported.

As can be realized, by browsing the RTT database, the most common form of RTT syndrome is the classic one. This form is well known and extensively reported. RTT variants are less easily diagnosed. In our experience the PSV variant is the commonest among all the variants. I will report the clinical description of a typical classic RTT patient and a PSV patient. More space will be given to the early seizure variant, less known and described in literature and for which we are now starting to understand the molecular cause.

![Graph of mutations](image)

**Figure 6.** Graph of mutations. This page contains a dynamic graph showing the position of the pathogenic mutations and their relative frequency. On the X axis of the graph a schematic representation of the protein with the main identified domains is reported. On the Y axis the frequency, expressed as percentage of mutated patients bearing a specific mutation, is reported. Mutations are positioned along the protein scheme according to their aminoacid numbering and they are represented as vertical columns; the height of the column represents the frequency of the mutation. The graph is managed by a dedicated program which read data in the table of mutations and uses these data to update the graph every time the table is updated.
A case with classic Rett syndrome

She is a 21 years old female (internal code 581). The mother had a normal pregnancy and delivery, and the child was born at term. She showed a normal development till the age of 15 months when her progress ceased. Afterwards a regression and an autistic behavior were noted (stage 2). She stopped saying the words she had gained and the ability to use her hands was overwhelmed by incessant hand washing activities. She also beat her hands together and showed tongue stereotypes. At four years she had the first epileptic episodes, which were difficult to control with various antiepileptic drugs and persisted during the following years. The EEG showed a diffuse slowing of the background rhythm and multifocal paroxismal activities. At the time of the examination (21 year-old) she was able to walk unaided, she showed microcephaly and scoliosis, and the stereotypic hand movements were still present (stage 3).

![Figure 7. Pedigree of patient #581. The case is sporadic. In the maternal branch of the family there is a male relative with mental retardation.](image)

The molecular analysis of the MECP2 gene was initially performed by PCR-DHPLC analysis searching for point mutations. This analysis resulted negative.

Gene deletions/duplication in females may be missed by PCR-DHPLC analysis due to the presence of the normal copy of the gene in the other X chromosome. In the last year we have validated an assay by Real Time PCR for the identification of deletions/duplications of MECP2. The Real-Time analysis allowed us to identify in this patient a MECP2 deletion (see article 1). The analysis of parents’ DNA permitted to establish that the MECP2 deletion was “de novo”. This result indicates that the mental retardation of her relative (the cousin of the mother) has a different etiology.
A case with preserved speech variant of Rett syndrome

There is general agreement that the inability to speak is a cardinal feature of RTT. However, the RTT phenotype is broader, including girls who recover the ability to speak in single words and using third person phrases, associated with improvement in purposeful hand movement at stage 3 of disease progression.

The first patients with preserved speech (PSV) were originally described by Zappella in 1992 (Zappella 1992). He reported three female patients who showed many clinical signs typical of RTT but with some peculiar differences. They showed developmental arrest, autistic symptoms, loss of acquired hand skills, dispraxia, and hand washing stereotypes and exceptionally all three patients were able to say numerous words and even able to make simple phrases. Zappella was convinced that these three girls could represent variants of RTT since one of them was the only sister of a classic RTT patient. Besides this lucky observation, also the molecular analysis proved that he was right. In fact, in 2001 a pathogenic mutation in the MECP2 gene was found in both sisters (Zappella et al. 2001). This was also one of the few familial RTT cases reported in the literature.

Zappella differentiated the PSV patients from the previously reported “forme fruste” cases asserting that the “forme frustes” have a late mild motor deterioration and only speak in single words compared to the girls reported by him. Since the appearance of the publication by Zappella in 1992, several other cases with symptoms of RTT in combination with varying degree of preserved speech have been described (Skjeldal et al. 1995; Zappella 1997; Zappella et al. 1998). In PSV patients are found only a subgroup of MECP2 mutations: late truncating mutations and missense mutations (De Bona et al. 2000; Zappella et al. 2001; Renieri et al. 2003).

The clinical description of one of the oldest PSV cases I had the opportunity to see is reported here.

*She is a 33 year-old female (internal code 612; fig.8). Her pedigree is represented in fig 9. The mother had a normal pregnancy, and the child was born*
after term by distocic delivery. No data concerning her auxological measurements at birth are available. She showed developmental delay: she could be able to sit unsupported at 8 months, she could walk and she said the first words at 2 years of age. Subsequently she lost the few words she had acquired and showed social withdrawal (stage 2). At around 6 years of age she recover the ability to speak in simple phrases, using the third person (stage 3). At 10 years, generalized convulsion of absence type, appeared. The epileptic episodes were difficult to control with various antiepileptic drugs and persisted during the following years. They got modified in time and at the time of the examination (33 years) tonic seizures were present 8-10 times a day. She was involved in educational classes since the age of 15. At that time she was able to wash and dress herself and to eat alone with very little help. She could also say simple phrases. Around that age she had a cognitive and motor deterioration (stage 4): she lost the ability to speak and to walk, she lost sphincters control and showed hand apraxia. Parents referred rare stereotypic hand movements of the rubbing type.

![Figure 8. Picture of the patient at present. Note the hands conjuncted in the mid-line and her overweight.](image)

![Figure 9. Pedigree of the patient. showing a female relative with mental retardation and epilepsy. Given the result of the molecular analysis the two disease are non correlated. The presence of consanguineity in parents could have delayed the diagnosis suggesting the possibility of a autosomal recessive condition.](image)
At the time of the examination (33 years old) she was not able to walk and to speak. During the genetic counseling she was sitting on a wheelchair with folded arms and she showed body rocking. Her head circumference was at the third percentile (52.5 cm), she had cold extremities and constipation was referred.

MECP2 point mutations were excluded by PCR-DHPLC analysis. Once again Real-Time PCR analysis has been crucial for the molecular diagnosis in this patient. In fact it allowed the identification of a duplication in the MECP2 gene in this PSV patient (see article 1).
Article 1

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.


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://mecep2.chwedu.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-Miltényi 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; Vaccaro 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 Hargberg and Skjeldal for RTT variants [Hargberg 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 [Aarnos and Vedeler, 2003]. We used the O2D2/280O method on a photometer to determine the appropriate DNA concentration [Sambruk 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′-TCG GAG GGT GTG GAG GTG AA-3′; MECP2 exon 4 reverse primer: 5′-TGG AAA ACG CAT CTT GAC AAG GA-3′; MECP2 exon 4 TaqMan probe: 5′-AGG GTC CTG GAG AAA AGT CCT GGC 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 RNAaseP kit as an internal reference (Applied Biosystems). This kit contains 20× RNAaseP Mix with a VIC-labeled probe and specific primers for the RNAaseP gene. We performed separate and multiplex primers for the detection 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× RNAaseP Mix, and HPLC pure water. A total of 100 ng of DNA (5 μl) was dispersed in each of the four sample wells for quadruplicate reactions. Thermal cycling conditions included a preheat 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 Microcarr 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 (dCt) 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 RNAaseP control gene. The threshold cycle number (Ct) was determined for all PCR reactions. The threshold was automatically set at 0.1 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 ΔCt 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 RNAaseP 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 RNAaseP 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. ΔCt very close to 0 (0.029), demonstrating that the efficiencies of the two systems are approximately equal (Fig 1B).

Using the comparative ΔCt 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: ΔΔCt = [ΔCt RNAaseP (calibrator sample) − ΔCt MECP2 (calibrator sample)] − [ΔCt RNAaseP (unknown sample) − ΔCt MECP2 (unknown sample)]. The relative gene copy number was calculated by the expression 2^([ΔΔCt]×2), which represents the SD of the difference calculated from the Ct SD of both MECP2 and RNAaseP. Using this calculation, a ΔΔCt ratio of about 1 for a diploid sample and about 0.5 for an haploid sample is expected.
RESULTS

Assay Development and Data Analysis

We performed separate and multiplex preruns, varying the concentrations of MEC2 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 MEC2 and RNase P 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 MEC2 and RNase P quadruplicates crossed the threshold at approximately the same point (Ct) (Fig. 2A). In a healthy control male, the Ct value of MEC2 showed an increase of approximately one cycle when compared with RNase P (Fig. 2B). We validated the method through the analysis of 15 male (MEC2 single copy) and 15 female (MEC2 double copy) healthy controls. Using the ddCt method, we quantified the MEC2 copy number. The values of the MEC2 ratio (2^(-ddCt)) 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^(-ddCt)). The second and the third column show the lower and the upper ranges of the ddCt ratio (2^(-ddCt)). From these results, we set the following threshold values for the ddCt ratios: a value
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; Vaccar et al., 2001]. We re-screened these patients by DHPLC, a more sensitive technique [Bujeste 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 RNaseP. 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 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 before 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-atatic 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 hypomorphic 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 ΔdCt 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; Aurskog and Vedeler, 2001].

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.

Duplication 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 cannot be established. Consequently, we cannot 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 3' 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.

ACKNOWLEDGMENTS

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REFERENCES


Two cases with highly functioning preserved speech variant of Rett syndrome

Comparing the phenotype of the two patients described before it comes natural to realize the different severity of the disease in those girls. The discovery that even patients with PSV carry a pathogenic MECP2 mutation demonstrates that the PSV phenotype belongs to the RTT spectrum (Renieri et al. 2003). In order to ascertain if this spectrum could be even broader we investigated the MECP2 gene in patients with many characteristic of RTT but with a more favorable progression of the disease.

During the course of my PhD program I had the opportunity to see the two patients (internal codes 362 and 386) described in the following article, who showed a striking preservation of their abilities in comparison with classic RTT and even PSV girls.

When I first see them they were 13 and 12 years old, respectively. The first girl was a great talker, she never stopped talking. Once she asked me my birth date and she realized that my sign was Cancer. The other girl spoke less but she could answer to simple questions in an appropriate way. During the examination she brought me around the room showing me the pictures hang to the wall that she preferred. Parents referred that she used to play with her dolls pretending to feed them as she was her mom. Unfortunately, on the last examination (15 years) her clinical condition worsened. She could not walk anymore and her social interaction was very poor.

A clinical review of these two patients revealed that they had a complete loss of autistic features through the years. Between the age 5-9, they acquired a behavior very similar to the phenotype reported as PSV and finally between 10-13 years they lost the PSV features in favor of a high degree of psychic development as a non-specific mental retardation with an IQ close to 50.

These highly functioning PSV seem to be very rare. This could be due to the fact that they have very shaded phenotypes compared to classic RTT and thus they are not recognized as RTT variants.
Article 2

Study of MECP2 gene in Rett syndrome variants and autistic girls.


Study of MECP2 Gene in Rett Syndrome Variants and Autistic Girls

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Mutations in MECP2 gene account for approximately 80% of cases of Rett syndrome (RTT), an X-linked severe developmental disorder affecting young girls, as well as for most cases of Preserved Speech Variant (PSV), a mild RTT variant in which autistic behavior is common. The aim of this study is to determine whether MECP2 mutations are responsible for PSV only or may cause other forms of autistic disorders. We screened for mutations by SSCP 19 girls with a clinical diagnosis of autism, two of them fulfilling the PSV criteria. A pathogenic mutation was found only in the latter two cases (R139C and R433X). A long follow-up of these two girls revealed a unique clinical course. They initially developed the first three stages of RTT, they were severely retarded and had autistic behavior. Over the years their abilities increased progressively and by early adolescence they lost autistic behavior, becoming adequately accustomed to people and reaching an IQ close to 45. These results confirm previous clinical studies suggesting that a wide spectrum of RTT exists including girls with mental abilities considerably higher than in classic RTT. We conclude that MECP2 mutations (missense or late truncating) can be found in girls with an IQ close to 45 and a clinical history of PSV of Rett syndrome. Furthermore, MECP2 mutations are not found in patients in which autism remains stable over the years.

KEY WORDS: RTT; PSV; preserved speech variant; mild mental retardation

INTRODUCTION

In the study of Autism, considerable attention has been given to syndromes where a strong association exists between a given syndrome and autistic behavior. In a limited number of cases, autism can be a feature of chromosomal, metabolic and dysmorphic disorders, usually with an IQ below 50 [Coleman, 1976; Gillberg and Coleman, 2000]. In most cases, however, Autism does not show such specific associations and appears as a disorder whose main features are relatively stable in time. Within this context, Rett syndrome (RTT, OMIM # 312750) shows a limited period of autistic behavior. RTT is a neurological disorder predominantly affecting females and showing a peculiar course structured in stages. After a few months of almost normal development, patients display a developmental arrest (stage 1) followed by a regression with loss of speech and purposeful hand use and appearance of postural microcephaly, stereotypic 'hand-washing' activities, ataxia, hand-apraxia, and abnormal breathing (stage 2). At this stage, similarities with autistic behavior are present. At stage 3 there is a limited amelioration followed in older girls by a final somatic and neurologic deterioration (stage 4). For a long time the relation between Autism and RTT was controversial. RTT was initially described in 1966 [Rett, 1966] but went almost unnoticed for many years and these girls were often inappropriately diagnosed as autistic [Witt-Engerstrom and Gillberg, 1987]. In 1983, when RTT was introduced into the mainstream literature, Autism was described as one of its main features [Hagberg et al., 1983]. Subsequently however, a distinction between Autism and RTT was made [Gillberg, 1986; The Rett Syndrome Diagnostic Criteria Work Group, 1988]. DSM IV notes that the diagnosis of Autism requires the absence of a diagnosis of RTT, which is, however, included among Pervasive Developmental Disorders (PDD). In fact, some similarities with autistic behavior are accepted for stage 2.

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A number of variants of RTT have been described. Among these, the Preserved Speech Variant (PSV) is characterized by a relatively benign course [Zappella, 1992]. It shows the same stage system and a number of symptoms (initial absence of speech, hand-washing stereotypic activities, etc.) common to classic RTT. However, during stage 3, these girls slowly improve the use of their hands and start to talk in short or longer phrases. Their mental abilities usually increase to levels between 2 and 4 years of mental age but their behavior and spoken language is autistic according to DSM IV criteria. Moreover, they differ from RTT in having normal head circumference, normal or even exceeding weight, slight kyphoscoliosis, and hardly ever epilepsy [Zappella et al., 2001]. The diagnosis of PSV, which has been considered as part of a 'complex' of disorders including classic RTT and other RTT variants [Zappella et al., 1998], rests on the inclusion criteria for RTT variants [Hagberg and Skjeldal, 1994] in addition to the course and clinical features described above. In summary, both RTT and its variants are correctly diagnosed as PDD-classic RTT girls have a transient autistic phase in stage 2 and most PSV show an autistic-like behavior often associated with a peculiar 'musical' aptitude.

RTT is usually due to de novo mutations in MECP2 gene [Amir et al., 1999]. MeCP2 is a nuclear protein that binds to methylated DNA and may act as a silencer of gene expression interacting with other proteins such as Sin3A and histone deacetylase complex. MECP2 mutations account for about 80% of RTT cases [Vacca et al., 2001]. We and others have shown that MECP2 mutations are found in PSV [Amir et al., 2000; De Bonne et al., 2000; Hupke et al., 2000; Zappella et al., 2001].

The opportunity to extend the search for MECP2 mutations in girls with Autism was a consequence of the above quoted studies: it is possible that girls with other forms of Autistic Disorders have MECP2 mutations or, alternatively, only girls with a clinical course and symptoms compatible with RTT have this genetic alteration. Until now, only two studies have attempted to address this question. In the first, analysis of 21 autistic females led to the identification of a putative splice site mutation in one case [Lum et al., 2000]. In the second, analysis of 59 autistic patients, 42 males and 17 females, failed to detect any mutation in MECP2 [Vourel et al., 2001]. However, a search for MECP2 mutations in 19 girls with Autism. Two of them had clinical features of PSV but reached over time an IQ close to 45.

MATERIALS AND METHODS

Patients

Nineteen girls ranging from 5 to 17 years with a diagnosis of Autism were studied. Autism was assessed by using DSMIV criteria [American Psychiatric Association, 1994], Autistic Behavior Checklist (ABC) [Krug et al., 1979], and Childhood Autism Rating Scale (CARS) evaluation [Schopler et al., 1980]. ABC score was over 57 in all except three cases in which a rating of 40-47 was found. CARS score ranged from 30 to 43 with a mean score of 37. These criteria for Autism were independently assessed by two child neuropsychiatrists and were concordant. IQ was obtained either by Wechsler scale (Wechsler Pre-school Performance Scale for Intelligence, WPPP, for children below 6, and Wechsler Intelligence Scale for Children-Revised, WISC-R, for children aged 6-18), or by the Leiter test, or the Stanford Binet L-M scale. Different tests were administered because the evaluation was realized independently in distinct centers. All patients were mentally retarded. Most had an IQ below 50 but four had values above 50, with the maximum IQ being 70. In 16 girls no metabolic or known genetic disorders were detected. Among the remaining cases, one had features of Sotos syndrome. In the other two patients, an accurate clinical follow-up suggested the possibility of a diagnosis of PSV. In these two patients, autistic behavior was present in infancy and early childhood but progressively remitted at the end of the first decade of life.

Genetic Analysis

Mutation screening of MECP2 gene was performed by SSCP as reported elsewhere [De Bonne et al., 2000]. DNA sequencing was performed with Big dye terminator cycle sequencing kit (Applied Biosystems) on an ABI 310 Automated Sequencer. X-chromosome inactivation analysis was performed as described elsewhere [Meloni et al., 2000].

RESULTS

Nineteen girls with a clinical diagnosis of Autism were screened for mutations in MECP2 gene by SSCP analysis. A pathogenic mutation was found only in the two cases that fulfilled the criteria for PSV. Case one had a c.1357C>T transition, which caused the replacement of arginine 453 with a stop codon in the terminal portion of MECP2 (p.R453X), skipping the last 34 amino acids. This mutation has never been reported before. Case two had the common missense mutation p.R133C in the methyl-binding domain of the protein, due to a c.397C>T transition. Both mutations were absent in the parents. X-chromosome inactivation studies revealed a borderline pattern in case one (70:30) and a balanced pattern in case two (55:45). Due to the unique clinical course, phenotypic description of these two cases will be given in more detail and is summarized in Table I.

Case one (#386): she is an only child, presently 12 years old. Pregnancy and delivery were normal. She said her first words at 18 months and walked alone at 21 months with a wide basis. At 18 months she appeared less responsive than before; she showed eye avoidance and would not turn if called by name. In subsequent months she became unable to hold objects in her hands and let them fall easily. At 20 months she lost her words and the use of her hands. At 2 years of age, she was severely mentally retarded, and hand-washing stereotypic activities began and remained active for most of the day in the following years. At 5 years of age, it was noticed that the use of her hands was slowly improving with an initial, awkward use of spoon and
TABLE I. Clinical Features of the Two Cases

<table>
<thead>
<tr>
<th>Feature</th>
<th>Case 1</th>
<th>Case 2</th>
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<tr>
<td>Normal pre-perinatal period</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Normal development in first year</td>
<td>+</td>
<td>+</td>
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<tr>
<td>First words at</td>
<td>12 m</td>
<td>12 m</td>
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<tr>
<td>Walked alone at</td>
<td>21 m</td>
<td>12 m</td>
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<td>Loss of hand use at</td>
<td>20 m</td>
<td>24 m</td>
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<tr>
<td>Loss of words at</td>
<td>20 m</td>
<td>24 m</td>
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<tr>
<td>Stereotypic hand washing at</td>
<td>24 m</td>
<td>4 y</td>
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<tr>
<td>Stereotypic hand washing fades at</td>
<td>9 y</td>
<td>-</td>
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<tr>
<td>Stereotypic hand clapping at</td>
<td>-</td>
<td>24 m</td>
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<tr>
<td>Improvement in hand abilities at</td>
<td>5 y</td>
<td>5 y</td>
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<tr>
<td>Starts again to say words at</td>
<td>5 y</td>
<td>5 y</td>
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<tr>
<td>Autism starts at</td>
<td>12 m</td>
<td>24 m</td>
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<td>Autism fades at</td>
<td>12 y</td>
<td>13 y</td>
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<tr>
<td>Severe mental retardation at 2–4 y</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Final IQ</td>
<td>49</td>
<td>39</td>
</tr>
<tr>
<td>Normal head circumference (constant)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Kyphosis (slight)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Scoliosis (slight)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Flat feet</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Genu valgum</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sleep EEG as in RTT</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Convulsions</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

m, months; y, years.

and she started again to say words. At 6 years she had been tested with WPPSI with an IQ of 45 (VIQ 60, PIQ 48), while on the Letter test she had an IQ of 36. On the Peabody Picture Vocabulary test her abilities corresponded to 3 years 8 months: she was frequently echolalic and had a tendency to repeat questions over and over again. Her ability to interact with her peers was very poor. She was seen by one of us (M.Z.) at 8 years, when hand-washing stereotypic activities were still frequently present. She was able to draw a sun and a face and she was playing with a doll like a normal 2-year-old girl. She fulfilled the criteria for Autism at the DSM IV (in group A she scored positive for (1) b, c; (2) b, c, d; (3) a, b, c, d, e and her ABC score was 58. Her head circumference (53 cm), weight (23 kg), and height (126 cm) were all in the normal range. She had flat feet; otherwise, her vertebral column was straight with no signs of kyphosis or scoliosis, and no genu valgum. She fulfilled the main criteria of a variant of RTT (Hagberg and Skjeldal, 1994) and was diagnosed as having a PSV. In the following year, at age 9, she was tested again with the Stanford Binet L-M scale and showed an IQ of 49. She was seen again at 12 years. She had no more signs of ataxia and moved around quite well. Her abilities had increased further: she was able to draw simple pictures (Fig. 1A) and spoke more correctly in long sentences. Her face had become more expressive and she was interacting appropriately with children in the ward. DSM IV for Autism was negative except items (2) b and (3) b, ABC was 34 and CARS 24.5 (Schopler et al., 1980). Hand-washing stereotypic activities had disappeared at 9 years and were now absent. Head circumference (55 cm), weight (39 kg), and height (149 cm) remained within the norm. An EEG showed the presence of sharp waves in the right parieto-temporal region, more intense during sleep. A MNR showed slightly asymmetric ventricles.

Case two (#562): she is a 13-year-old girl with a healthy older sister. Pregnancy and delivery were normal. She walked alone and said her first words at 12 months. In the second year of life her development is described as slow. At 2 years there was a regression: she lost the use of her hands concomitantly with the appearance of hand-clapping stereotypic activities, became avoidant and isolated, and lost the few words she had been able to say, in a context of severe mental retardation. Her parents report that at 4 years contin-

Fig. 1. Pictures of the two cases with MECP2 mutation. Case one at the age of 12 years while she is drawing a house (panel A); case two at the age of 13 years while she is writing single letters of her name (panel B).
uous hand-washing stereotypic activities became evident together with hand-clapping and persisted in subsequent years. At 5 years she started again to say some words at a slower rate and her vocabulary was unchanged. She showed an increased interest in the following year; similar progress was reported in her manual abilities and at seven she was able to say some short sentences and to feed herself with a spoon and fork. At 9 years she had two generalized convulsions and was put on a regular valproate treatment. An EEG showed sharp waves in the left hemisphere and in the right parieto-frontal regions. A CT of the brain was normal. At this age (nine), she was seen in our department. She showed a persistent hand-washing activity and spoke in short sentences, frequently echolalic, with an altered tone of speech mainly out of context. She did not play with dolls but was able to draw triangles and squares. A difficulty in opposition movements of thumb and index finger was noticed bilaterally; nerve conduction appeared to be slower, both in the sensory and motor component, in the abducens and opponent nerves of both thumbs. Evaluated with the DSM IV for Autism, she had all items positive in group A, except (3) d, and with CARS (35) she scored in the moderate range of an autistic disorder. She had flat feet, genu valgum, and a slight kyphoscoliosis. She fulfilled the main criteria for a variant of RTT (Hagberg and Skjeldal, 1994) and a diagnosis of IV was made. She was seen again at 11 years 7 months when her progress had advanced considerably: she was speaking in long correct sentences, although with some out of context expressions, had achieved the capacity of symbolic play and interacted better with her peers. An EEG showed a basic rhythm of seven c/s with frequent slow bursts of five c/s and occasional sharp waves in the right centro-temporal regions in sleep. Her head circumference (52.6 cm), weight (51.5 kg), and height (142 cm) were within normal values. At present, at 13 years, she has further improved her mental abilities. A WISC-R test was administered scoring below 40, with a VIQ below 45 and a PIQ below 45. Her ABC is 17, at CARS she scores 23 and at DSM IV for Autism in group A she has only (3) c as a positive item. She is a lively girl, speaks a lot, has a sense of humor, likes to sing, and is able to engage in a fully reciprocal conversation using long phrases. She knows some of the common metaphors of a child with her mental age, can write some words in capital letters (Fig. 1B) and is able to read and understand single words. Her hand-clapping stereotypic activities are still occasionally present, and she has become obese with a weight of 70 kg.

A clinical review of the remaining 17 patients revealed that Autism and mental retardation had remained stable over the years in all. None showed the criteria suggested by Hagberg and Skjeldal (1994) for RTT variants. One of them had Sotos syndrome, a co-morbidity which had already been reported [Zappella, 1990]. MECP2 analysis revealed a likely non-pathogenic variant in two cases. One girl (#196) had a homozygous c.1202G>A transition causing the replacement of Serine 401 with Asparagine in the C terminal portion of the molecule. The same change was found in the hemizygous father and in the heterozygous state in the paternal grand-mother, in a paternal aunt and in the mother. Another girl (#184) had a heterozygous transversion, c.720C>G, leaving Threonine 240 invariant. The change was inherited from the unaffected mother, who did not show a skewed X-inactivation (not shown). Neither of the two changes was found in 50 healthy controls, suggesting that they are not common polymorphisms but instead private variants.

**DISCUSSION**

In the present study a MECP2 mutation was not found in 16 girls with Autism and in one girl with Sotos syndrome and Autism. A mutation was found only in two girls which fulfilled the main criteria for RTT variants [Hagberg and Skjeldal, 1994], had a diagnosis of PSV, and lost autistic behavior by early adolescence increasing cognitive and social skills. These two girls shared with other PSV a typical course structured in stages as in RTT, accompanied by hand-apraxia in stage 2 and by a parallel development of hand-washing stereotypic activities that continued along stage 3. Five of the six main criteria for RTT variants [Hagberg and Skjeldal, 1994] were present in both, and sleep EEG abnormalities were also compatible with this definition. Other clinical features were relatively milder than in most PSV: an improved use of body and of their hands along the years was observed in both and no evidence of scoliosis was found (only a slight kyphosis in case two). Head circumference was normal as in the majority of the other recently reported cases of PSV with MECP2 mutation [De Bona et al., 2000; Zappella et al., 2001]. From a neurological point of view, both girls had convulsions and one showed signs of peripheral neuropathy. The main difference between these two cases and those reported previously lies in the higher intelligence level reached at the end of the first decade of life, and in a parallel improvement in interpersonal behavior with marked reduction of previous autistic traits. However, RTT, PSV and the two cases described here all appear part of a continuum in the improvement of their abilities.

Two main features are noteworthy in these two girls: (1) The progressive disappearance of autistic features. (2) The improvement of cognitive level after the regression/deterioration period with a final IQ close to 45. These data confirm, in part, previous observations, conducted on clinical grounds only, which already showed some PSV girls with moderate mental retardation but with persistent autistic behavior [Zappella et al., 1996]. The two girls described here in their younger years displayed a moderate but clear autistic picture with abnormalities in speech and interaction typical of this behavior, as usual in PSV. Parallel to the general improvement, in early adolescence they lost most autistic features, no longer fulfilling the DSM IV criteria [American Psychiatric Association, 1994] and the usual tests such as ABC [Krug et al., 1979] and CARS [Schopler et al., 1980] where the cutoff scores for Autism are, respectively, 6 items in group A for DSM IV, and a score of 37 for ABC and 30 for CARS. The possibility of this positive evolution of behavior in PSV outlines the
difference of these girls from most cases of Autism where abnormal behavior remains constant over time.

The other girls with Autism described in this study, who did not have MECP2 mutations, maintained stable autistic behavior and mental retardation over time. In a study of 21 autistic females, a MECP2 mutation was found only in one girl, aged 4 years [Lam et al., 2000]. It was a putative 5′ splice site mutation in intron 2 but it has not been proven to be pathogenic. Unfortunately, authors gave a very limited clinical description of this case and did not have the opportunity to re-evaluate her later. In another recent study, neither mutations nor polymorphisms were identified in 59 autistic patients (42 males and 17 females) [Vourc'h et al., 2001]. In our 17 patients with stable autistic behavior, a clear pathogenic MECP2 mutation was not found. It is more difficult to understand the role of the inherited MECP2 rare variants found in two cases. The change e.720C > G (patient 184) does not cause an amino acid change and it is very unlikely that it exerts an even mild variation of MeCP2 function. However, a splice effect and/or influence on mRNA stability cannot be excluded without mRNA analysis. Likewise, a slight variation of MeCP2 function cannot be excluded for the p.S410N change (patient 196) in that Serine 410 is conserved in mouse, and is substituted by a neutral amino acid (Glycine) in Xenopus (while in chicken the entire C terminal domain is missed). At present a role of MECP2 as modifier gene in the context of a potential polygenic and multifactorial disorder like Autism cannot be ruled out.

Three types of mutations are reported in MECP2 gene: missense mutations determining an amino acid change, early truncating mutations leading to a very short protein, and late truncating mutations leading to a protein with some preserved domains. It is important to note that the mutations reported here are one missense (p.R135C) and one late truncating mutation (p.R455X). The p.R135C is common to both RTT and PSV while p.R455X was never reported before, even if it was predicted to be the fifth Arginine which can change to a stop codon by T transition in CpG dinucleotide [Wan et al., 1999]. Both missense and late truncating mutations lead to a protein able to translocate to the nucleus where it may exert some residual function. On the contrary, early truncating mutations lead to a protein lacking the nuclear localization signal and part of the transcription repression domain; consequently, this protein is predicted to be inactive and this might determine a more severe prognosis. We and others reported that missense and late truncating mutations lead to both classic RTT and PSV, while early truncating mutations only to classic RTT [Obata et al., 2000; Auranen et al., 2001; Umansky et al., 2001; Zappella et al., 2001]. The two cases described here, one with a missense and one with a late truncating mutation, are in line with this rule and strengthen the hypothesis that a missense or late truncating mutation is necessary to improve the behavioral phenotype and reach a diagnosis of PSV, while a skewed X inactivation in blood cells is not necessary [Auranen et al., 2001; Nielsen et al., 2001]. A partially preserved MeCP2 function may, however, not be sufficient and the improvement may be favored by social facilitation and educational support, and/or by the presence of one (or more) modifier genes.

Limitations

One limitation of this study is the reduced size of the sample analyzed (19 patients). A larger sample size would have strengthened the conclusions. A second limitation concerns the short time span during which the two PSV girls have been observed. In fact, in other reported cases a final mental and motor deterioration occurred, even in the second decade [Zappella et al., 2001]. Consequently, we can not exclude that one or both girls may undergo a progressive deterioration with loss of the present improvements in the future years. Another limitation of this study is represented by the sensitivity of the mutation screening technique. In fact SSCP has a sensitivity of about 80% and consequently some mutations could have been missed. We can not exclude that the employment of more sensitive techniques, like DGGE and DHPLC, would lead to the identification of additional mutations [Buyse et al., 2000]. However, a recent DGGE analysis on 59 autistic patients failed to detect MECP2 mutations [Vourc'h et al., 2001].

Clinical Implications

Both the present and previous studies suggest that MECP2 mutations are not likely to cause Autism in girls. This is likely to be a different complex of genetic disorders in which more than one susceptibility gene is involved [Persico et al., 2001]. Furthermore, our data suggest that MECP2 mutations can be found in PSV girls reaching, in time, a moderate mental retardation and losing previous autistic features.

From a clinical point of view, signs such as mild stereotypic hand activities and moderate dispraxia (possibly following a developmental regression occurred at early age) deserve attention and careful assessment for underlying causes. In these cases molecular investigations (i.e., search for MeCP2 mutations) may lead to the correct diagnosis and appropriate educational activities.

In conclusion, while MECP2 mutations are not seen in Autism, they could have relevance to understand the genetic basis of autistic behavior, albeit transient.

NOTE ADDED IN PROOF

Case one: Following the last visit, this girl developed a severe epileptic syndrome, difficult to treat and accompanied by profound mental deterioration with an extensive loss of language abilities which, one year later, were reduced to short phrases and single words.

ACKNOWLEDGMENTS

This work was supported by grants to AR from the Emma e Ernesto Rullo foundation and from Telethon (grant no. GGP02372). We thank Dr. Marco Seri for referring case one.
REFERENCES


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Four cases with the early onset seizure variant of Rett syndrome

Clinical description

The early onset seizure variant of RTT was first described by Hanefeld in 1985 (Hanefeld 1985). He described a female patient who presented with atypical infantile spasms with hypsarrythmia and later on developed symptoms consistent with the diagnosis of RTT. Early onset severe seizures (in one case reminiscent of infantile spasms) were also reported in two girls who later developed atypical variants of RTT (Goutieres and Aicardi 1986). Four further cases with early onset of seizures (one of whom had infantile spasms) were published: one by Hagberg and Witt-Engerström in 1987 and three by Maia et al in 1986 (Maia et al. 1986; Hagberg and Witt-Engerstrom 1987). Since then several other cases have been diagnosed. In this variant the characteristic RTT presentation is blurred by the epileptic disorder but later on patients develop characteristics typical of RTT.

Almost a year ago I had the opportunity to visit two unrelated female patients showing many characteristics of RTT (Fig.10). However, the clinical course of the disease was not typical of RTT, since the normal period of development was blurred by the presence of seizures.

![Patient 1](Image1) ![Patient 2](Image2)

**Figure 10.** Pictures of patient 1 (left) and patient 2 (right) at 9 and 8 years, respectively. Note the hands’ position in the mid-line. Both girls have been shot while having stereotypic hand movements.

These patients are two girls presently aged 9 and 8 years, respectively. Both patients were first hospitalized very early in life due to the occurrence of
epileptic episodes. Patient 1 (internal code 849) had the first seizures at 1 month and half of age, while in patients 2 (internal code 902) epilepsy occurred at 10 days of life. In patient 1 the seizures resembled infantile spasms, while in case 2 infantile spasms were presumed by parents’ report and we have no data about the clinical details. Although infantile spasms occurred, the EEG did not show hypersrrhythmia. Both showed developmental delay since the first months of life. At the time of the last examination, patient 1 (9 years) was able to walk unaided even if on a broad base, while patient 2 (8 years) was unable to walk without support. Both patients showed various degree of reduction of hand skills. At the time of the last examination patient 1 was able to briefly hold an object in her hands, dropping it shortly afterwards, while patient 2 could hold a biscuit and some bread in her hands and eat them. Both girls showed stereotypic hand movements, but less frequently than in classic RTT patients (fig.10). During the genetic counselling both patient showed a good preverbal interaction. No sings of autonomic dysfunction were present in either patient. Patient 1 showed bruxism and breathing irregularities with hyperventilation episodes. While case one showed post-natal microcephaly, patient 2, at the time of the examination, showed a OFC around the 10º percentile. No data about her head growth are available. Parents referred a normal birth OFC, thus a reduction of head growth can not be ruled out for this patient.

Considering the phenotype of these two patients, we made a diagnosis of early onset seizure variant of RTT for both. Even if no MECP2 mutations have been reported in patients with this RTT variant, we decided to analyze the MECP2 gene in these two cases and we did not find any mutation. In particular, we excluded the presence of MECP2 point mutations (including exon 1) and gross rearrangements by PCR-DHPLC and qPCR, respectively.

Given that these girls had infantile spasms and that initially for patient 1 a diagnosis of West syndrome was made, we decided to test in the two patients both ARX and CDKL5, the two genes currently involved in West syndrome. Analysis of the ARX gene, the principal gene involved in the syndrome, did not reveal any pathogenic mutation, while, CDKL5 analysis revealed a “de novo” frame-shift deletion in exon 5 and exon 18 of the gene, in patient 1 and 2, respectively (see article 3).
The analysis was subsequently extended to 19 classic RTT, 15 PSV, 5 early seizure variants, 5 “forme fruste” variants and 4 congenital variants (tab.2). We identified 2 novel mutations in two unrelated patients with the early onset seizure variant, while no mutations were found in the other variants of RTT (see articles 3 and 4). Thus, among 7 patients with the early onset seizure variant, 4 were positive for a CDKL5 mutation (57%).

**Table 2.** Total of patients analyzed for CDKL5 mutations.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>N° of patients</th>
<th>N° of mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Classic Rett</td>
<td>19</td>
<td>0</td>
</tr>
<tr>
<td>Preserved Speech Variant</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>Early Onset Seizure Variant</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>“Forme fruste” Variant</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Congenital Variant</td>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>

The two additional female patients positive for a CDKL5 mutation had a similar clinical course to the previously reported patients: they had seizures in the first months of life and subsequently developed recognizable RTT features (fig.11).

**Figure 11.** Pictures of patient 3 (left) and patient 4 (right) at 7 and 4 years, respectively. Note the hands’ position in the mid-line. Both girls have been shot while having stereotypic hand movements.

These patients are presently aged 7 and 2 years, respectively. The first episodes diagnosed as seizures were noted in the first days of life in case 3 (internal code 505) and at 6 months in case 4 (internal code 950). In both, seizures resembled infantile spasms although, as for the previously reported patients, the EEG did not show hypsarrhythmia. Only in the younger patient (case 4) the psychomotor development was normal in the first 6 months, while patient 3 showed developmental delay since the first months of life similarly to
the other two CDKL5 mutated patients. While patient 3 has never been able to walk, patient 4 acquired the ability to walk unaided at 20 months and at the time of the last examination she could walk alone even if she showed truncal ataxia. Both cases showed various degree of reduction of hand skills. Case 3 has been always unable to use her hands to take objects. In the younger patient (case 4) hand skills were better preserved. In fact, she was able to hold light objects for long time. Similarly to the other two patients they showed stereotypic hand movements (Fig.11). While patient 3 was able to interact at a preverbal level, patient 4 showed reduced social skills and autistic features. Patient 4 occasionally uttered some words, while case 3 has never uttered a word. In addition, bruxism and cold extremities were present in patient 3 and stypsis was present both in case 3 and 4. Gastroesophageal reflux was present in case 3 at the time of the last examination while in patient 4 it was reported in the first months of life. Both patient had a normal OFC at the time of the last examination.

In the following table (tab 3) the major clinical RTT features of all four patients are summarized.

<table>
<thead>
<tr>
<th>Patient</th>
<th>1 (internal code 849)</th>
<th>2 (internal code 902)</th>
<th>3 (internal code 505)</th>
<th>4 (internal code 950)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>9 years</td>
<td>8 years</td>
<td>7 years</td>
<td>2 years</td>
</tr>
<tr>
<td>Age at first seizure</td>
<td>1.5 months</td>
<td>10 days</td>
<td>first days of life</td>
<td>6 months</td>
</tr>
<tr>
<td>Infantile spasms</td>
<td>yes</td>
<td>presumed by the parents' report</td>
<td>Yes</td>
<td>yes</td>
</tr>
<tr>
<td>Hypsarrhythmia</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Epilepsy controlled by therapy</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Normal first 6 months</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Hand skills</td>
<td>+/-</td>
<td>+/-</td>
<td>-</td>
<td>+/-</td>
</tr>
<tr>
<td>Stereotypic hand movements</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>Preverbal interaction</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>Language</td>
<td>+/-</td>
<td>+/-</td>
<td>-</td>
<td>+/-</td>
</tr>
<tr>
<td>Bruxism</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Stypsis</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cold extremities</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Gastroesophageal reflux</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Breathing dysfunction</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Microcephaly</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

41
In conclusion, our experience suggests that CDKL5 mutations are associated only with a particular RTT phenotype: the early onset seizure variant. However, two recent papers reported the identification of “de novo” point mutations in the CDKL5 gene in slightly different phenotypes (Tao et al. 2004; Weaving et al. 2004). Since these patients have been seen by different clinicians part of this phenotypic variability may be due to different clinical “sensitivity”. Otherwise, similarly as for MECP2 that is associated with classic RTT and the highly functioning PSV, CDKL5 may cause both the early onset seizure variant of RTT and a less defined phenotype ranging from autism and mental retardation.

Since MECP2 and CDKL5 mutations cause a similar phenotype we investigated whether the two proteins belong to the same molecular pathway (see article 4). At first, we compared the expression patterns of Mecp2 and Cdkl5 in embryonic and postnatal mouse brains. We then investigated whether the two proteins interact at the transcriptional level in humans by Real Time qPCR in lymphoblastoid cell lines. Finally, we wanted to verify whether MeCP2 and CDKL5 interact in vitro and in vivo. The whole experimental work permitted us to conclude that CDKL5, that was originally identified as a kinase by sequence similarity searches, can autophosphorylate and it mediates MeCP2 phosphorylation. 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 is limited to phosphorylation.
Article 3

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


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

E Scala, F Ariani, F Mari, R Caselli, C Pescucci, I Longo, I Meloni, D Giachino, M Bruttini, G Hayek, M Zappella, A Renieri

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 spasms type.

Retinoid acid receptor (RTR; 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. RTR variant has 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). These variants present some symptoms of RTR, 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.

Approximately 80% of patients with classic RRT have a mutation in the methyl CpG binding protein 2 gene (MECP2; OMIM # 300005). MECP2 mutations have also been identified in about 50% of PSV cases and in a lower percentage of other variants. In the variant with early development of convulsions described by Hanefeld, MECP2 mutations have not been published.

The Hanefeld variant of RRT 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 aristless related homeobox (ARX) gene, which maps to Xp21.3-p22.1. 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.

Recently, a second gene has been found to be involved in ISSX, the cyclin-dependent kinase-like 5 gene (CDKL5; STK9; NM_003159). These authors characterised two unrelated female patients with an apparently balanced translocation, 46,XX, t(X;7)(p22.3; p15) in one case and 46,XX, 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 RRT patients with early onset of convulsions. The analysis was subsequently extended to 19 classic RRT and 15 PSV patients.

METHODS

Patients: We investigated two patients, aged 9 and 8 years respectively, with early development of convulsions, who later developed many characteristics of RRT. They both fulfilled the criteria for the early seizure variant of RRT.

We then investigated 19 classic RRT and 15 PSV patients. The girls with classic RRT were diagnosed according to the international criteria. The PSV girls fulfilled the criteria of Hagberg and Skjeldal for RRT variants. In particular, the PSV

Abbreviations: DHPLC, denaturing high performance liquid chromatography; ISSX, infantile spasm syndrome; X linked; PSV, preserved speech variant; R5, retinoschisis; RRT, 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 transgenicomic 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 Pagonaro et al. Intensity of silver stained bands was measured using the Diversity Database program (Biorad) and the values were corrected for preferential allele amplification. RNA isolation from lymphoblastoid and CDNA synthesis were performed according to standard protocols. We used primers designed to form cDNA products spanning exons 4–6 (4RTF: 5'-GAAAACATGAAATTGTCGCCG-3'; 6RTF: 5'-GTGAATACGCTTACTAGTGCAG CTCG-3') and 17–18 (17RTF: GAGAAGATCTGACATGTCCGCAG; 18RTF: AGCTGAGGCTGCTGAGCGCG). 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 4600 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 aminoacidemia, 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 8.3 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 (5–7th centile). Scoliosis, kyphosis, cold extremities, and constipation were not present. The EEG showed sharp waves in the central and occipital regions.

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

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<th>Exon</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<th>PCR annealing temp (°C)</th>
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CDKL5/STK9 is mutated in RTT syndrome variant with infantile spasms

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 hypotonia, 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 centromedial 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 hypotonia 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). Seizures, 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 caramazepine. 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. Based on the partial clinical overlap between the Henecka variant and 1SSX, 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_001190). 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 peptide 1 serine active site, as predicted by the ScanProsite program (http://prosite.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 active site is represented by the redacted 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 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 hypotonia, and stereotypic hand activities. 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. It should be noted that only a few cases of this disorder have been reported 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 4-8 (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. 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 KIAA1RE and KIAAMRE and their orthologues. 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). In addition, a Thr-Xaa-Tyr motif was identified, and the dual phosphorylation of these Thr and Tyr residues has been shown to be essential for activation of the MAP kinase group.

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. This finding was derived from a study of two severely affected ISSX female patients with apparently de novo balanced Xautosome 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. 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 (GTSMCPFL), 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 peptides. CDKL5 probably does not have a peptidase activity, as it lacks other conserved residues necessary for the catalytic activity. However, we cannot exclude that CDKL5 might form a complex with other proteins that harbour the other essential domains.

A terminal deletion of CDKL5 has already been reported by Huopaniemi et al 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 5’ than that reported here, generates a milder phenotype.

Finally, it is important to understand why MECP2 and CDKL5 mutations lead to a similar phenotype. McCP2 and CDKL5 could belong to the same signalling pathway. As it has demonstrated that McCP2 is subjected to phosphorylation and that CDKL5 has a kinase domain, it is possible that McCP2 is directly phosphorylated by CDKL5. However, at least in patient 1, CDKL5 kinase activity is abolished, and a reduced degree of McCP2 phosphorylation would lead to a reduction in its dissociation from methylated DNA and to a gene silencing increase. However, it is known that MECP2 mutations presumably cause a reduction of gene silencing. Alternatively, CDKL5 might phosphorylate a second protein that could dephosphorylate McCP2. Additional studies are necessary to determine whether McCP2-CDKL5 interaction really exists and to unravel the complex mechanisms underlying the above phenotypes.
ACKNOWLEDGEMENTS
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Competing interests: none declared.
The first two authors contributed equally to this work.

REFERENCES
Article 4

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


CDKL5 belongs to the same molecular pathway of MeCP2 and it is responsible for the early 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 no. 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-axapria and abnormal breathing. In 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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†The authors wish to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

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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 and 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 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 CDKL5 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 the 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 expression developmental pattern, protein interaction and functional activity.

Here, we demonstrate that Cdk5 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 bitemporal 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
second year of life. Her head circumference is presently 51.3 cm (25–50th). She is able to interact at a preverbal level. Generalized convulsions characterized by tonic–clonic jerks, eyes retraction 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, bioelectric analyses and screening for metabolic disorders were normal. She presently shows autistic features according to DSM-IV. 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, 50th cent). 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). In case 1, we identified a 1 bp deletion in exon 16 (c.2343delG, p.S781fsX783) leading to protein truncation in position 783 (Fig. 1B). Case 2 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).

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
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. 3B with F). Cdkl5 expression is first observed in neural cells that have reached their final position in the cortical plate (Fig. 3B) (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 neuronal maturation and synaptogenesis as already proposed for Mecp2 (21,29). On the same line, the delay in the increase of Cdkl5 expression in the dentate gyrus (dg) with respect to the hippocampal area may follow the different phases of neurogenic maturation of these two fields (inserts in Fig. 3C and D). However, not all the brain regions show a comparable levels of Cdkl5 and Mecp2 expression. For instance, at P10 in the cerebellum, the two genes show different levels of expression in specific cerebellar domains. In fact, a strong Mecp2 but weak Cdkl5 staining is detected in the Purkinje neurons; whereas high Cdkl5 and low Mecp2 expression levels were found in granular cells (Fig. 3I–L). This indicates that different Cdkl5 and Mecp2 expression levels may coexist in the same cells suggesting independent mechanisms of gene regulation in such tissues.

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

To understand whether MeCP2 and CDKL5 belong to the same molecular pathway, we went ahead analyzing the possibility that the two proteins may even be directly interacting. We addressed this point performing a classical glutathione S-transferase (GST) pull-down assay in which a GST–MeCP2 fusion protein, expressed in *Escherichia coli*, was immobilized on a Glutathione-Sepharose resin and challenged with bCDKL5 translated in vitro. As shown in Figure 4A, CDKL5 is retained on the GST–MeCP2 resin (lane 4), whereas no CDKL5 is
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
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 factor 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 extract 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 sensitivity; 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 35S-labeled CDKL5 in the presence of γ-32P]ATP. In order to discriminate between 35S- and 32P-signals, the dried SDS–PAGE was exposed to a series of X-ray films of which the one closest to the gel receives the 35S- and 32P-signals, whereas the one farthest away picks up only the signal from 32P. As seen in Figure 6A, the signal derived from incorporated [35S]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 [γ-32P]ATP, was incubated with [γ-32P]ATP for 30 min at 30°C. Proteins were fractionated on SDS-PAGE, and 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 35S and 32P, 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 32P. Input shows [γ-32P]-labeled CDKL5 (lanes 1 and 3) whereas 1+γ-ATP shows CDKL5 incubated with 32P (lanes 2 and 4). The 32P 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-32P]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).

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-32P]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 visible 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 CRK kinase (31), and assayed its capability to mediate MeCP2 phosphorylation. The obtained results indicated that in our experimental conditions, this kinase maintains a strong autophosphorylated 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 seizure onset 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 seizures 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 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 Meep2 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, Meep2 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 worthwhile 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 Bhd 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 way 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).
This phenotype largely overlaps that previously described as early-onset seizure variant of RTT. Usually in the classic RTT phenotype, seizures and epileptic signs appear in the 80% of cases and only in the third pseudostationary 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 mutations (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 (Qugen). DNA samples were screened for mutations in the four exons coding for MECP2 using Trangeneomic 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 the patients 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-CDKL5F: CTGAGCAGATGAGAACCTTTCTCAGT
EX10-CDKL5R: TGAGGATGTTAACACGCTGGAA
PROBE: 6-FAM-TCTCTTCCTCATTGAGCTCCG-TAMRA

The CDKL5 probe contained a fluorophore 5′-FAM as reporter and a 3′-TAMRA as quencher. 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 concentration and temperature to optimize the 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 primer, 2× MECP2 kit, 2× GAPDH and HPLC pure water. A total of 100 ng of RNA was dispersed 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 MeCP2α) comprising four exons, with translation start site in exon 2 and the transcript MeCP2B (or MeCP2β) lacking exon 2, with translation start site in exon 1. We have tested only the isoform MeCP2A (or MeCP2B) 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.R556X74 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 ΔΔCt 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 0.05% was used for the comparison between control and mutated samples.

**Plasmid construction**

The cDNA encoding hCDKL5 was obtained from RZPD, Germany (RATp790G1233D). 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 reaction. pGFP–CDKL5 was cloned by inserting the entire CDKL5 cDNA into BglII and EcoRI 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 BamHI site of pGEK-4T-1. pGST–Ct was obtained by PCR cloning the whole C-terminal domain (residues 311–486) into the BamHI site of pGEK-4T-1; pGST-TRD-Ct was produced inserting a PCR fragment coding for the human residues 201–486 into the EcoRI/SalI sites of pGEK-4T1. pGST-ΔND and pGST-ΔC1 have been described elsewhere (37). pSGS-FLAG-MeCP2 was cloned by inserting a BamHI-digested PCR fragment, amplified from the human cDNA, in frame with an N-terminal FLAG tag in pSGS (Stratagene). All constructs based on PCR were verified by PCR. The pCDNA3 vectors encoding for the CRK 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–MeCP2A199–300) was generously given by Dr Jan 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 35S-labeled CDKL5 for 2 h at 4°C in PBS; 1 mM PMFS. 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 35S-labeled CDKL5 derivatives, produced using the TNT T7 Quick for PCR DNA (Promega) using hCDKL5 as template. Incubation, 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 7.5). Before hybridization, the slides were washed twice in 2x 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, 5x 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 0.5x 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 0.5x SSC, 20% formamide at 65°C and for 20 min in 0.2x SSC and blocked for 1 h at room temperature in 1% blocking reagent (ROCHE) in MABT. A 1:500 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). CDK5 and MeCP2 probes were obtained by *in vitro* transcribing the two full-length murine cDNAs containing untranslated regions.

**Commmunoprecipitation experiments**

For communoprecipitation, 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 immunoprecipitate 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

CDK5 autophosphorylation was revealed incubating 10 μl of in vitro translated CDK5 in 30 μl of kinase buffer (20 mM HEPEs pH 7.4, 10 mM MgCl₂, 0.5 mM DTT, 200 μM sodium orthovanadate) in the presence of 50 μM ATP; 5 μCi [γ-32P]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 received the mixed 32P- and 35S-signals, whereas the last picks up only the 32P signal.

To detect MeCP2 phosphorylation, total cell extracts were prepared and immunoprecipitated 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 manufacturer’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-32P]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; 32P-labeled protein was detected by autoradiography.

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Conflict of Interest statement. None declared.

REFERENCES


Genetic counselling in Rett syndrome

As I could realize during my experience, genetic counselling in RTT is important to suggest the correct diagnostic test in order to provide a definitive diagnosis: classic RTT or a variant of the RTT spectrum. For this purpose is important to apply the correct molecular test according to the patient’s phenotype. The MECP2 analysis should be requested for classic and PSV patients, while from 2004 in the early seizure variant of RTT CDKL5 analysis can be applied. Furthermore, genetic counselling gives the family an estimation of the recurrence risk, specific for each single case allowing us to differentiate between “de novo” mutations (low recurrence risk), inherited mutations (recurrence risk of 50%) and germ-line mosaicism (intermediate recurrence risk). In some instances, genetic counselling can also offer a prognostic evaluation of the molecular test.

Usually RTT is a sporadic disease and it is caused, as already mentioned, by “de novo” mutations in the MECP2 gene or in the CDKL5 gene. Once we have verified that the mutation is not present in parents DNA the recurrence risk for the couple in the case of future pregnancies is considered low, for the reasons I will discuss in the next paragraphs. For the relatives of proband the recurrence risk is considered null, according to present day knowledge. However, even if not indicated by the clinical geneticist, a molecular test cannot deny maternal uncles or sisters of a RTT girl with a known mutation. This consideration arises from the fact that the scientific knowledge about RTT is not to be considered definitive.

Even if RTT is considered a sporadic disease some exceptions to this rule do exist. In fact, among the 137 cases with a molecular diagnosis of RTT, we have collected in the RTT database, two were familial (http://www.bank.unisi.it). In both cases, probands were two sisters with a different phenotype: a classic RTT girl and a PSV girl (Zappella et al. 2001) and unpublished data). In the first family the asymptomatic mother carried the pathogenic MECP2 mutation (Fig.12a). In the second case neither parent carried the mutation (Fig.12b). For the first case the recurrence risk is about 50%, also the second couple has a high recurrence risk even if not easily predictable.
Among the remaining 135 sporadic cases, parents had a wild type genotype in 134 cases. In one instance the mother was a carrier (Fig.12c). In this case the theoretical low recurrence risk ranged from nearly 0% to 50%. This experience strengthens the importance to always test parents, even in sporadic cases.

The identification of an apparently “de novo” mutation is not enough to exclude a recurrence risk for the couples who desire a future pregnancy. During the genetic counselling, it has to be stated that a risk of germ-line mosaicism, even if very low, does exist. The experience of the first nine cases of prenatal diagnosis, led to the identification of a case of germ-line mosaicism (see article 5). A couple with a RTT daughter came to my attention and the MECP2 analysis was performed. As soon as the pathogenic mutation was identified, the analysis of parents’ DNA permitted us to state that the mutation was probably “de novo” and a low recurrence risk was given to the couple. The couple decided to perform prenatal diagnosis through villocentesis for the next pregnancy and the analysis revealed that the fetus carried the same mutation of the RTT daughter (Fig.12d). This is the first case of germ-line mosaicism identified by prenatal diagnosis in a couple with a RTT daughter with an apparently “de novo” mutation. This experience strengthens the importance of performing prenatal diagnosis on all couples with a RTT daughter.

**Figure 12.** Schematic representation of four RTT pedigrees showing carrier mothers (a,c) and germ-line mosaicism (b,d). White symbols stand for asymptomatic subjects, black symbols represent classic RTT cases while gray symbols represent PSV patients. For each subject is indicated the genotype and the specific MECP2 mutation. a and c represent RTT cases who inherited the MECP2 mutation from the asymptomatic mother, b and d represent two familial cases where germ-line mosaicism occurred.
Another important task for the clinical geneticist is to define a prognosis. This is a very delicate aspect and one of the most important for the family. In fact, parents frequently ask the clinical geneticist what will happen to their daughter in the future. Will they improve in some skills or not? Or will they get worse? It is very hard to give an answer to this question. Many factors can influence the prognosis of this syndrome, among them the X-inactivation status in the cerebral tissue and the status of modifier genes (Renieri et al. 2003; Longo et al. 2004). These factors are not easily detectable due to the tissue variability between blood and cerebral tissue of X-inactivation and since the modifier genes are not presently known. Besides these factors, the type of the MECP2 mutation can be remarkable. Even though there is not a strict genotype-phenotype correlation, it is possible to give a prognostic value to a MECP2 mutation. In fact, while in classic RTT patients every type of mutation has been found, PSV patients carry only late-truncating or missense mutations. Therefore, the presence of a late-truncating or missense mutation, together with some clinical signs, such as absence of microcephaly or growth delay can predict a more favorable course of the syndrome (Tab.3). It is too early to define a possible prognosis for a CDKL5 mutation given the low number of reported patients. For the same reason a genotype-phenotype correlation for the CDKL5 mutations has to be deferred to a time when a larger number of different mutations and patients will be accessible.

**Table 3.** Criteria for predicting a more favorable disease course (reproduced with permission from Zappella et al. 2001.

<table>
<thead>
<tr>
<th>Three of the following:</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MECP2</strong> late truncating mutation (leaving intact the MBD and TRD)</td>
</tr>
<tr>
<td><strong>MECP2</strong> missense mutation (especially R133, T158 and R306 residues)</td>
</tr>
<tr>
<td>Normal head circumference</td>
</tr>
<tr>
<td>Normal weight or overweight</td>
</tr>
<tr>
<td>Normal height</td>
</tr>
<tr>
<td>Scoliosis mild or absent</td>
</tr>
<tr>
<td>Persistence of a few words even in the Regression period (stage 2)</td>
</tr>
</tbody>
</table>
**Article 5**

Germline mosaicism in Rett syndrome identified by prenatal diagnosis.


Short Report

Germline mosaicism in Rett syndrome identified by prenatal diagnosis


Rett syndrome is an X-linked neurodevelopmental dominant disorder that affects almost exclusively girls. The vast majority of cases are sporadic and are caused by de novo mutations in the MECP2 gene, located in Xq28. Only few familial cases have been reported: in four cases, the mother was an asymptomatic carrier and in other four cases, the germline mosaicism in the mother was postulated. Owing to the above reported cases of germline mosaicism, we decided to offer prenatal diagnosis to all expectant mothers with a Rett daughter despite the absence of the causative mutation in parents’ blood. We describe here the outcome of the first nine cases of prenatal diagnosis followed by our center. In eight cases, the fetus did not carry the mutation. In one case, the female fetus did carry the same mutation of the affected sister. The couple decided to interrupt the pregnancy and to devolve fetal tissues for research purposes. Our results indicate that prenatal diagnosis should be proposed to all couples with a Rett daughter, even when the mutation is apparently de novo. Moreover, one positive prenatal test among the first nine cases indicates that germline mosaicism may be seriously considered for the assessment of recurrence risk during genetic counseling.

As soon as the gene responsible for Rett syndrome (RTT) was discovered, we started performing molecular analysis of MECP2 gene in the RTT females who were admitted to the Child Neuropsychiatry of the University of Siena (1). We also started to collect parents’ samples in order to verify whether the mutation was de novo or inherited. In 115 apparently sporadic cases diagnosed from 1999 till today, the mutation was not found in parents’ DNA except for one case where the same MECP2 mutation was found in both a RTT girl and her unaffected mother (unpublished data). As germline mosaicism was reported in RTT, we decided to offer prenatal diagnosis in case of a second pregnancy of the above reported couples even with a de novo mutation. This suggestion was clearly stated in the counseling report. During the last 4 years, nine couples decided to have a pregnancy and accepted the suggestion to perform a prenatal diagnosis. These nine prenatal diagnoses were performed by chorionic villous sampling in five cases and by amniocentesis in the other four. In eight cases, the fetus DNA was normal and pregnancies were successfully delivered. Five girls and three boys were born and every child, aged from 6 months to 4 years, is presently healthy. In one case, we detected in the fetus the same MECP2 mutation of the affected sister (Table 1).

Case report

We first met the family for genetic counseling during an hospitalization of the affected daughter in the Child Neuropsychiatry, University of Siena.
Table 1. Prenatal tests performed from 2000 till today

<table>
<thead>
<tr>
<th>Year</th>
<th>MECP2 mutation type in the affected daughter</th>
<th>Prenatal test</th>
<th>Sex of the fetus</th>
<th>Result</th>
<th>Mother's age (years)</th>
<th>Father's age (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2000</td>
<td>p.Y141X</td>
<td>Chorionic villous sampling</td>
<td>Female</td>
<td>Negative</td>
<td>34</td>
<td>40</td>
</tr>
<tr>
<td>2000</td>
<td>p.R435X</td>
<td>Amniocentesis</td>
<td>Female</td>
<td>Negative</td>
<td>39</td>
<td>45</td>
</tr>
<tr>
<td>2002</td>
<td>p.R206C</td>
<td>Amniocentesis</td>
<td>Male</td>
<td>Negative</td>
<td>36</td>
<td>39</td>
</tr>
<tr>
<td>2002</td>
<td>p.T156M</td>
<td>Chorionic villous sampling</td>
<td>Male</td>
<td>Negative</td>
<td>31</td>
<td>Unknown</td>
</tr>
<tr>
<td>2003</td>
<td>p.R168X</td>
<td>Amniocentesis</td>
<td>Male</td>
<td>Negative</td>
<td>37</td>
<td>51</td>
</tr>
<tr>
<td>2003</td>
<td>p.P152R</td>
<td>Chorionic villous sampling</td>
<td>Female</td>
<td>Negative</td>
<td>30</td>
<td>36</td>
</tr>
<tr>
<td>2003</td>
<td>p.R133C</td>
<td>Amniocentesis</td>
<td>Female</td>
<td>Negative</td>
<td>31</td>
<td>36</td>
</tr>
<tr>
<td>2003</td>
<td>p.R270X</td>
<td>Chorionic villous sampling</td>
<td>Female</td>
<td>Negative</td>
<td>33</td>
<td>39</td>
</tr>
<tr>
<td>2003</td>
<td>c.567insA</td>
<td>Chorionic villous sampling</td>
<td>Female</td>
<td>Positive</td>
<td>38</td>
<td>38</td>
</tr>
</tbody>
</table>

(Fig. 1a). The proband (No. 709) is the second child of non-consanguineous parents and she was 3 years old at the time of the counseling. She had a normal development in the first 6 months. Then her progress ceased. The ability to use her hands was overwhelmed by incessant hand stereotypes, and hyperventilation and groundless smiles were referred. At 3 years of age, she still had palliation and she was not able to walk. Her head circumference was 45 cm (<3rd cent). She fulfilled the criteria for RTT (2). As a collateral finding, sarcosin, which is not normally detectable in urine and plasma, was detected in this patient in both urine (6.53–9.66 mmol/l) and plasma (0.16–0.17 mmol/l).

We offered a molecular analysis of MECP2 gene to the family. Blood was collected from the proband and her parents after informed consent. The genomic DNA was extracted and coding exons 2–4 of MECP2 gene were amplified and directly sequenced. In the DNA of the proband, the c.567-568insA mutation was found in heterozygous state. Either of the parents did not carry the mutation and there was no evidence of low-grade mosaicism by denaturing high performance liquid chromatography (DHPLC) analysis (Fig. 1b). The suggestion for prenatal diagnosis in case of a future pregnancy was clearly stated during the second session of genetic counseling together with the explanation of the molecular results.

One year after the genetic counseling, the parents, 38 years old each, decided to have a third pregnancy and asked for prenatal diagnosis. The couple decided to go through chorionic villous sampling at 13 weeks + 5 days of gestation. The extracted DNA was analyzed for the presence of the c.567-568insA mutation by DHPLC and direct sequencing. The DNA of the female fetus was found to have the same mutation of the RTT sister (Fig. 1b). In agreement with the couple, we decided to repeat the analysis on a second chorionic villous sampling on the sixteenth week of gestation. The result was confirmed and the couple decided to abort the fetus on the seventeenth week + 1 day of gestation. The fetus was aborted through prostaglandin induction. On the DNA extracted from the umbilical cord, we again confirmed the presence of the c.567-568insA mutation (data not shown).

The whole fetus was devoted to our institute for research purposes. We collected samples from different areas of the brain and from other organs (thymus, liver, spleen, placenta, kidneys, heart, adrenal glands, lung, esophagus, stomach, intestine, and pancreas) for tissue culture, molecular biology tests,
Mari et al.

and histological analysis. Morphological analysis of the brain showed a normal picture. However, the detection of subtle differences in the architectural pattern should require a comparison with a fetus of the same gestational age. This control sample is difficult to obtain, as in voluntary abortion usually pregnancy is interrupted in an earlier gestational age.

Discussion

Rett syndrome is an X-linked neurodevelopmental dominant disorder that affects almost exclusively girls. The vast majority of cases is sporadic and is caused by de novo mutations in MECP2 gene. Only few familial cases, with a documented MECP2 mutation, have been reported. For some of them, the explanation resides in the fact that the mother is an asymptomatic carrier (3–6). In other cases, four in all, germline mosaicism in the mother was postulated. Wan et al. (3) reported of a woman with motor-coordination problems and mild learning disabilities, her RTT sister, her RTT daughter, and her son who died of encephalopathy. All the four individuals carried the same MECP2 mutation. Her parents did not carry the mutation suggesting germline mosaicism (3, 4). In 1999, Amir et al. (7) reported of two half sisters with clinical and molecular diagnosis of RTT. The mutation was not present in their mother suggesting germline mosaicism. Another identical case was reported by Villard et al. (8). Yaron et al. reported of a RTT girl and her brother with severe neonatal encephalopathy, carrying the same MECP2 mutation. The asymptomatic mother did not carry the mutation (9).

In the four familial cases reported above, the MECP2 mutation was maternally derived. Trappe et al. observed that in sporadic cases of RTT, the origin of the MECP2 mutation was almost exclusively paternal (10). Yaron et al. state that we should be more careful in defining a risk of recurrence in those cases where the mutation is maternally derived (9). In order to strengthen this hypothesis, it would have been useful to establish the origin of MECP2 mutation in our case. Unfortunately, in our family the MECP2 mutation origin could not be derived.

So far, expression studies in human tissues have used adult RTT brain only (11). The availability of this fetal brain will allow us to study MECP2 expression, its localization, and gene-expression profiling in a particular developmental stage. Furthermore, this material will allow us to study the possible effects of MECP2 absence on brain structure and on neuronal morphology and plasticity.

This is the first reported case of mosaicism found after a prenatal diagnosis. The frequency of germline mosaicism in RTT is at present unknown. The small number of cases (nine) does not allow to derive a correct percentage (1/9 = 11%) useful for genetic counseling. However, taking into account our experience, mosaicism should not be considered so rare. Despite the fact that the precise rate of germline mosaicism of MECP2 mutations remains unknown, our results strongly indicate that the opportunity to perform a prenatal diagnosis should be discussed with all couples with a RTT daughter despite the apparently de novo mutation.

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This work was also supported by the Ministero della Salute (Progetti di Ricerca Finalizzati NEMEC) to L.L.

References


MECP2 mutations and male patients with mental retardation

Until 2000, it was known that MECP2 mutations could be responsible for a RTT like phenotype in males if associated with Klinefelter syndrome or if they were present in a somatic mosaic state (Schwartzman et al. 2001; Topcu et al. 2002). Constitutional MECP2 mutations in 46,XY males were known to be responsible for a severe neonatal encephalopathy. In order to reveal if the MECP2 gene could be responsible for other phenotypes, we analyzed the gene for point mutations in 21 mentally retarded male patients. Eight of these male patients were familial cases in linkage with a region which included Xq28. MECP2 analysis was performed by single strand conformation polymorphism. A MECP2 mutation was found in one patient belonging to a family linked to Xq28 (see article 6).

The family was not evaluated in our center and a detailed clinical description and pictures of the affected males have been reported by Claes et al. in 1997. A brief summary of the phenotype of the family is given below.

The affected males were a 6 years old boy and his maternal uncle, who died at the age of 39 from pneumonia (Fig.13). Both males showed delay of psychomotor development from infancy. They had severe mental retardation and neurologic signs such as ataxic gait, spasticity and coreoathetotic movements. The 6 year-old boy had his first epileptic episodes at the age of 2, while the maternal uncle developed a grand mal seizure at the age of 27. The carrier females (mother and maternal grandmother of the 6-year-old boy) had borderline intelligence without neurological signs.

![Figure 13. a) The 6 years old boy, b,c) maternal uncle. Note the general habitus of both and the facial hypotonia of the maternal uncle. They have an identical clinical picture. Modified by Claes et al, 1997](image)
This report has contributed to the expansion of the phenotypic spectrum of *MECP2* mutations. After our first report, in the last few years, constitutional *MECP2* mutations have been reported in an increasing number of male patients with a broad spectrum of neurodevelopmental disorders. Further screening of various cohorts of mentally retarded males reported in the literature have allowed to assess that *MECP2* mutations are responsible for MR of various degrees, mostly in combination with neurological features, sometimes associated with psychiatric disorders (Renieri et al. 2003).

However, the initial suggestion that *MECP2* mutations might be a significant cause of MR in males, comparable even to *FMR1* mutations (Couvert et al. 2001), could not be confirmed by further studies as reviewed in detail recently (Ylisaukko-Oja et al. 2005). In the latter study, no pathogenic mutation was found in a group of 103 males with unspecific MR.

In conclusion, according to present day knowledge, *MECP2* mutations are considered an infrequent cause of mental retardation in males.
Article 6

A mutation in the Rett Syndrome gene, MECP2, causes X-linked mental retardation and progressive spasticity in males.


A Mutation in the Rett Syndrome Gene, MECP2, Causes X-Linked Mental Retardation and Progressive Spasticity in Males

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Heterozygous mutations in the X-linked MECP2 gene cause Rett syndrome, a severe neurodevelopmental disorder of young females. Only one male presenting an MECP2 mutation has been reported; he survived only to age 1 year, suggesting that mutations in MECP2 are male lethal. Here we report a three-generation family in which two affected males showed severe mental retardation and progressive spasticity, previously mapped in Xq27.2-qter. Two obligate carrier females showed either normal or borderline intelligence, simulating an X-linked recessive trait. The two males and the two obligate carrier females presented a mutation in the MECP2 gene, demonstrating that, in males, MECP2 can be responsible for severe mental retardation associated with neurological disorders.

Several reports have shown that heterozygous de novo mutations in the MECP2 gene (MIM 300005) cause Rett syndrome (MIM 312750), a sporadic severe neurodevelopmental disorder of young females (Bienvenu et al. 2000; Cheadle et al. 2000; De Bona et al. 2000; Hupke et al. 2000; Kim and Cook 2000; Xiang et al. 2000). The few known familial cases are due to the normal fitness of nonpenetrant carriers who either bear the mutation in a mosaic state or have a nonrandom X inactivation (Amir et al. 1999; Wan et al. 1999). There have been no reports of males with MECP2 mutations surviving beyond age 1 year, which suggests the hypothesis of lethality in males. However, since mutations in the same gene often cause different phenotypes, we tested whether MECP2 mutations may be found in males in whom mental retardation segregated as an apparently X-linked recessive trait.

In one of the families, segregating a very severe mental retardation mapped in Xq27.2-qter, an MECP2 mutation was found. In this family, mutations in other candidate genes (L1CAM, GDI, 16A, and SEX) were excluded (Claes et al. 1997). Screening of the MECP2 gene was performed by SSCP followed by direct sequencing of PCR products showing abnormal migration, as reported elsewhere (De Bona et al. 2000). A C→T transition in position 1216 was found in exon 3. The mutation causes a substitution of glutamine (CAG) 406 with a stop codon (TAG). Since the mutation introduces an ApaI restriction site, we tested all family members by restriction analysis (fig. 1). The mutation was present in the two affected males, as well as in the carrier females, and was absent in all unaffected males in the family. The mutation was never described in Rett cases and was not found among 100 chromosomes, analyzed by ApaI digestion, from unaffected females.

Nonsense and frameshift mutations are supposed to act in two different ways. In some cases, they have been shown to cause production of a truncated protein. Alternatively, they may cause a null phenotype because of the so-called nonsense-mediated mRNA decay, a mechanism that recognizes and degrades mRNAs containing nonsense mutations. To define the exact consequence of the mutation C1216T, we performed semiquantitative RT-PCR on RNA from lymphoblastoid cell lines from one of the affected males and two unaffected controls. After 35 PCR cycles, in the exponential phase of amplification, the amount of transcript was identical in the patient and in the controls (fig. 2), indicating that the
transcript is not degraded and that a truncated protein may be produced.

In the few familial cases of Rett syndrome, nonpenetrant females have nonrandom X inactivation. We studied X inactivation in DNA from blood cells of both carrier females (Allen et al. 1992). The assay showed balanced X inactivation in both females (fig. 3). Band intensities were measured: the ratio between the two alleles was 56:44 in one female and 60:40 in the second.

A detailed phenotypic analysis of the family has been reported elsewhere (Claes et al. 1997). In brief, males showed delayed development (first steps at 2.5-5.5 years) and were never able to speak. They showed facial hypotonia and sialorrhea and a habitus suggesting complicated spastic paraplegia; their head circumferences were at the 75th-90th percentile. One of them had choreoathetotic movements in the right arm, a global bradyarrhythmia as indicated by electroencephalogram, and bilateral juvenile cataract; he was confined to a wheelchair and died from pneumonia at age 39 years. A thorough phenotypic analysis of the family and comparison with signs of Rett syndrome reveals some similarities, including absence of language, ataxic gait, seizures, grinding of teeth, and sialorrhea (table 1). Moreover, spastic paraparesis is a frequent end-stage finding of most cases of Rett syndrome. Among salient differences are the absence (1) of growth retardation, (2) of loss of acquired purposeful hand skills, and (3) of acquired microcephaly. The latter is one of the major diagnostic criteria and contrasts with the macrocephaly of this family. Moreover, stereotypic hand movements versus choreoathetotic movements (present in only one male) and constipation versus diarrhea are notable differences.

The only documented case of a male with an MECP2 mutation is an infant bearing an early truncating mutation (806delG) who died, at age 1 year, from congenital encephalopathy (Wan et al. 1999). Two other reported cases were presumed to carry the same mutation segregating in the family (Wan et al. 1999; I. Meloni, M. Zappella, and A. Renieri, unpublished data). However, direct evidence was hampered by the absence of biological samples from both boys. We now demonstrate that hemizygosity of an MECP2 mutation may be compatible with life.

To our knowledge, the mutation described in the present report has never been described in Rett patients. The presence of a normal amount of MECP2 mRNA in the patient analyzed suggests that a shorter protein, lacking 80 amino acids at the C terminus, is synthesized. The abnormal protein maintains the methyl-binding domain, the transcription repression domain, and only part of the highly conserved histidine and proline-rich C-terminal domain (Huppke et al. 2000). This C-terminal region of the protein shows an overall homology of 35% identity and 46% positivity in a 100-amino acid region with two brain-specific factors, BF-1 and FKH4, which are members of the fork head gene family (Murphy et al. 1994). Moreover, the portion between amino acids 404 and 467 has been shown to facilitate MeCP2 binding to DNA (Chandler et al. 1999).

Altogether, our results suggest that a late truncation of the MeCP2 protein (at amino acid 406) is compatible

Figure 2  Quantitative RT-PCR of total RNA from lymphoblastoid cells of patient (OE) and male (XY) and female (XX) controls. Primers were: MECP-2 RT-S: 5'-GCC TCC ATC ATC GCT GAC-3'; MECP-2 RT-A: 5'-TCT GCC AGT TCC TGG ACC-3'; GAPDH-F: 5'-AAC ACA GTC CAT GCC ATC AC-3'; GAPDH-R: 5'-TCC ACC ACC CTG TTG CTA-3. The RT was done with (+) and without (−) retrotranscription, as described elsewhere (Bonne et al. 1996), using 1 µg of total RNA. We amplified 2.5 µl of the RT reaction in a 50-µl PCR reaction. C = PCR control without template DNA. PCR conditions were the following: GAPDH, 5 min at 94°C; 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C for 25 cycles; MECP-2, 5 min at 94°C; 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C for 35 cycles.
with some of the functions of MeCP2 and allows survival of the patients while nevertheless causing very severe neurological defects. How the mutation described in this paper is similar to the group of late frameshift MECP2 mutations clustered between amino acids 384 and 455 cannot be determined at this point; unfortunately, no studies could be performed on RNA stability or the presence of a truncated protein in those patients.

The absence of a skewed X inactivation in carrier females was perhaps the most striking result of this study. Since a notable variation between tissues is reported, the presence of a completely skewed X inactivation in relevant tissues cannot be excluded. The milder effect of the mutation described here could, however, explain the lack of skewed pattern of X inactivation in females. Alternatively, Rett syndrome may be considered a digenic disease that develops when a de novo mutation in the MECP2 gene occurs in the presence of a mutation in another gene.

Following this digenic model, the MECP2 mutation alone produces a recessive phenotype (as in this family), whereas a mutation in the second gene alone may produce no phenotypic effect at all. The model fits with the evidence that, in familial and sporadic Rett cases, the X-inactivation pattern plays a central role in modulating the disease (Amir et al. 2000; Van den Veyver and Zoghbi 2000).

In conclusion, we suggest that mutations in the MECP2 gene account for a subset of cases of apparently recessive, very severe X-linked mental retardation associated with neurological disorders and that mutation analysis of the MECP2 gene should also be performed in mentally retarded males with progressive encephalopathy. Extensive analysis of the MECP2 gene in such cases will give an indication of the percentage of X-linked mental retardation due to an MECP2 mutation.

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**Electronic-Database Information**

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**References**


Amir RE, Van den Veyver IB, Schultz R, Malicki DM, Tran...
Cohort of male patients with mental retardation

In 2003 an Italian collaborative project on X-linked mental retardation, which, at present, includes 28 clinical centers and 12 laboratories, spread all-over Italy, has been conceived. The aim of this network is to establish the percentage of Italian XLMR patients with mutations in the known genes and to set up rapid and sensitive enzymatic and/or biochemical tests for mutation analysis. A number of 72 sporadic, 9 likely familial, 35 familial mentally retarded males negative for FRAXA, chromosomal, and subtelomeric rearrangements have been collected. In order to obtain accurate clinical data and to fasten data sharing between centers a database has been constructed and is available at http://xlmr.unisi.it (fig. 14).

Figure 14. Login page. Here you can find the description and the aims of the network. If you are a clinician with a password you can access the site to insert patients and verify the status of the molecular tests performed on your patient. If you are a laboratory center with password you can access the site and insert and update the molecular results.

In all the collected cases the following genes are currently analyzed: RSK2, ARX, IL1RAPL1, XNP, OPHN1, FACL4, PAK3, AGTR2, ARHGEF6, FMR2, SLC6A8, MECP2, GDI1, and the two genes found involved in autism (NLGN3 and
NLGN4). Whenever possible, the screening is performed using enzymatic assays (FACL4, RSK2 and PAK3) or dosage of metabolites, such as creatine (SLC6A8). For FACL4 the assay has been already available, the assays for the other two proteins is not available at the moment. All other genes are analyzed with standard mutation analysis techniques. When mother’s biological sample is available, X-chromosome inactivation is tested. A skewed X-inactivation strengthens the hypothesis of XLMR, even in sporadic cases, and mutations in specific genes (FACL4, ARHGEF6, XNP, GDII). Patients with possible contiguous genes deletion syndrome will be tested by Array-CGH using a micro-array of BAC clones of X chromosome developed in The Netherlands (Veltman et al. 2004). Since the majority of cases are sporadic (72 cases out of 116), we decided to include the Array-CGH analysis of the whole genome among the available tests. When two affected individuals are available, locus exclusion/compatibility for specific MRX loci is tested. In larger families, classic linkage analysis is performed. General information is publicly available (number of inserted patients, pedigrees, references). Specific information is accessible only to participants and includes: 1) Detailed clinical data; 2) Location of biological samples (DNA, lymphoblastoid cell lines, plasma, urine etc.); 3) Laboratory results. Patients’ insertion is possible only to participant members through the use of a password. For each record the electronic form is divided in three parts (Parts 1, 2 and 3). Part 1 is filled by the clinician who has visited the proband and includes: Pedigree (to be attached as a JPEG file), Clinical data on relatives (free text), Anamnestic information (free text), I.Q. (value, age, scale, Wechsler scale preferred), Adaptive behavior (value, age, text, scale)(Vineland scale preferred), Behavior (value, age, text, scale)(CBCL scale preferred), Other examinations (free text), EEG (free text and/or JPEG file), MRI (free text and/or JPEG file), Karyotype (banding resolution, result), FRAXA (result), Subtelomeric rearrangement (probes, result), Physical examination (free text), Dysmorphic examination (free text), Photos (to be attached as a JPEG file), Movie (up to 20” movie by digital camera), Neurologic examination (free text), Physician depository of consent form (full address). Part 2 is filled by the laboratory/ies recipient of biological samples and includes: DNA, Lab depository (full address), Leukocyte in DMSO medium, Lab depository (full address), Lymphoblastoid cell
line Lab repository (full address), Plasma Lab repository (full address). When molecular analyses have been performed, results are inserted in the patients’ record part 3, which includes: Mother X-inactivation (result, laboratory). FACL4 enzymatic assay: (result, laboratory). PAK3 enzymatic assay: (result, laboratory). RSK2 enzymatic assay: (result, laboratory). Creatine analysis: (result, laboratory). RNA analysis: (gene name, result, laboratory). SNP analysis: (result, laboratory). Array-CGH analysis (result, laboratory). Mutation analysis: (gene name, result, laboratory).

As it can be seen in Fig.15 the inserted cases are 116 and they are numbered from 1 to 130.

**Figure 15.** A user with password once entered the site can insert new patients. Both a clinical center and a laboratory center can see the list of all the inserted patients and see some clinical details of each patient who is anonymous. Each center can modify or remove from the archive its cases.

In the following sections of this thesis I will report two cases among those I have personally seen during my genetic counselling activity (corresponding to case 1 and case 127 of the XLMR database).

In 2002 I visited two brothers with microcephaly and mental retardation. Clinical investigation of healthy females revealed the presence of microcephaly in some of them. The apparent X-linked semidominant inheritance made the family
suitable for linkage analysis. In October 2004, I visited a patient with high and broad forehead, epichantal folds, ear abnormalities and severe mental retardation. The case is one of the 72 sporadic mentally retarded patients inserted in the database. Despite his enrollment in a X-linked database, a “de novo” interstitial deletion of chromosome 3 was found by whole genome array-CGH analysis.

The extensive use of the array-CGH analysis on the whole genome will probably unravel other autosomal mental retardation cases among those inserted in the database. All these case will enrich our bank which could become a resource also for autosomal MR.
A family with microcephaly and mental retardation

Here, it is reported a three generation family in which an X-linked semi-dominant trait seems to segregate. Two male patients show microcephaly and mental retardation while some females show isolated microcephaly. Analysis of some of the genes currently screened in the context of the XLMR network has been performed. Following the hypothesis of an X-linked disorder a linkage analysis was also performed with markers spanning the entire X-chromosome.

**Figure 16.** Pedigree of the family.

Clinical description

Proband (III-7, fig.16) was born pre-term (7° month of gestation). Birth weight was 1700 gr. and OFC was 26 cm (10° percentile). At 5 months of age he showed microcephaly (OFC 33 cm; <<3° percentile). An ophthalmologic examination performed at that time was negative. At 4 years the child was hyperactive, he had attention deficit disorder and showed auto aggressive episodes and language delay. At 10 years he had movement coordination difficulties, which affected both deambulation and manual abilities. At the time of the examination he was 29 years old (fig. 17). He showed microcephaly (OFC 42.5 cm; <<3° percentile), hypotelorism, height of 182 cm (90° percentile),
weight of 50 Kg, moderate-severe mental retardation, language delay. He was not able to read but he was able to write his name. He was attending a community for disabled people and he practiced several sports. Brain MRI showed a global reduction of the brain volume especially in the supratentorial region; thin brain stem; normal corpus callosum; undergrowth of frontal lobes and temporal pachygiria. Cardiac ultrasound revealed a mild tricuspid valve insufficiency. Auditory evoked potential showed normal hearing. Ultrastructural examination of the hair was negative.

The younger brother (III-9, fig. 16) was born at term: birth OFC was 26.5 cm (<<<3° percentile), birth weight was 2090 gr. (<<3° percentile), birth length was 43 cm (<<3° percentile). At birth cleft lip/cleft palate was noted. He could be able to walk at 18 months and he said his first words at 3 years of age. Parents referred that he has been always hyperactive, and sometimes showed an auto and etero-aggressive behavior. An ophthalmologic examination performed at 3 years of age was negative. At the time of the examination he was 20 years old (fig. 17). He showed microcephaly (OFC 41 cm; <<<3° percentile), hypotelorism, severe mental retardation, height of 160 cm (<3° percentile), language delay.

Figure 17. Patients’ pictures at the time of the last examination (III-7 was 29 years old and III-9 was 20 years old). Note receding forehead in both brothers. Note the surgical scar for cleft lip/cleft palate in III-9.
He was not self-governing, he could not read or write. He practiced several sports. Cardiac ultrasound revealed a mild tricuspid and pulmonary valve insufficiency. Auditory evoked potential showed sensory neural hearing loss of moderate degree for medium-high frequencies. Ultrastructural examination of the hair was negative.

Clinical investigation of healthy females revealed the presence of isolated microcephaly in their sister (OFC 50.5; <3° percentile), their mother (OFC 48.5; <3° percentile), their two maternal aunts (OFC 52.5; <10° percentile) and OFC 52; <3° percentile) and two of their female cousins (OFC 50.5 e 52; <3° percentile).

Until now the ARX, FMR2, NLGN3, NLGN4, PQBP1, AGTR2 and MECP2 genes have been analyzed in these patients and no pathogenic mutations have been identified. In particular, given the presence of microcephaly as a cardinal clinical feature in this family, MECP2 point mutations (exons 2-3-4) were excluded by direct sequence analysis. The X-inactivation status analysis in DNA from blood cells of the mother showed balanced X-inactivation. Array-CGH analysis on the X-chromosome with a resolution of 200 Kb revealed no deletions/duplication of the X-chromosome.

Linkage analysis

The presence of microcephaly and mental retardation in two brothers and the presence of isolated microcephaly in related females prompted us to hypothesize an X-linked semi-dominant disorder in the family. We analyzed 18 microsatellite spanning the entire X-chromosome in the available subjects of the family (fig. 18). Linkage analysis was performed by LINKAGE 5.1 package. Two-point LOD scores between the disease locus and the 18 microsatellite markers are presented in table 4. No significant two-point LOD scores were obtained with any markers. The marker DXS8043 gave a LOD score between −2 and 3 at any theta (tab 4). In fact, it was not informative in probands’ mother (genotype 2.2, fig. 18). Given the above described results we decided to test three more microsatellite flanking the non informative DXS8043 microsatellite (DXS8106, DXS8045 and DXS998). The obtained LOD scores of the flanking markers DXS8106, DXS8045 and DXS998 were <-3 for any recombination fraction. In
fact, both affected males did not share any allele at these loci (fig. 18). The linkage result was expected since there were not markers among the 21 tested which were shared by the affected males and the microcephalic females (fig. 18).

**Figure 18.** Pedigree of the present family, showing alleles for each microsatellite of each subject. Boxed loci are not comprised in panel 28, but they have been taken from panels 84, 85 and 86.
<table>
<thead>
<tr>
<th>Patient</th>
<th>Present case</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
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<td>6m</td>
<td>fetus</td>
<td>5y</td>
<td>1y</td>
<td>3y/2m</td>
<td>fetus</td>
<td>?</td>
<td>1m</td>
<td>22m at death</td>
<td>fetus</td>
<td>23d</td>
<td>22m</td>
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<tr>
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<td>+</td>
<td>+</td>
<td>?</td>
<td>+</td>
<td>+/-</td>
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<td>+</td>
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<td>-</td>
<td>+</td>
<td>n.s.</td>
<td>+</td>
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<td>n.s.</td>
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<td>+</td>
<td>n.s.</td>
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<tr>
<td>Heart defect</td>
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<td>n.s.</td>
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<tr>
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<td>n.s.</td>
<td>+</td>
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<td>n.s.</td>
<td>n.s.</td>
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<td>Atresia of the choanae</td>
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<td>n.s.</td>
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<td>n.s.</td>
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<td>-</td>
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<td>n.s.</td>
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<td>n.s.</td>
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<td>-</td>
</tr>
<tr>
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<td>+</td>
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<td>n.s.</td>
<td>+</td>
</tr>
</tbody>
</table>
Materials and methods

DNA analysis

Blood samples were obtained for the nine subjects of the family available for the study, after informed consent. Genomic DNA was extracted from whole blood using standard procedures. Initially a set of 18 fluorescently labeled microsatellite markers spanning the X chromosome was used for primary mapping (ABI Prism set, version 2, panel 28 from Applied Biosystem). This set comprises microsatellite markers dispersed at intervals of ~10 cM over the entire X chromosome. Then three further polymorphic microsatellites located at Xq27, namely DXS8106, DXS8045 and DXS998 (Applied Biosystem), were used to study a possible candidate region.

Polymerase chain reaction (PCR) amplification of microsatellite markers was performed according to the manufacturer’s recommended protocols (Applied Biosystem). ABI PRISM 310 Genetic Analyzer was used to determine the size of each allele.

Linkage analysis

Two-point linkage analyses between each marker and the disease locus were performed using MLINK of the computer program LINKAGE 5.1 package assuming X-linked dominant inheritance. Full penetrance of the X-linked gene was assumed and the frequency of the disease allele was chosen as 0.0001. Allele frequencies were assumed to be equal for each microsatellite.
Finding a gene for microcephaly and mental retardation

Despite our expectation, the linkage analysis performed on the family with microcephaly and mental retardation did not reveal any candidate region on the X chromosome. However, given the clinical description of the family and the characteristics of the pedigree, the more probable inheritance of the disease in this family still strongly seems X-linked.

We excluded deletions/duplications in the X-chromosome larger than 200Kb by X-chromosome array-CGH analysis. Point mutations and intragenic deletions of some of the XLMR genes were also ruled out. The more likely possibility is that this family carries a point mutation or an intragenic deletion in a gene located on the X-chromosome different from those already screened.

Given the X-linked inheritance of the disease in this family two possible explanations for the result of the linkage analysis could be hypothesized. The first one is an incorrect clinical classification of some females of the family since they were classified on the basis of OFC measurements. A follow up of the entire family is necessary in order to verify the OFC measurements. Another possible explanation is that we could have missed the candidate region since the 21 microsatellites used for the analysis are dispersed at intervals of almost 10-15 Mb over the entire X chromosome. Actually, the available panels for microsatellite markers are five. We have analyzed one panel and three more microsatellites belonging each one to a different panel. The complete analysis of all panels will narrow the intervals between each analyzed marker to 4-5 Mb, getting more probable to achieve the goal.

An alternative strategy is to search for candidate genes on the basis of the phenotype. Clinical hints are limited to MR and microcephaly which are not tightly specific signs. One of the two males has cleft lip/cleft palate. On the website of the Journal of Medical Genetics, among the list of papers accepted for publication, there is an article which will report mutations in the PHF8 gene located on the X-chromosome associated with MR and cleft lip/cleft palate. The PHF8 gene could be a candidate gene to be tested in this family.
A male case with mental retardation and dysmorphisms

Clinical description

Proband is a 26 years old male. Pedigree is silent for genetic diseases. The mother had a normal pregnancy and delivery, and the child was born at the 38° week of gestation. Birth weight was 2520g (3°-10°percentile) and length was 46 cm (10°-25° percentile). no data about birth OFC is available. Motor and mental development was delayed: he was able to sit at 1 year and to walk unaided at 2 years. He said his first words at 2 years of age. He showed growth delay with disproportion between height and weight. In fact, while his height has always been below the 5° percentile since the age of 3 month, he has always been overweight.

![Family tree](image)

Figure 19. Pedigree of the family.

On physical examination at the age of 26 years he showed: turricephaly with OFC of 56 cm (75° percentile), height of 155 cm (<5° percentile) and weight of 70 Kg (75° percentile, overweight if adjusted for the height, BMI = 29). He showed some dysmorphic facial features: he had high and broad forehead,
epichantal folds, strabismus, thick and arched eyebrow, dysmorphic ear with hypoplastic lobules and folded helix, wide interalar distance with hypoplastic alae nasi, large mouth and high vaulted palate. He had small hands (16.5 cm, 3º percentile) and feet (21 cm, << 3º percentile), 5º finger camptodactyly, ulnar deviation of both hands, and calcaneovalgus feet.

Figure 20. a) Proband’s facies at present (26 years); b) proband’s hands, showing 5º finger camptodactyly and ulnar deviation of both hands; c) calcaneovalgus feet.
To better ascertain the facial phenotype of the patient we asked the parents to show us picture of their son when he was younger. As it can be appreciate comparing the pictures at different ages of proband, some facial differences are present. Epichantal folds were more evident in the pictures when proband was younger while other features have become more evident with the age like the long face and the shape of the nose.

![Figure 21](image). Proband at 5 months, 1 year and 2 years old (from the left). Note the arched eyebrows and the high and broad forehead evident since the age of 5 months.

At the last examination (26 years) he showed severe mental retardation being impossible to perform IQ testing in this range. Language was absent. He was able to walk unaided on a broad base. During the examination he showed an aggressive and self-injuring behavior. However, his parents referred that at home he was a very peaceful boy.

EEG, performed at 2 years, was normal. RX of the wrist showed a bone age equivalent to the chronological age. Brain MRI, performed at 14 years, showed hyperplasia of corpus callosum. Recent cardiac and abdominal ultrasonography were normal. On the Vineland Adaptive Behavior Scale, performed at 26 years, he functioned in the lowest range of the severe mental retardation spectrum especially in the communication and socialization areas with a very little better preservation of daily living and motor skills.
Cytogenetic and molecular cytogenetic analysis

High resolution karyotype revealed the presence of a 3p interstitial deletion: 46,XY.del(3)(p14.3;p14.1). Whole genome array CGH analysis confirmed an interstitial deletion of about 8 Mb of chromosome 3. Both the karyotype and the array-CGH analysis were performed in the General Biology and Medical Genetics of the University of Pavia.

Figure 22. Plot Ratio plot from array data for chromosome 3 for the proband. Each ratio plot is comprised by normalizing data from two independent arrays. Normalized data from the array in which the test sample was labeled with Cy3 is shown in red while the normalized data from the array in which the test sample was labeled with Cy5 is shown in blue. Individual spots along the ratio plot represent the normalized ratio of individual clones in linear order with the left-most clone being consistent with the p-arm terminus, while the right-most clone is consistent with the q-arm terminus. As the normalized Cy5: Cy3 ratio is computed for both arrays, a loss of a particular clone is manifested as the simultaneous deviation of ratio plots from the modal value of 1.0, with the red ratio plot showing a positive deviation (upward), while the blue ratio plot shows a negative deviation (downward) for the same clone. Conversely, DNA copy number gains show the opposite pattern. Chromosome 3 shows a seven-clone deletion (from clone RP11-754F19 to clone RP11-88H12), on the p-arm.

Karyotype at a banding resolution of 400 bands was performed on both parents. The cytogenetic study revealed no abnormalities suggesting a “de novo” origin of the deletion. Microsatellite analysis aimed to determine the parental origin of the deletion is ongoing. In order to definitively exclude a recurrence risk for the asymptomatic 30 year-old sister more studies are necessary. As soon as the parental origin of the deletion will be ascertained, FISH analyses with specific probes on chromosome 3, will be performed to exclude that the deletion has originated from a cryptic chromosomal rearrangement, such as inversions, not visible at the cytogenetic study.
Materials and Methods

Array-CGH

Genomic DNA was extracted from peripheral blood using a QIAamp DNA Blood Maxi kit (QIAGEN S.p.A., Milano, Italy). Patient and control DNA samples were digested with EcoRI to produce a homogeneous smear DNA extending from app. 600bp to 2Kb. DNA samples were then purified using a DNA Clean and Concentrator kit (Zymo Research, Orange, CA). We used commercially available genomic DNA array slides (Human BAC Array, Spectral Genomics, Houston, TX), containing 2600 BAC clones spotted in duplicate with a resolution of 1 Mb. Labeling and hybridization were performed according to the manufacturer’s protocol for Spectral Genomic Chip. Both forward and dye swap labeling experiments were done for the patient. Following hybridization, slides were scanned on a GENEPIX 4000B scanner (Axon Instruments, Union City, CA) and the TIFF images captured using GENEPIX Pro Software. The images were analyzed using SPECTRALWARE BAC Array Analysis Software v 2.0 (Spectral Genomics). The software recognizes the regions of fluorescent signal, determines signal intensity and compiles the data into a spreadsheet that links the fluorescent signal of every clone on the array to the clone name, its duplicate position on the array and its position in the genome. The software was also use to normalize the Cy5:Cy3 intensity ratios for each slide and each data point. The normalized Cy5:Cy3 intensity ratios were computed for each of the two slides and plotted together for each chromosome. The ratio plot is arbitrarily assigned such that gains in DNA copy number at a particular locus are observed as the simultaneous deviation of the ratio plot from a modal value of 1.0, with the blue ratio plot showing a positive deviation while the red ratio plot shows a negative deviation at the same locus. DNA copy number losses show the opposite pattern. The linear order of the clones is reconstituted in the ratio plots consistent with an ideogram, such that the p terminus is to the left and the q terminus is to the right of the plot.
Comparison with other published cases

Deletions of the short arm of chromosome 3 are rare and most of them have been found to involve the terminal segment, from 3p23 or 3p24 to 3pter. A total of 13 cases of proximal interstitial deletion of chromosome 3 have been published so far, including four prenatally detected and aborted fetuses (Kogame and Kudo 1979; Wyandt et al. 1980; Sichong et al. 1981; Mitter et al. 1984; Neri et al. 1984; Short et al. 1986; Hertz et al. 1988; Naritomi et al. 1988; Karimi-Nejad et al. 1990; Crispino et al. 1995; Wieczorek et al. 1997; Pfeiffer et al. 1998; Schinzel et al. 1998). Figure 23 summarizes the cytogenetic findings of the reported deletions in comparison with our patient’s deletion (present case and cases 1-13). All of them were identified by standard karyotype. For this reason, it is likely that the deleted segments differ among all patients, even if, in some patients, the breakpoints are reported in the same bands (see for example patient 3 and 5 or 9 and 10). Some of them are slightly larger deletions totally missing the band p14 and the others partially overlap extending toward the centromere. The deletion found in our patient is the smaller one and comprises the G-negative band 3p14.2 and part of dark G-bands 3p14.1 and 3p14.3. Common to all the first 13 cases, including the present case, seems to be part of the dark G-band 3p14.1. One more case with proximal interstitial deletion of 3p has been reported in 2003 by Petek (case 14) (Petek et al. 2003). In this case the deletion did not overlap the deletion found in our patient. This case was included since useful for the discussion.

In two reported cases the chromosomal rearrangements was more complex: the 3p deletion was associated with a translocation. The patient reported by Kogame in 1979 (case 7) had a 3p deletion associated with a reciprocal translocation between chromosomes 3 and 18, while Crispino reported a patient (case 13) with a 3p deletion and a t(3;20) reciprocal translocation.

In six cases (cases 1, 5, 6, 11, 12 and 14) the origin of the deletion has been investigated and in all these five cases the deletion originated in the paternal chromosome. In two of them the father carried a silent rearrangement on
chromosome 3. In the case reported by Wyandt (case 6) the asymptomatic father carried an inverted insertion of band p12 from the proximal short arm of chromosome 3 into the distal short arm at the approximate position of band p25.5. The father of case reported by Pfeiffer (case 1) had the paracentric insertion ins(3)(24.1p12.1p21.31).

![Figure 23](image-url)  

Table 5 summarizes the major phenotypic abnormalities in cases of proximal interstitial deletion of 3p including our patient. Several clinical information is lacking for some patients. However, from the available data, patients 1-13 and present case have some clinical features in common, especially
some minor facial anomalies. Apart from growth and mental retardation, the clinical picture seems to be characterized by high and broad forehead, epicanthal folds and ear abnormalities. The specificity of these anomalies is reinforced by the phenotype of case 14, even if also this patient presents ear abnormalities.

According to us the facial phenotype is remarkably similar especially when picture of the reported patients are directly compared with the pictures of our patient when he was younger. In fact, since all reported cases were younger than 5 years and our case was adult, the resemblance was more impressive if comparing patients of the same age (fig. 24).

Face is characterized by high forehead, arched eyebrows, epicanthic folds, broad nasal bridge, short stubby nose with anteverted nares and dysplastic ears. Other two major manifestations of the proximal 3p deletion phenotype seem to be limitation of joint movements and deformities including ulnar deviation of hands, camptodactyly and calcaneovalgus feet.

Some of the reported cases, have a severe phenotype compared to our patient, including intestinal malformation, heart defects or genital abnormalities. These associated malformation seem to be variable at a high degree and some seem to be specific for each patient. Probably, this variability can be related to the different extension of the deletion and thus to the involvement of specific bands.

In conclusion, there seem to be in the present patient and in those previously described a sufficiently typical combination of minor congenital anomalies to allow the delineation of a proximal 3p deletion syndrome. However, it could be useful to deeply investigate the phenotype-genotype correlation through a collection of clinical follow-up data and biological samples of the previously reported cases. I am already in contact with prof. Albert Schinzel, who is one of the clinician who reported a 3p deleted patient. He agreed to send us biological samples of patient and parents and to inform us about the clinical follow up data of patient. Besides collecting biological samples and clinical data of already described patients, widely use of array-CGH may lead to the identification of additional microdeletions, further delineating a specific syndrome.
Figure 24. Comparison between the facial phenotype of our patient when he was 5 months, 1 and 2 years old (left panel) and the available pictures of the reported patient with a 3p deletion overlapping that of our patient (upper right panel). Patients are numbered according to figure 23. Case 2 is 5 months old, case 5 is 5 years old, case 6 is 1 year old and case 10 is 1 month old. In the lower right panel, frontal, lateral and total view of the patient (22 months) with an interstitial 3p deletion non overlapping with that of our patient. Note the lip scar after surgical correction of the cleft lip.
<table>
<thead>
<tr>
<th>Patient</th>
<th>Present case</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
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<tbody>
<tr>
<td>Age</td>
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<td>fetus</td>
<td>4m</td>
<td>6m</td>
<td>fetus</td>
<td>5y</td>
<td>1y</td>
<td>3y/2m</td>
<td>fetus</td>
<td>?</td>
<td>1m</td>
<td>22m at death</td>
<td>fetus</td>
<td>23d</td>
<td>22m</td>
</tr>
<tr>
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<td>+</td>
<td>?</td>
<td>+</td>
<td>+</td>
<td>?</td>
<td>+</td>
<td>+/-</td>
<td>+</td>
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<td>+</td>
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<td>?</td>
<td>+</td>
</tr>
<tr>
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<td>n.s.</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>n.s.</td>
<td>+</td>
<td>n.s.</td>
<td>+</td>
<td>n.s.</td>
<td>+</td>
<td>n.s.</td>
<td>-</td>
</tr>
<tr>
<td>Heart defect</td>
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<td>+</td>
<td>+</td>
<td>n.s.</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>n.s.</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Coloboma of iris or retina</td>
<td>-</td>
<td>n.s.</td>
<td>+</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>-</td>
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<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>Atresia of the choanae</td>
<td>-</td>
<td>n.s.</td>
<td>+</td>
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<td>-</td>
<td>n.s.</td>
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<td>-</td>
</tr>
<tr>
<td>Genital abnormalities</td>
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<td>n.s.</td>
<td>+</td>
<td>-</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
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</table>
FUTURE AIMS AND PERSPECTIVES

RTT has been always for me a fascinating syndrome either from a molecular and a clinical point of view. I tightly think that the direct contact with RTT girls and their families has enriched both my moral and clinical qualities.

The majority of classical RTT patients (93 out of 116, 80%) I have seen carries a MECP2 mutation. However, neither an extensive point mutation analysis nor the Real Time PCR analysis, was able to demonstrate a MECP2 mutation in 23 patients (20%). These cases might bear intronic/splicing mutations in MECP2 itself. Attempts to study MECP2 mRNA in these patients are ongoing. The hypothesis of a second gene is less likely although not completely excludible.

In the RTT variants the percentage of the identified mutations is lower. Approximately only the 50% of Preserved Speech Variant (19 out of 39) bear a MECP2 mutation. It is more difficult to give a percentage for the other variants due to the low number of patients. In our cohort we have identified a MECP2 mutation in 6 patients with the “forme fruste” variant. No mutations in the congenital variant have been identified although I have seen only 3 cases. As in the classic form some patients might bear intronic/splicing mutations in the MECP2 gene. Alternatively they might have alterations in one or more additional genes, as suggested by the identification of a chromosomal rearrangement in one patient (Pescucci et al. 2003).

Our work has demonstrated genetic heterogeneity in RTT by the identification of CDKL5 mutations in patients with the early onset seizure variant of RTT. For this variant things are now starting to become more clear. So far CDKL5 mutations have not been found in the classic RTT phenotype. We thus suggest to deserve CDKL5 mutation analysis for RTT patients with early onset of seizures since CDKL5 seems to be specific for this variant. In our cohort of patients we have found a CDKL5 point mutation in four out of seven patients with the early onset seizure variant of RTT. The phenotype of the remaining three girls is very similar to the clinical pictures of the four patients with a CDKL5 mutation. We are attempting to set up a Real Time PCR assay for CDKL5
deletions/duplications scanning, since, as happened for the MECP2 gene, some of these patients could bear CDKL5 deletions or duplications.

As underlined in the aims of this thesis, the opportunity to directly see patients is of great importance to get hints for research. As described in article 3 the clinical features of infantile spasms have driven our mutation analysis toward CDKL5 (Scala et al. 2004). If we could not have visited all the RTT and RTT-like patients we actually have seen, we could not have proven the involvement of the CDKL5 gene in RTT. Since not all the researchers working on RTT have this opportunity, thanks to the RTT bank, they could benefit from this precious cohort of RTT patients.

In a section of this thesis I have also reported my experience of genetic counselling in RTT. I have reported the exceptional out-come of one of the first nine cases of prenatal diagnosis of RTT followed by our center. In eight cases the prenatal diagnosis resulted negative, while in one instance the female fetus did carry the same mutation of the affected sister. The couple decided to interrupt the pregnancy and to devolve fetal tissues to our Institute for research purposes.

So far, MeCP2 expression studies in human tissues have used adult RTT brain only (Colantuoni et al. 2001). The RTT female fetal brain tissues will consent us to study MeCP2 expression and localization and gene expression profiling in a particular developmental stage. A fetal age-matched control tissue is necessary for the comparison with the RTT fetus in each experiment that will be designed. We are making attempts to obtain the control fetus through an agreement between our Department and the Department of Gynecology, Perineonatology and Human Reproduction of the University of Florence. Since a perfectly age-matched control fetus is very hard to obtain we have considered an alternative strategy. This strategy consists in using, as control cells, cells of the RTT fetus itself, which have inactivated the X chromosome with the mutation.

With the collaboration of the department of Anatomy and Pathology of Siena we are designing immunocytochemical experiments with two mouse-monoclonal antibodies, one designed against the N-terminus region of MeCP2 and the other against the C-terminus (Sigma). These two antibodies will allow us to discriminate the cells where the wtMECP2 is expressed from the cells where
the mutated MECP2 is expressed. In fact, the mutation c.567insA, carried by the RTT fetus, will result in a protein lacking the C-terminus domain. The truncated protein will be recognized only by the N-terminus antibody. We will previously verify if the mutated mRNA undergoes RNA decay through the retrotranscription of the RNA extracted from fetal material and subsequent direct sequencing. After this passage we will instantly dissect positive and negative single cells. For the microdissection we will use the laser “capture” technique (one of the two available techniques for single cell dissection). The cells removed will be used for the isolation of RNA. After RNA isolation we will test the expression of specific genes by Real Time PCR. First of all, we will study the CDKL5 gene and the MAP2 gene. The latter is one of the more promising pathogenetic genes deleted in the above mentioned RTT-like patient who carries a de novo 2q34 deletion (Pescucci et al. 2003). Furthermore additional promising genes will be analyzed (Horike et al. 2005; Pescucci et al. 2005).

If the candidate gene approach will not give results, we will perform microarray studies. For the microarray technology we will need to amplify the RNA of about a million fold to reach a suitable RNA amount. In fact, protocols for labeling of probes for microarrays specify the use of 100-200 of total RNA (approximately 3-6 µg of mRNA) and a single cell has approximately 25 pg of total RNA (0.5 pg of mRNA). The amplified antisense technology, a linear amplification procedure which guarantee no differential amplification, will be used.

Future perspectives of the two cases with MR have been discussed in dedicated sections of the thesis. For the familial case a linkage analysis with additional markers will be performed in order to find a possible candidate region for XLMR associated with microcephaly. For the sporadic case with the interstitial 3p deletion, a more detailed clinical and molecular comparison with the other published cases and the discovery of additional 3p14 deleted patients will clarify if there is a 3p deletion syndrome.

Besides the discovery of new XLMR loci and/or X-microdeletion the aim of the XLMR project is to collect a large number of potential XLMR patients, which will be analyzed for mutations in the known MRX genes, in order to establish
the mutation rate in the Italian population. Presently, most of mentally retarded males, both sporadic and familial, are not screened for known MRX genes due to the time consuming and high cost of molecular diagnosis. The establishment of mutation frequencies will have important relapse in the management of mentally retarded patients. Genes with a mutation frequency higher than 1% should be introduced in the routine diagnosis of mentally retarded males besides the fragile-X. As it has happened for the patient with 3p deletion, the cohort of patients we are collecting in the XLMR bank could also become an important resource for autosomal mental retardation.
ACKNOWLEDGEMENTS

First of all my thanks go to “chance”. I often say that my having become a geneticist is the result of a series of circumstances, even if, as a youth, I found myself fascinated by the extraordinary intuition of Mendel and the theories of Darwin.

At this time, however, I would like to express specific gratitude to prof. Alessandra Renieri, director of the Medical Genetics Unit, for all she taught me and for her supervision throughout. I am also very grateful to her for having provided me with the opportunity to gain experience during these years.

I would like to thank all those people who so generously shared with me their gifts of knowledge. And I extend thanks to all my colleagues, so many of whom are female colleagues, for having worked with me, and with whom I continue to work. I thank them for their lively exchange of ideas and for their having made me feel an integral part of a solid group....... not only limited to work.

And now......“all the others”......I thank you for your loyal support, always.
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