MECP2 is highly mutated in X-linked mental retardation

Philippe Couvert1, Thierry Bienvenu1,2, Cécile Aquaviva2, Karine Poirier1, Claude Moraine3, Chantal Gendrot3, Alain Verloes4, Christian Andrès3, Anne Celine Le Fevre2, Isabelle Souville2, Julie Steffann2, Vincent des Portes1, Hans-Hilger Ropers5, Helger G. Yntema6, Jean-Pierre Fryns7, Sylvain Briault3, Jamel Chelly1,+ and Beldjord Cherif1,2

1INSERM Unité 129-ICGM, CHU Cochin 24 Rue du Faubourg Saint Jacques, 75014 Paris, France, 2Laboratoire de Biochimie et Génétique Moléculaire, CHU Cochin, Paris, France, 3CHU de Tours, Service de Génétique, Hopital Bretonneau, 37044 Tours Cedex, France, 4Centre Universitaire Wallon de Génétique, CUWG CHU Sart Tilman Domaine Universitaire, Liège B-4000, Belgium, 5Max-Plank-Institute for Molecular Genetics, Ihnestrasse 73, Berlin-Dahlem, Germany, 6University Hospital Nijmegen, 417 Department of Human Genetics, 6500 HB Nijmegen, The Netherlands and 7Center for Human Genetics, Clinical Genetics University, UZ Gasthuisberg, Herestraat 49, B-3000 Leuven, Belgium

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Following the recent discovery that the methyl-CpG binding protein 2 (MECP2) gene located on Xq28 is involved in Rett syndrome (RTT), a wild spectrum of phenotypes, including mental handicap, has been shown to be associated with mutations in MECP2. These findings, with the compelling genetic evidence suggesting the presence in Xq28 of additional genes besides RabGDI1 and FMR2 involved in non-specific X-linked mental retardation (MRX), prompted us to investigate MECP2 in MRX families. Two novel mutations, not found in RTT, were identified. The first mutation, an E137G, was identified in the MRX16 family, and the second, R167W, was identified in a new mental retardation (MR) family shown to be linked to Xq28. In view of these data, we screened MECP2 in a cohort of 185 patients found negative for the expansions across the FRAXA CGG repeat and reported the identification of mutations in four sporadic cases of MR. One of the mutations, A140V, which we found in two patients, has been described previously, whereas the two others, P399L and R453Q, are novel mutations. In addition to the results demonstrating the involvement of MECP2 in MRX, this study shows that the frequency of mutations in MECP2 in the mentally retarded population screened for the fragile X syndrome is comparable to the frequency of the CGG expansions in FMR1. Therefore, implementation of systematic screening of MECP2 in MR patients should result in significant progress in the field of molecular diagnosis and genetic counseling of mental handicap.

INTRODUCTION

The MECP2 gene, located in Xq28, comprises three coding exons and encodes a 486 amino acid protein that was identified in 1992 based on its selective binding to methylated CpG dinucleotides in mammalian genomic DNA (1). It is widely expressed and alternative polyadenylation in the 3′-UTR results in a highly expressed 10 kb transcript in the fetal brain and a 5 kb transcript in the adult brain (2). MeCP2 contains two functional domains, an 85 amino acid methyl-cytosine-binding domain (MBD) that binds to DNA bearing one or more symmetrically methylated CpGs, and a 104 amino acid transcriptional repression domain (TRD). The TRD interacts with the transcriptional corepressor SIN3A to recruit histone deacetylases. Interaction between this transcriptional repressor complex and chromatin-bound MeCP2 causes deacetylation of core histones resulting in transcriptional repression (1,3–5).

Recently, mutations in the MECP2 gene have been found in patients with Rett syndrome (RTT), a severe neurological condition occurring almost exclusively in females (6). Further reports showed that mutations in MECP2 account for 70–80% of RTT cases and are also involved in a broad spectrum of phenotypes, including mild intellectual difficulties in females and neonatal encephalopathy in males surviving to birth (7–16). More recently, two interesting mutations associated with severe forms of mental retardation (MR) were reported in two families. The first, an A140V mutation, was identified in a small family in which two females are affected with mild MR and four males with severe MR (17). The second mutation, E406X, was identified in a three-generation family in which two affected males exhibit severe MR and progressive spasticity (18). Together, these findings strongly suggested that the MECP2 gene is a potential candidate for non-specific X-linked MR (MRX). In this study, we report for the first time mutations in MECP2 associated with recessive MRX in families where linkage studies excluded the whole X chromosome except an

*To whom correspondence should be addressed. Tel: +33 1 44 41 24 10; Fax: +33 1 44 41 24 21; Email: chelly@icgm.cochin.inserm.fr
interval in Xq28 encompassing MECP2. Also, we present evidence to show that the frequency of MECP2 mutations in MR populations is comparable to that of CGG expansions in FMR1 responsible for the fragile X syndrome.

RESULTS

MeCP2 is involved in recessive non-specific MRX

The wide spectrum of neurological phenotypes resulting from mutations in MECP2 prompted us to assess its involvement in non-specific MRX, a very common and vastly heterogeneous group of disorders in which MR appears to be the only consistent manifestation. We screened the MECP2 gene in the proband of the large MRX16 family localized to Xq28 and in probands of 29 MR families with at least two affected males. Coding exons of the MECP2 gene were screened for mutations by denaturing gradient gel electrophoresis (DGGE) analysis followed by direct sequencing of fragments exhibiting abnormal migration profiles. Primers used to amplify the nine fragments corresponding to the three coding exons of MECP2 and DGGE conditions were as described previously (7). The screening showed the presence in two patients of an abnormal DGGE migration pattern of the fragment 3A bearing the mutation, showing the cosegregation of the mutation with the phenotype. Heteroduplexes were formed by mixing normal and mutated PCR products. To exhibit the status of the maximum number of individuals, PCR products of some unaffected males were mixed before loading. Direct sequencing of PCR products showing the mutation is presented below the DGGE results. The underlined nucleotides and arrows indicate the mutated nucleotide. The mother was found to be heterozygous and both unaffected boys inherited the normal allele.

Figure 1. Identification of the A→G mutation (E137G) in MRX16 and C→T mutation (R167W) in T36 families, respectively. Open squares, unaffected males; closed squares, affected males; open circles, unaffected females; dotted symbols, phenotypically normal carrier females. (A) Pedigree of the MRX16 family and DGGE of PCR products corresponding to the fragment 3A bearing the mutation, showing the cosegregation of the mutation with the phenotype. Heteroduplexes were formed by mixing normal and mutated PCR products. To exhibit the status of the maximum number of individuals, PCR products of some unaffected males were mixed before loading. Direct sequencing of PCR products showing the mutation is presented below the DGGE results. The underlined nucleotides and arrows indicate the mutated nucleotide. (B) Pedigree of the T36 family and DGGE results showing the cosegregation of the mutation (PCR products of the proband IV-20 were loaded twice) and direct sequencing of PCR products showing the mutation. Unaffected III-28 and III-29 boys and their mother were also investigated for the presence of the mutation. The mother was found to be heterozygous and both unaffected boys inherited the normal allele.
tion (E137G) was found in the MRX16 family (19) (Fig. 1A) and the latter was found in a three-generation family with four non-specific X-linked mentally retarded males (T36; Fig. 1B). These base substitutions were absent in 500 X chromosomes from unrelated control individuals.

The large four-generation MRX16 family was diagnosed with non-specific MR. Clinical and neuropsychological data were previously reported (19). The degree of MR was variable, ranging from profound to mild, and was associated with speech handicap in most of them. All obligate carrier females had normal intelligence. Affected patients did not show a history of regression of higher brain functions after an initial normal development. Linkage studies excluded the whole X chromosome except an interval in Xq28 encompassing the MECP2 gene. The XLMR locus in this family (MRX16) was mapped to an 8 cM interval in Xq28 between DXS1113 and the Xq telomere. Maximum LOD scores of 5.43, at θ = 0.00, were obtained with DXS1108 (19). Rab GDI (RabGDI1), a dissociation inhibitor known to be involved in MR, and IL-1 receptor-associated kinase (IRAK), two genes located in this interval, were previously investigated by the same approach and no mutation has been found (20 and data not shown). The X-inactivation pattern using DNA prepared from peripheral blood leukocytes was assessed as described by Allen et al. (21) and a normal X chromosome inactivation pattern was found in all tested females, including those carrying the mutation (data not shown).

Following the detection of the R167W mutation in family T36, a recent clinical examination has been performed. The four affected boys, aged 25–50 years, have a moderate MR (IQ between 50 and 70), resting tremors (3/4), moderate to severe obesity (3/4) and aggressive behavior in one patient. None of the patients had autistic-like phenotype or a history of regression of higher brain functions after an initial normal development. All obligate carrier females had normal intelligence but two of them showed a non-progressive slight resting tremor (III-21, III-26; Fig. 1B). To further consolidate the relevance of the identified mutation, we genotyped DNA of available members of the family with microsatellite markers evenly distributed on the X chromosome. Pairwise and multipoint LOD scores analyses allowed us to exclude most of the X chromosome except the Xq28 region.

The altered glutamate (E137G) is located in the MBD, whereas the altered arginine (R167W mutation) is situated in the region between the MBD and TRD (3). Interestingly, mutations identified in these two families have not been found in RTT.
### Table 1. MECP2 variants found in MR patients

<table>
<thead>
<tr>
<th>Family/ patient</th>
<th>Nucleotide change</th>
<th>Amino acid change</th>
<th>Domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRX16 A→G at 410</td>
<td>E137G</td>
<td>MBD</td>
<td></td>
</tr>
<tr>
<td>T36 C→T at 499</td>
<td>R167W</td>
<td>Between MBD and TRD</td>
<td></td>
</tr>
<tr>
<td>Sporadic cases</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P1 C→T at 419</td>
<td>A140V</td>
<td>MBD</td>
<td></td>
</tr>
<tr>
<td>P2 C→T at 419</td>
<td>A140V</td>
<td>MBD</td>
<td></td>
</tr>
<tr>
<td>P3 C→T at 1196</td>
<td>P399L</td>
<td>C-terminus domain</td>
<td></td>
</tr>
<tr>
<td>P4 G→A at 1358</td>
<td>R453Q</td>
<td>C-terminus domain</td>
<td></td>
</tr>
<tr>
<td>P5 A→G at 850</td>
<td>K284E</td>
<td>TRD</td>
<td></td>
</tr>
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MBD, methyl-CpG-binding domain; TRD, transcription repression domain.

**Frequency of MECP2 mutations among the mentally retarded patients screened for the fragile X syndrome**

The fragile X syndrome is, so far, the most frequent MRX disorder caused by an expanded CGG repeat (>200) in the first exon of the FMR1 gene. This expansion is accompanied by hypermethylation of the repeat and its upstream region, resulting in a shutdown of transcription and absence of the FMR1 protein. The prevalence of the fragile X syndrome was estimated at 1/4000 to 1/6000 (22, 23). Most molecular diagnosis units offering diagnosis of the fragile X syndrome by DNA analysis estimated the frequency of pathogenic expansions at ~3–4% among mentally handicapped individuals. Similar figures were found by our diagnosis unit at Cochin Institute (data not shown). To assess the frequency of MECP2 mutations, we screened the coding sequence of the MECP2 gene in a cohort of 185 mentally retarded patients found negative for CGG expansions. DNA samples of male patients were randomly selected among 2000 samples. In total, this investigation of the nine fragments covering the coding part of MECP2 identified abnormal DGGE migration patterns in nine unrelated patients. Direct sequencing of abnormal PCR products revealed silent polymorphic variants in four cases: C→G at 849 (A283), C→T at 897 (T299), A→G at 1035 (K345) and C→T at 1160 (P387); all were unique. In the five remaining cases we identified the following nucleotide variants: P399L, R453Q, a recurrent A140V (n = 2), and a CT→GT in position 22 of the second intron (IVS + 22), suggesting the creation of a potential GT splice donor (Table 1). None of the base substitutions corresponding to these mutations were detected in 500 normal controls. The latter mutation (A140V) was previously described by Orrico et al. (17) as responsible for a dominant form of RTT. In this study all mutations were identified in sporadic cases of moderate to severe non-specific MR (IQ between 60 and 40), except in one patient where MR was associated with psychiatric features (Materials and Methods). Segregation studies were performed in the two families with the A140V mutation. In both families, the healthy mothers were found to be carriers. The absence of phenotype in the mother carrying the A140V mutation prompted us to study the X inactivation pattern in these families using DNA prepared from peripheral blood leukocytes. The X chromosome inactivation pattern was assessed as previously described (21). The assay showed balanced X inactivation in the mothers and her daughters who did not inherit the mutated allele (data not shown). For the three other mutations, IVS + 22CT→GT, P399L and R453Q, parents’, DNA was unavailable for analysis. RNA or cell lines for the patient with the intronic variant were also unavailable to study the consequences on the maturation of MECP2 transcripts.

**DISCUSSION**

MECP2 on Xq28 was identified as the disease-causing gene for RTT, an X-linked dominant neurodevelopmental disorder affecting 1/10 000 to 1/15 000 girls. Various missense, nonsense and frameshift mutations were identified in 70–80% of patients with RTT (6–16). Most of these mutations occurred as de novo mutations in unrelated patients, reflecting the hypermutability of this gene. MECP2 mutations have also been found to cause a variety of phenotypes other than classical RTT (8, 17, 18), but very little is known about its potential involvement in recessive forms of non-specific MRX, a common and vastly heterogeneous group of disorders in which affected patients do not have any distinctive clinical or biochemical features in common apart from cognitive impairment (24). Seven genes have been identified so far and each account for <0.5% of MR (25–28).

In this study we report compelling evidence that demonstrates involvement of MECP2 in recessive MRX. Firstly, mutations that we identified in MRX16 and T36 families co-segregate with non-specific MR phenotypes which affect only males, in families where linkage studies excluded the whole X chromosome except for an interval in Xq28 encompassing MECP2. Secondly, both missense mutations change strictly conserved, from mammals to Xenopus laevis (6), amino acids in the MBD (E137G) or in the vicinity of this domain (R167W) which were not found in typical RTT patients. Thirdly, these mutations were not detected in 500 X chromosomes from unrelated control individuals. Given the relevance of all these findings, we conclude that mutations in MECP2 are the cause of non-specific MR observed in these two families. It is worth noticing that in both families affected males did not show a history of regression of intellectual performance after an initial normal development.

In view of the data demonstrating the involvement of MECP2 in non-specific MRX and in order to evaluate the prevalence of MRX related to MECP2, we have screened a cohort of 185 mentally retarded patients found negative for CGG expansions in FMR1. Patients were randomly selected by examining the fragile X case record of our diagnosis laboratory. Interestingly, we identified four nucleotide variations resulting in amino acid substitutions, one of them (A140V) being a recurrent mutation that has been detected in two unrelated patients and previously described in a dominant form of MRX (17). In this study, we showed that MR patients inherited mutations from their non-affected mothers, who have a random X chromosome inactivation pattern (at least in peripheral blood leukocytes). Although phenotypic heterogeneity between females carrying this mutation is striking and neuronal mosaicism for MeCP2 expression resulting from
X-inactivation is likely to be involved in these differences, it is relevant to point out that in hemizygous males this mutation is so far always associated with an MR phenotype. The preserved position of this alanine in the MBD and the predicted effect of the substitution on the structure of the α-helix length (17) are additional arguments for the deleterious effect of this mutation.

Besides this recurrent mutation we have identified, in three sporadic cases of MR, three nucleotide variants: two potential missense mutations (P399L and R453Q) and one intronic mutation (IVS + 22 CT→GT). These three variants were not found in any of the reported screening studies of RTT cohorts, nor in the 500 control individuals analyzed in this study. The arginine in position 453 is conserved from mammals to X.laevis, whereas the proline in position 399 is replaced in X.laevis by an asparagine. Though further investigations are required to ascertain the pathogenic effects of these variants, in view of all the data reported in this study and elsewhere, it is reasonable to suggest that these potential missense mutations are involved in the MR phenotypes. The sequence GTCT-ACAG, resulting from the IVS + 22 CT→GT variant, does not resemble the consensus splice donor sequence, GTAAAGTAN, therefore this variant will not be included in the assessment of the frequency of mutation involved in MR.

The missense mutations, P399L and R453Q, are located outside the two functional domains (MBD and TRD) and they will be extremely useful for assessment of the role of the region in binding and transcription repression activities of the MeCP2 protein. The specific association in hemizygous males between MR phenotype and some mutations which were not described in RTT suggests that these mutations might have minor deleterious effects on the transcription repressor complex in which MeCP2 is involved. However, these data also suggest that MeCP2 could have another function, yet to be known, disruption of which is responsible for only MR.

In addition to the unambiguous data showing the involvement of MECP2 in MRX, this study suggests that mutations in MECP2 are responsible for ~2% (4/185) of MR. Interestingly, this figure is comparable to the 3–4% of CGG expansions associated with the fragile X syndrome. The relatively high frequency of mutations in MECP2 contrasts with the very low frequencies (~0.5%) of mutations in previously described MR genes such as OPHN1, GDI1, IL1RAPL, PAK3 and RhoGEF (25). As these figures were obtained by screening cohorts of MR patients referred to our diagnosis laboratory for fragile X syndrome diagnosis. CGG expansion in the FMR1 gene was tested by Southern blot analysis using DNA digested with EcoRI/EagI endonucleases and SsB12-3 probe corresponding to the FRAAX locus (29). Patients were identified by examining the fragile X case report of the diagnosis unit and only patients with pathogenic CGG expansions (>200) were excluded for MECP2 gene screening. Further enquiries concerning the four patients with mutations in MECP2 revealed that all patients were affected with moderate to severe MR (IQ between 60 and 40) except one patient who showed, at the age of 12, a sudden severe psychotic state including auditory and visual hallucinations, insomnia, anxiety and agitation. An electroencephalogram, head computerized tomography (CT) scan and routine blood tests were normal. Investigation of the four families showed that mentally retarded patients are sporadic cases and their mothers have normal intellectual performance.

**Mutation analysis**

Primer sequences and parameters for amplification of the MECP2 gene fragment and DGGE conditions were as previously described (7). Briefly, for DGGE, DNA was extracted from peripheral blood leukocytes or lymphoblastoid cells and the three exons and the flanking intronic sequences of the MECP2 gene were separately PCR amplified from genomic DNA using the primers described by Bienvenu et al. (7). DGGE conditions were chosen according to the Meltmap program, kindly provided by L. Lerman and colleagues. PCR products were subjected to DGGE electrophoresis as described previously (7).

For mutation identification, PCR products showing an abnormal migration pattern on DGGE analysis were directly sequenced on an automated sequencer (ABI 373; Perkin-Elmer) using the Dye Terminator method.

**X-inactivation study**

Lymphocyte DNA was extracted from peripheral blood by standard procedures. X chromosome inactivation was determined as described by Allen et al. (21), using PCR analysis of the androgen receptor gene, which contains two methylation-sensitive sites (HpaII and HhaI) flanking a polymorphic trinucleotide repeat in the first exon. PCR amplification products were electrophoresed on a 6% denaturing polyacrylamide gel, transferred onto a nylon membrane and analyzed, but all families include at least two affected males. Clinical and linkage data for the MRX16 family was reported by Gandrot et al. (19). Recent investigation of four affected males (aged 25–50 years) of the T36 family revealed the presence of a moderate MR (IQ between 50 and 70). Both verbal and non-verbal performances are reduced. Physical examination and neuroclinical investigation showed no abnormalities except resting tremors (3/4), moderate to severe obesity (3/4) and aggressive behavior in one patient. None of the patients had autistic-like phenotype or a history of regression of higher brain functions after an initial normal development. All obligate carrier females had normal intelligence and two of them showed a non-progressive slight resting tremor (III-21, III-26; Fig. 1B). Also, no brain malformation was observed by imaging investigation.

Large screening for mutation was carried out in 185 mentally retarded males referred to our molecular diagnosis laboratory for fragile X syndrome diagnosis. CGG expansion in the FMR1 gene was tested by Southern blot analysis using DNA digested with EcoRI/EagI endonucleases and SsB12-3 probe corresponding to the FRAAX locus (29). Patients were identified by examining the fragile X case report of the diagnosis unit and only patients with pathogenic CGG expansions (>200) were excluded for MECP2 gene screening. Further enquiries concerning the four patients with mutations in MECP2 revealed that all patients were affected with moderate to severe MR (IQ between 60 and 40) except one patient who showed, at the age of 12, a sudden severe psychotic state including auditory and visual hallucinations, insomnia, anxiety and agitation. An electroencephalogram, head computerized tomography (CT) scan and routine blood tests were normal. Investigation of the four families showed that mentally retarded patients are sporadic cases and their mothers have normal intellectual performance.

**MATERIALS AND METHODS**

**Families and patients**

Families investigated in this study are affected with non-specific MR. Low intelligence quotient (IQ <70) is the only common feature for all patients. The level of the deficit is heterogeneous, even within the same family. The linkage to the X chromosome was not systematically confirmed by linkage analysis, but all families include at least two affected males. Clinical and linkage data for the MRX16 family was reported by Gandrot et al. (19). Recent investigation of four affected males (aged 25–50 years) of the T36 family revealed the presence of a moderate MR (IQ between 50 and 70). Both verbal and non-verbal performances are reduced. Physical examination and neuroclinical investigation showed no abnormalities except resting tremors (3/4), moderate to severe obesity (3/4) and aggressive behavior in one patient. None of the patients had autistic-like phenotype or a history of regression of higher brain functions after an initial normal development. All obligate carrier females had normal intelligence and two of them showed a non-progressive slight resting tremor (III-21, III-26; Fig. 1B). Also, no brain malformation was observed by imaging investigation.

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then hybridized overnight at 42°C with a (32P)dCTP-labeled poly-CAG probe; the pattern of inactivation was determined by visual comparison of the two bands and was scored as random (ratio 50:50–60:40), moderately skewed (ratio 70:30–80:20) or extremely skewed (ratio >90/10).

**Mutation nomenclature**

Nucleotides were numbered from the first base of the translation initiation ATG codon (GenBank accession no. X99686) in accordance with Cheadle et al. (11).

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