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Phenotypic and Genotypic Variability in Four Males With *MECP2* Gene Sequence Aberrations Including a Novel Deletion

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ABSTRACT: *The MECP2* gene mutations cause Rett syndrome (RTT) (OMIM: 312750), an X-linked dominant disorder primarily affecting girls. Until RTT was considered lethal in males, although now approximately 60 cases have been reported. Males with *MECP2* mutations present with a broad spectrum of phenotypes ranging from neonatal encephalopathy to nonsyndromic mental retardation (MR). Four boys (aged, 3–11 y) were evaluated for MR. Patient 1 had autistic features. Patients 2 and 3 were brothers both presenting with psychomotor delay. Patient 4 showed dysmorphic features and behavioral problems reminiscent of FXS. All patients had a normal 46, XY karyotype and three were tested for FXS with negative results. *MECP2* gene analysis of exons 3 and 4 was performed using methods based on the PCR, including Enzymatic Cleavage Mismatched Analysis (ECMA) and direct sequencing. Patient 1 presented somatic mosaicism for the classic RTT p.R106W mutation and patient 4 carried the p.T203M polymorphism. Analysis of the mothers in both cases revealed normal DNA sequences. Patients 2 and 3 had a novel deletion (c.1140del86) inherited from their unaffected mother. *MECP2* gene mutations may be considered a rare cause of MR in males although great phenotypic variation hinders genotype-phenotype correlation. **(*Pediatr Res* 67: 551–556, 2010)**

Rett syndrome (RTT) (OMIM: 312750) is a severe X-linked dominant neurodevelopmental disorder, typically affecting females. It is characterized by apparently normal psychomotor development during the first 6 months of life, followed by gradual loss of acquired skills, deceleration of head growth, initiation of stereotypic hand movements, and autistic behavior by the age of 3–4 y (1). During the course of the disease multiple clinical problems emerge such as seizures, scoliosis, breathing abnormalities, and severe mental retardation (MR) (1). The prevalence of RTT is 1:10–15.000 live births per year and 99% of the cases are considered sporadic (1).

Although the diagnosis of classic RTT is based on well-defined criteria, several atypical RTT forms also exist (2). In the majority of cases, the syndrome is caused by heterozygous mutations in the *MECP2* gene, which is located on chromosome X (Xq28) and encodes a methyl-CpG binding protein 2 (*MeCP2*). The protein is thought to selectively bind methyl-CpG islands in the mammalian genome, functioning as a regulator (mostly a repressor) of gene expression. Mutations of the *MECP2* gene have been reported in a broad phenotypic spectrum of mentally retarded individuals (3). The first case was molecularly documented in 1999 after the discovery of the *MECP2* gene. Generally, males were considered not to survive due to the X-linked mode of inheritance of the disease (4), but to date, more than 60 male patients with *MECP2* mutations have been reported (5), suggesting that mutations in this gene may be included among the causes of MR in males. Recent data show that the frequency of *MECP2* mutations among mentally retarded males is approximately 1.3 to 1.7% (5).

The clinical phenotype in males displays great variability and ranges from severe neonatal encephalopathy with early lethality to mild unexplained MR with a longer life span (3). However, males with *MECP2* mutations can be grouped into three main categories: 1) male infants with severe neonatal encephalopathy, usually siblings of classic RTT female patients (6) who characteristically have microcephaly, axial hypotonia, limb rigidity, movement disorders, and seizures without a structural brain anomaly; 2) males with *MECP2* mutations with features of classic RTT either carrying an extra X-chromosome (Klinefelter syndrome) (5) or presenting somatic mosaicism (5,7); 3) patients with variable MR (8) often segregating to other family members (X-linked mental retardation, XLMR) (8). The observed mutations in this latter category are usually different from those causing classic and atypical RTT.

At the molecular level, *MECP2* comprises four exons and has two isoforms as a result of alternative splicing of exon 2 and the use of two alternative start codons (9). The more abundant second isoform (*MECP2* B or *MeCP2-a*) has only recently been described (9,10) and has led to the re-evaluation of exon 1 (10), which is now being thoroughly studied for mutations. Exon 1 is observed to contribute approximately 0.5% of the overall mutations and only in female patients (11) whereas exon 2 has never been observed with mutations. The majority of causative alterations (including missense, nonsense, and frameshift mu-

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Abbreviations: CEL, endonucleases isolated from celery; **ECMA,** enzymatic cleavage mismatch analysis; **FXS,** fragile X syndrome; **MBD,** methyl binding domain; **TRD,** transcriptional repression domain; **XCI,** X chromosome inactivation

tations) occur in exons 3 and 4, which encode the *MeCP2* functional domains, the MBD and the TRD domain, respectively (12). The C-terminal region located downstream of the TRD is prone to deletions of various sizes resulting in the production of truncated proteins (13). Large rearrangements encompassing even whole exons and more specifically duplications ranging from 400 to 800 kilobases (Kb) have also been detected in RTT patients, accounting for ~10% of RTT cases. Their detection is facilitated by the application of methods such as Multiple Ligation Probe Amplification (MLPA) (14).

In this study, we present three different nucleotide variations in the *MECP2* gene among four male cases with MR, including two likely pathogenic mutations and one previously reported benign polymorphism (15). These findings extend the observations in males with *MECP2* mutations, and highlights the value of *MECP2* molecular testing in males with neurodevelopmental disorders, especially when other studies have failed to provide diagnostic information.

PATIENTS AND METHODS

Patients. The patients in this study were retrospectively selected from among 2 different patient groups. Group 1 included 11 male patients who had been referred with suspected symptoms of RTT syndrome, and Group 2, which included 104 male patients presenting fragile X features but with negative molecular testing. Standard karyotype analysis by G-banding technique was normal for all patients. Informed consent was obtained from the guardian of each participant while approval from our Institutional Review Board was also provided.

Patient 1 (from group 1), was born at term after an uneventful pregnancy and delivery. His birth weight was 3,800 g (50–75th centile), with a body length of 53 cm (50–75th centile) and a head circumference of 35.5 cm (50th centile). Apgar scores were 9 at 1 min and 10 at 5 min. Parents were nonconsanguineous and of normal intelligence. The boy was reported to develop normally until the age of 18 mo when he began to display some autistic features such as monotonous playing, stereotyped and clumsy hand movements, and social withdrawal. Regression of acquired skills such as speech development became gradually apparent. As a toddler, he also demonstrated recurrent episodes of acute otitis media and urinary tract infections. Asperger or RTTs were the most probable diagnoses at the first neurologic evaluation at the age of 6. He was referred to the Medical Genetics Department at the age of 11 where clinical examination found all somatometric parameters above the 97th centile (weight: 86 kg, height: 162 cm, head circumference: 58.5 cm, and body mass index: 32.77). He was hyperactive and presented disruptive behavior and attention-deficit disorder. Follow up at the age of 14 showed persistent obesity, stereotypic movements, and obsessive behavior with frequent temper tantrums. He never experienced seizures. Speech was limited to one word, while he was able to read isolated phrases from texts. Although presenting autistic-like behavior and social withdrawal, he managed to attend and complete his studies in an elementary school for children with "special educational needs."

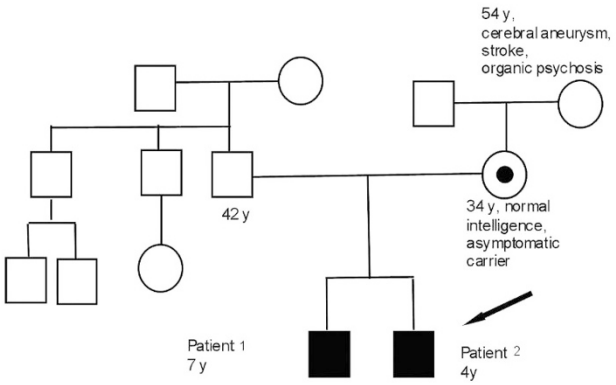

Figure 1. Pedigree of the second family.

Patients 2 and 3 (from group 2), were siblings born from healthy unrelated parents. The only family member with adverse family history was the maternal grandmother who had a premature vascular ischemic attack at the age of 35 y, due to a cerebral aneurysm (Fig. 1). Patient 2, the elder sibling, was first examined at the age of 7 because of severe psychomotor delay. He also presented pyramidal signs, spasticity, inability to walk, and stereotypic movements of the trunk. The brain MRI scan showed mild cerebral atrophy especially in the frontal lobes. His younger brother, patient 3, was born at the 40th week of gestation by normal delivery. His birth weight, length, and head circumference were normal (3,400 g, 52 cm, and 34 cm, respectively). During the first year of age, he was able to use some words, however, he could not walk unaided until the age of 18 mo. At the age of 4, he presented speech stagnation, no sphincter control and gait disturbance. He dragged his left leg and showed brisk tendon reflex of the left lower extremity. Morphologic facial features such as downslanting parpebral fissures, hypertelorism, a deep nasal bridge, and a protruding lower lip were observed. Extensive biochemical investigations including serum and urinary amino acids analysis, urinary glycosamino glycans, serum lactic and pyruvic acid, and thyroid hormones were negative. Fundoscopy, audiogram, and orthopedic examinations were normal. Although the patient had never presented seizures, the sleep EEG showed mild diffuse slowing and some atypical spikes (F_3, C_3). The MRI of the brain revealed mild frontal cortical atrophy.

Patient 4, (from group 2) was born after an uneventful pregnancy weighing 2,850 g (10th centile), with a height of 50 cm (50th centile) and head circumference of 34 cm (10–25th centile). He was initially evaluated for developmental delay at the age of 7. He demonstrated behavioral problems, such as aggressiveness, hyperactivity, and attention deficit, and his long face with protruding ears were reminiscent of Fragile-X syndrome. As there was no follow-up, further clinical data were unavailable.

All patients and their mothers underwent molecular analysis of the *MECP2* gene. Genomic DNA was extracted from peripheral blood leukocytes according to the protocol of an automated robotic system (QIAGEN BioROBOT M48, QiaGen, Hilden, Germany).

Exons 3 and 4, which include the majority of the *MECP2* sequence variations, were analyzed in five overlapping fragments. Polymerase chain reaction (PCR) amplification used primer pairs designed with the aid of the Integrated DNA Technologies software (http://www.idtdna.com/Scitools/Applications/Primerquest/) (Table 1).

The PCR fragments were analyzed initially using ECMA, a simple enzymatic protocol we have previously described, in which PCR reaction products are heat treated to induce heteroduplex formation (which forms

Table 1. *The primers and PCR conditions designed for the analysis of exons 3 and 4 of the* MECP2 *gene*

Exons	Fragment	Primers	Product size	Tm (°C)
3	3.1F	5′-AAG ATC TGA GTG TAT GAT GGC CTG GG-3′	428bp	60
	3.1R	5′-TTT GCT TAA GCT TCC GTG TCC AGC-3′		60
	3.2F	5′-AAG AGA AAG AGG GCA AGC ATG AGC -3′	405bp	60
	3.2R	5′-AAG CAC ACC TGG TCT CAG TGT TCA -3′		60
4	4.1F	5′-CAG TTT GTC AGA GCG TTG TCA CC A CCA T-3′	626bp	62
	4.1R	5′-TGA CGG AGT ACG GTC TCC TGC ACA GAT-3′		62
	4.2F	5′-CAG TTC CTG GGA AGC TCC TTG TCA AGA T-3′	616bp	62
	4.2R	5′-TGA CTC CTC TGG GCA TCT TCC CTC TTT-3′		62
	4.3F	5′-CAG TGG GAA AGG ACT GAA GAC CTG TAA G-3′	566bp	60
	4.3R	5′-TGA CCA GTT AAT CGG GAA GCT TTG TCA G-3′		60

when there are both wild-type and mutant alleles present in the DNA sample being analyzed), followed by digestion with a mismatch-specific DNA plant endonuclease from the CEL family. Under specific digestive conditions surveyor nuclease recognizes and cleaves both DNA strands at mismatched nucleotide positions (Surveyor Mutation Detection Kit, No 706020, Transgenomics Inc.) (16).

The PCR-generated fragments, including also those without an abnormal ECMA result, were also directly sequenced with the Thermo Sequenase Primer Cycle Sequencing kit (GE Healthcare, UK), according to the manufacturer's instructions, run on Long Read Tower System automated sequencer (VisGen, Ontario, Canada) and analyzed using GeneObject Software (Visible Genetics Inc, Ontario, Canada).

"In silico" analysis with the PolyPhen (http://genetics.bwh.harvard.edu/pph/) and SIFT softwares (http://sift.jcvi.org/) was carried out to evaluate the likely effect on protein structure of the nucleotide variations identified by DNA analysis.

RESULTS

DNA sequencing in patient 1 revealed a substitution of a cytosine by thymidine at position 316 in exon 3, predicted to result in an arginine to tryptophane amino acid change in the MBD at the position 106 of the *MeCP2* protein (R106W) Surprisingly, the sequencing chromatogram showed two nucleotide peaks at the variant base position, suggesting the existence of two cell lines and thus a possible somatic mosaicism. To further evaluate the mutation and mode of inheritance the mother was also tested, revealing a normal sequence (Fig. 2). In addition DNA from the hair roots of patient 1 (representing the same embryonic origin as the brain) was analyzed, revealing a normal sequence. No other tissues such as fibroblast or buccal cells were available for analysis.

Patient 2 carried a deletion of 86 basepairs in exon 4, in the C-terminal region of the *MeCP2* protein (c.1140del86), predicted to result in an abnormal protein lacking 29 aminoacids and also a shift to the reading frame by one amino acid. DNA sequencing analysis was then performed on both his brother (patient 3) and their mother. The same deletion was detected in both, confirming the diagnosis of a RTT-like condition in patient 3 and revealing the mother as a carrier (Fig. 3).

Patient 4 carried the C to T mutation at codon 608 in exon 4, predicted to result in the substitution of threonine by methionine at amino acid 203 (http://mecp2.chw.edu.au/mecp2). The T203M change is located in the region between the *MeCP2* functional domains (MBD and TRD) and has been previously described as a polymorphism (15). In silico analysis using the SIFT software evaluated the T203M substitution as likely deleterious to the protein, while the Polyphen program did not yield an abnormal result. The patient's mother had a normal DNA sequence.

DISCUSSION

MeCP2 is a member of the family of methyl-binding proteins (MBPs) and preferentially binds to symmetrically methylated CpG dinucleotides (17). Furthermore, *MeCP2* recruits co-repressor complexes such as Sin3a and Ski, which remodel and repress chromatin and subsequently inhibit the transcription of certain neurodevelopmental genes (18). The mechanism of inhibition not only includes the transcription repression at gene promoters but also influences the chromatin structure through the formation of silent chromatin loops (19). The RTT neurobiology has been proven complicated as the expression of multiple genes is influenced in variable stages and at different dosages during the neuronal maturation (20).

RTT phenotypes in females display great variability and often extend beyond the boundaries of the classical clinical criteria described (2). Further phenotypic heterogeneity, which has been well documented (5,6,7,21,22), can also be attributed to variable X chromosome inactivation (23), along with the interaction of other disease-causing genes such as

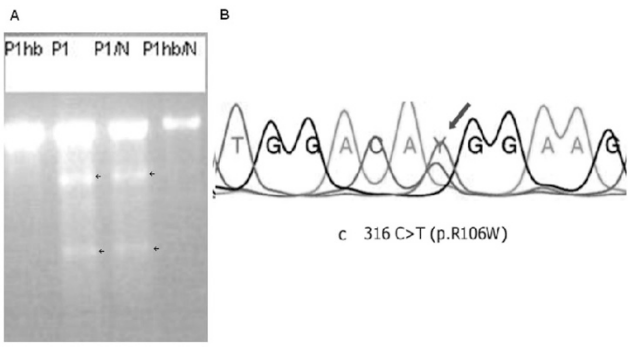

Figure 2. A. ECMA analysis of MECP2 exon 3 showed the presence of three products in patient 1 (one from the undigested PCR product and two coming from the cleavage of mismatched sites) indicating the occurrence of a mutation, and one product in patients 1 hair bulbs. Pt1hb: patient 1 hair bulbs (DNA), Pt1: patient 1 (DNA), Pt1/N: heteroduplex resulting from DNA samples of patient 1/normal control, Pt1HB/N: heteroduplex from DNA samples of patient 1 hair bulbs/normal control. B. Direct sequencing analysis of patient 1 DNA sample showing the c.316 C>T (p.R106W) transition. The presence of a second normal allele suggests somatic mosaicism. The PCR template was generated using M13-tailed (underlined bases) exon 3-MECP2 gene-specific primers (F:5′- *GTAAAACGACGGCCAGT* AAGAGAAAGAGGGCAAGCATGAGC -3′, R:5′- *CAGGAAACAGCTATGAC* AAGCACACCTGGTCTCAGTGTTCA -3′).

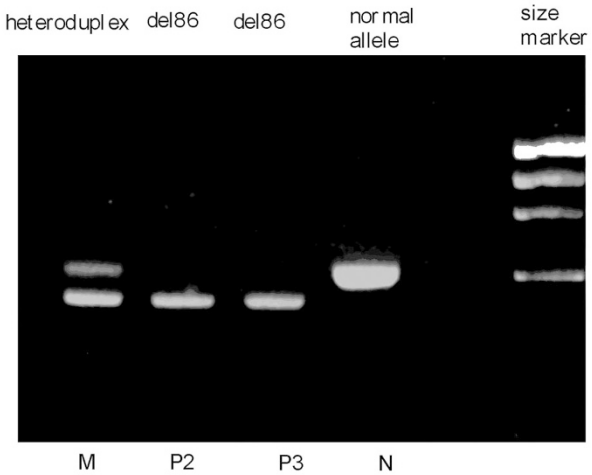

Figure 3. PCR analysis showing different product sizes in patients 2 and 3 and their unaffected mother due to the c.1140del86 deletion in the CTS portion of *MECP2* (*M*, Mother; *P2*, Patient 2; *P3*, Patient 3; *N*, Normal). The primers used for the PCR amplification of the peripheral leukocyte-derived genomic DNA were designed with the aid of the Integrated DNA Technologies Bioinformatic tool (http://www.idtdna.com/Scitools/Applications/Primerquest/) and were the following: Forward: 5′-CAG TGG GAA AGG ACT GAA GAC CTG TAA G-3′, Reverse: 5′-TGA CCA GTT AAT CGG GAA GCT TTG TCA G-3′ (Tm: 60°C).

CDKL5 (24). Compared with RTT females, males with *MECP2* mutations could provide further information about genotypic-phenotypic correlations, because they are haploid for the *MECP2* gene status. Indeed, our patients appeared with variable clinical phenotypes and molecular findings.

Patient 1 exhibited the R106W (c.916 C>T) missense mutation, among the eight most common mutations in the *MECP2* gene (12), and causing the full RTT phenotype in girls. Gene expression studies suggest that the R106W mutation has multiple impacts on the putative *MeCp2* protein, inhibiting the initial binding to the heterochromatin via the MBD, and resulting in the compromise of the subsequent events of nucleosome-nucleosome interaction and final maximal chromatin condensation (19). Given the severe loss of function of *MeCP2*, early lethality or neonatal encephalopathy could be expected in a male proband. However, at the age of 6, patient 1 had phenotype that was reminiscent of classical RTT, although he mainly demonstrated autistic features and behavioral disturbances. Up to the age of 11 y, he had no major neurologic problems, unlike females with classical RTT syndrome who at the same age are profoundly intellectually impaired and have neurologic problems including seizures. This could be explained either by the presence of a supernumerary normal X chromosome or by somatic mosaicism. An extra X chromosome was excluded by chromosomal analysis, but the results of sequencing analysis of leukocyte DNA strongly supported the evidence of somatic mosaicism. The absence of the *MECP2* mutation in hair roots indicates that, even if the R106W mutation is present in the brain, it is not widespread. It is known that somatic mosaicism is not always easily detected and requires analysis of a large number of cells derived from different tissues. Because the number of hair roots available for analysis was rather small (<10), the failure to detect the R106W mutation could have been technical, and thus the occurrence of this mutation in the brain cannot be ruled out. In addition, as the analysis of the maternal DNA disclosed a normal sequence, maternal inheritance was excluded. This is consistent with the *de novo* occurrence of the mutation, and the presence of somatic mosaicism, which possibly arose at the postzygotic phase of the early embryonic development. Somatic mosaicism with a normal blood karyotype has been previously observed in other RTT-like males described with the hotspot mutations R270× and T158M, a novel missense mutation (R133H) and finally a novel early truncating 166del2 mutation (5,7).

Patients 2, 3 and their mother represent an example of MR due to inherited *MECP2* mutations. The identified mutation was an 86 bp deletion in the deletion-prone region at the C-terminus of the fourth exon (1140del86). The boundaries of this deletion hot-spot region are located between nucleotides 1050 and 1200, giving rise to intragenic deletions of various sizes and accounting for approximately 10% of RTT mutations in females (13). Although the late truncation of the *MeCP2* protein results in intact methyl-binding and transcriptional repression capacity, it nevertheless prevents the highest level of chromatin compaction (19). The phenotype is considered to be milder in terms of preservation of some cognitive functions, however, motor skills seem to be more seriously affected (13,25). Severe spine deformities due to dystonia and extra-

Table 2. MECP2 *C-terminal affecting deletions and point mutations identified in families with MR male members so far*

Case	CTS molecular defect	Phenotype	Affected family members	Reference
	Deletions			
1	1140 del 86 (V380fs)	7 y, MR, spasticity of lower limbs,	4 y, brother, MR, mild dysmorphic features, neurologic symptoms unaffected carrier mother	Present study (patients 2 and 3)
2	1154 del 32 (p384fs)	Congenital encephalopathy. Died at 21 mo	Asymptomatic carrier mother Also affected brother who died at 18 months	26
3	1158 del 44 (P388X)	4 y, MR + neurological problems	Unaffected carrier mother	27
4	1161 del 240 (del 388–467) in-frame deletion	Mild to severe MR (one male 24 y, with mild attention deficit, one male 55 y with aggression and dysarthria problems, and another one 52 y with limited language abilities and oligophrenia)	4 males affected in 2 generations, one asymptomatic female carrier	28
5	1415–1416 del (E472fs)	10 y, moderate MR, mild dysmorphic features, hypotonia, obesity, gynaecomastia	De novo	29
	Point mutations			
6	p.P322S (c. 964 C>T)	6 y, moderate MR + epilepsy	Unaffected mother with borderline intelligence	5
7	Q406X (C1216T)	Severe MR + neurological symptoms	Carrier female with borderline intelligence and mild hypotonia, also affected maternal uncle	5
8	K417M (c.1250A>T)	Congenital encephalopathy, died at 14 mo	Unaffected carrier mother	5
9	R453Q (c.G1358A)	Moderate MR	NK	5

pyramidal signs are often the final outcome when *MECP2* truncating mutations are present (13,25).

Males with C-terminal deletions that have been reported to date are summarized in Table 2. The clinical features range from severe neonatal encephalopathy and classic RTT manifestations (hand stereotypes and developmental regression) to nonspecific MR. Truncal hypotonia, ataxia, and rigidity of the lower limbs seem to be common features in this group of patients and were also observed in our patients (26,27,28,29). Yntema *et al.* (30) described a case where the deletion was in-frame, and the symptoms were even more atypical. Healthy carrier mothers were detected in all but one case (Table 1). The mother of our patients 2 and 3 did not manifest any symptoms and had a normal intellect. Although the molecular analysis on the X inactivation pattern of the mother was not available, her normal presentation could be attributed to an extremely skewed XCI pattern toward the expression of the normal *MECP2* allele. Alternatively, it could be that the particular truncation mutation has an unexpectedly mild consequence on the function of the *MeCP2* protein.

Patient 4 who manifested virtually unexplained MR and some mild dysmorphic features was characterized to have the *de novo* missense variant T203M (c. 608 C>T). This variant has been reported as a benign polymorphism based on its observation in the unaffected father of an RTT female, and furthermore it is not located in an evolutionary conserved region (15). However, our analysis with bioinformatics tools yielded contradictive results.

The Polyphen software analysis predicted that the substitution of the threonine residue at position 203 with a methionine exerts a benign effect to the *MeCP2* protein, whereas SIFT regarded this variation as "probably damaging." Because the T203M variant has not been previously reported in a male patient with MR, the findings in this case should be interpreted with caution and it remains inconclusive as to whether the T203M is causative for MR.

Altogether, our results further demonstrate the wide spectrum of *MECP2* mutations and clinical heterogeneity can be observed in carrier males. This study includes examples with a classic RTT mutation, which is attenuated by somatic mosaicism, a C-terminal deletion leading to residual protein functionality and a previously classified missense polymorphic variant. The mutations themselves are probably directly responsible for the long survival in our patients, along with the absence of multiple neurologic complications, aside from MR. A number of different potentially disease causing mutations of *MECP2* has been reported in males affected by neurologic disorders. Those include mutations detected in females with RTT, as well as unclassified novel or previously reported variants observed in single families only (5). The molecular testing of mothers of patients and all first degree female relatives with RTT syndrome is important for defining the likely pathogenecity of any *MECP2* mutations identified.

In conclusion, the existence of various *MECP2* mutations in males is becoming well documented. They can be associated with a wide spectrum of states ranging from severe neonatal encephalopathy and lethality on one hand, a full RTT phenotype or mild MR. *MECP2* mutations should be suspected in any males with unexplained MR and neurologic problems.

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