Mutational Analysis of Methyl-CpG Binding Protein 2 (*MECP2*) Gene in Indian Cases of Rett Syndrome

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Rett syndrome (RTT) is an X-linked postnatal neurological disorder, primarily affecting females and characterized by regression, epilepsy, stereotypical hand movements, and motor abnormalities. Its prevalence is about 1 in 10,000 female births. RTT is caused by mutations within methyl CpG-binding protein 2 (*MECP2*) gene. Over 200 individual nucleotide changes in the gene, which cause pathogenic mutations, have been reported; however, eight most commonly occurring missense and nonsense mutations account for almost 70% of all mutations. RTT cases have also been reported from India. The phenotype (classical and atypical inclusive) has many differentials. However, a genetically based confirmed diagnosis would help in management and counseling. In this pilot study we have analyzed *MECP2* mutations in ten Indian sporadic patients diagnosed clinically as having

RTT. Two mutations and one novel variant in *MECP2* have been detected. Missense mutations p.R133C and c.806delG have been detected. The missense mutation p.R133C was the part of eight hotspots reported in Rett patients. This patient met all the essential criteria except delayed onset of regression. The other c.806delG mutation positive patient also fulfilled all the obligatory criteria of classical RTT. Another clinically atypical Rett patient showed a novel mutation p.C339S in *MECP2* gene. The preliminary result necessitates a large-scale study of RTT patients to determine more precisely the influence of *MECP2* mutations in Indian patients and their correlation with clinical phenotypes. J. Clin. Lab. Anal. 27:137–142, 2013. © 2013 Wiley Periodicals, Inc.

Key words: DNA sequencing; *MECP2*; mutation; Rett syndrome; RTT

INTRODUCTION

Rett syndrome (RTT, OMIM#312750) is a progressive neurological disorder with X-linked dominant inheritance. The reported prevalence is about 1 in 10,000–15,000 female births (1). Typically, RTT is characterized by a period of nearly normal development followed by regression with loss of social, motor, and communication skills combined with the occurrence of specific features including hand stereotypies, microcephaly, autonomic disturbances, or epilepsy.

Clinically, RTT can be classified into classical and atypical based on the clinical presentation even though there is a lot of overlap among the symptoms. In 1999, Amir et al. showed the involvement of methyl-CpG binding protein 2 (*MECP2*) gene mutations in these cases (2). *MECP2* gene

located on Xq28, encodes a broadly expressed nuclear protein of 486 amino acids that participate in transcriptional silencing by binding to methylated DNA in nucleosomes and chromatin. It contains functional domains, a methyl-CpG binding domain (MBD) of 85 amino acids

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that binds to methylated CpG islands, and a transcriptional repression domain (TRD) of 104 amino acids that interacts with the transcriptional repressor Sin3A, which recruits histone deacetylases (3). In addition, MeCP2 has a nuclear localization signal (NLS) within the TRD and the function of NLS is to facilitate the transport of MeCP2 into the nucleus. Any disease-causing mutations in the gene lead to alteration in the MeCP2 protein encoded by it, which in turn, can affect normal function of the protein.

Majority of *MECP2* mutations are located either within the MBD or TRD, encoded by exons 3 and 4 in patients with classic and atypical RTT (4, 5). This is useful in molecular diagnosis within the first year of life in suspected cases, before the typical developmental regression stage occurs. It is postulated that the mutations in these domains result in alterations in DNA binding or protein binding to its target molecule. To date, over 200 individual nucleotide changes that cause pathogenic mutations have been described (RettBASE: mecp2.chw.edu.au and mecp2.org.uk). However, eight most commonly occurring missense and nonsense mutations causing either altered or premature termination of protein account for almost 70% of all mutations. Small deletions associated with deletion hotspots in the C-terminal region of MeCP2 protein account for an additional 9% of pathogenic mutations (6). The genotype–phenotype correlation is, however, complicated due to the phenomenon of X chromosome inactivation that confounds the phenotype.

There are only a few reports regarding mutational analysis of *MECP2* from India. Khajuria et al. (2011) have reported a novel variant p.P430S in a boy with Rett-like phenotype and congenital blindness and the same variant was also shown to be present in his family (7). In another report Mittal et al. (2011) reported de novo deletion in *MECP2* gene in a family with affected brother–sister pair with symptoms of RTT (8). In this report we demonstrate the presence of *MECP2* mutations in patients with clinically diagnosed RTT from the Indian populace.

MATERIALS AND METHODS

Patients Recruitment

We studied ten isolated cases with clinical characteristics of RTT, both classical and atypical. Clinical diagnosis was based on essential and supportive criteria reported by Hegberg et al., (9). The essential criteria for inclusion were normal prenatal and perinatal period, apparently normal psychomotor development through the first 6 months, normal head circumference (HC) at birth, loss of purposeful hand skills between 6–30 months. Other causes of mental retardation, evidence of intrauterine growth retardation, evidence of prenatally acquired brain damage were excluded. Patients were recruited over a period of one year

(Jan 2010–Feb 2011). Table 1 shows the age and clinical findings in all the above-mentioned cases. The study was approved by Institutional Ethics Committee for Clinical Studies and samples were collected after obtaining written informed consent (Institutional Ethics approval No 168/2009).

Genomic DNA Extraction

Whole blood samples from RTT patients were collected in tubes containing ethylenediaminetetraacetic acid (EDTA) as anticoagulant. Genomic DNA was isolated from 2.0 ml of blood collected from the patients using QIAmp DNA extraction kit (Qiagen, GmbH, Germany). After isolation, integrity of the DNA was checked by running on 0.8% agarose gel electrophoresis.

Allele-Specific Oligonucleotide PCR

Four different hotspot mutations (p.R133C, p.R168X, p.R255X, and p.R294X) were targeted for allele-specific PCR. Primers for the allele-specific PCR were used as per Baris and Battaloglu (2009) (10). Two forward primers with a common reverse primer were used for identification of each mutation: one forward primer that amplifies the normal allele (wild type) and the other forward primer that amplifies the mutant allele (mutant type). The sequences of both forward primers were same except the mutant primer carried a nucleotide at the 3' end corresponding to the specific mutation. The sequences of the primers have been provided in Supplementary Table S1. We have targeted four different mutations (p.R133C, p.R168X, p.R255X, and p.R294X) for allele-specific PCR for identification in a multiplex format. All the four sets of respective primers amplify fragments of different sizes to resolve the products in gel electrophoresis. PCR was performed in a total volume of 25 μ l containing approximately 60 ng of genomic DNA, 2.5 mM MgCl₂, 0.2 mM dNTPs, and 1.5U of Taq DNA polymerase. The PCR program on the thermal cycler was modified slightly from the reported program as follows: an initial denaturation step at 94°C for 5 min; 5 cycles of PCR consisting of 30 sec at 94°C, 45 sec at 63°C, and 1 min at 72°C and 5 cycles of PCR consisting of 30s at 94°C, 45 s at 61°C and 1 min at 72°C followed by 20 cycles consisting of 30 s at 94°C, 45 s at 59°C and 1 min at 72°C; and a final extension step of 10 min at 72°C. A total of 5 μ l from each PCR product was run on 2.5% standard agarose gel at 100 V for 30 min. The fragments were visualized by ethidium-bromide staining on a UV transilluminator.

PCR Amplification for Sequencing

For sequencing, PCR amplification was performed in 50 μ l of 10 mM Tris HCl (pH 8.3) containing 50 mM

TABLE 1. Clinical Characteristics of Patients with RTT

Patient ID	Age (years)	age till onset (months)	HC (cm)	Hand stereotypies	Gross motor skill	Epilepsy/ Seizures	Bruxism	Type of Rett	MECP2 mutation observed/ not observed
P1	7	36	49	+	Delayed	—	+	Atypical	p.R133C
P2	2.5	Since birth	43	+	Delayed	+	—	Atypical	Not observed
P3	1	Since birth	43	No hand use	Delayed	—	—	Atypical	p.C339S
P4	0.9	Since birth	43	+	Delayed head holding	—	—	Atypical	Not observed
P5	1.5	3	47	+	Cannot walk	—	—	Atypical	Not observed
P6	2	Since birth	43	+	Delayed	—	+	Atypical	Not observed
P7	2	Since birth	NA	+	Delayed	—	—	Atypical	Not observed
P8	1.7	Since birth	NA	+	Delayed	—	—	Atypical	Not observed
P9	1.5	6	45.5	+	Delayed	—	+	typical	c.806delG
P10	2	6	43	+	Delayed	+	—	Atypical	Not observed

NA, not available.

KCl, 1.5 mM MgCl₂, 200 mM of each dNTPs (dATP, dCTP, dGTP, and dTTP), 20 pmol primers, 250 ng template DNA, and 1.5 units of Taq DNA polymerase (MBI-Fermentas, MD). All the four exons of *MECP2* gene were amplified separately using specific primers designed from the standard *MECP2* sequence. PCR was cycled 35 times; each cycle consisted of denaturation for 1 min at 94°C, annealing for 45 sec at 56–59°C and extension for 1 min at 72°C. After amplification, 3 µl of PCR product was subjected to electrophoresis on 1% agarose gel for 45 min at 100 V in TAE buffer and bands were stained with ethidium bromide (0.5 mg/ml).

Sequencing and Sequence Analysis of *MECP2* Gene

For sequencing, the PCR products were gel-purified using QIAquick Gel extraction kit (QIAGEN, GmbH, Germany) according to manufacturer protocol. The gel-purified PCR products were sequenced using gene specific primers (supplementary Table S2) on ABI PRISM 3130xl version 3.1 DNA Sequencer (Applied Biosystems, Foster City, CA). Sequences were aligned with reported normal sequence of *MECP2* using the MegAlign program of Lasergene software.

RESULTS

The results of allele-specific PCR could be visually identified on the agarose gel. Products of different sizes corresponding to the four targeted mutations were obtained. The primers for p.R133C amplified a product of 141 bp whereas p.R168X, p.R255X, p.R294X primers amplified products of 247 bp, 504 bp, and 625 bp sizes respectively (Supplementary Table S1). We have analyzed all the ten patient samples with this multiplex allele-specific PCR. Patient P1 amplified a fragment of 141 bp using mutant primers whereas the corresponding frag-

ment with wild-type primer was not amplified (Fig. 1). This 141-bp fragment corresponds to p.R133C mutant primer, which led us to suspect the presence of p.R133C mutation in this case. In all the other nine patients, a normal pattern of amplification was seen with the wild-type primer indicating absence of all four targeted mutations (p.R133C, p.R168X, p.R255X, and p.R294X) (Supplementary Figure). The mutation p.R133C in patient P1 was confirmed by PCR sequencing of *MECP2* gene (Fig. 2A).

In rest of the nine mutation-negative cases, all four exons were screened for known and novel mutations by direct sequencing in two steps. As majority of mutations have been reported to be restricted to the functional MBD and TRD domains of *MECP2* gene that are encoded by exon 3 and 4 (4, 5), these two exons were initially sequenced. In absence of any mutations in these two exons, the two other exons 1 and 2 were also sequenced. Primers for exon 3 amplified a fragment of 596 bp. The exon 4, in view of its large size, was amplified in two overlapping fragments. The first primer pair for exon 4 amplified a fragment of 841 bp and primer pair 2 amplified a fragment of 932 bp with an overlap of 468 bp (Fig. 3). All the PCR products of exon 3 and 4 were gel purified and subsequently subjected to sequencing.

We have identified one missense mutation (p.R133C) in patient P1 (Fig. 2A) and a 1 bp deletion (c.806delG) (Fig. 2B) in P9. The heterozygous c.806Gdel was identified based on the sequence chromatogram of exon 4, which is evident through the frameshift of one nucleotide in one of the alleles at the position showed by arrow (Fig. 2B). Out of these two mutations, p.R133C is part of eight hotspot mutations reported in classical RTT. The other mutation c.806delG was found in an atypical patient. In P3, we identified a novel variant (p.C339S) in exon 4 (Fig. 2C). No mutation was detected in seven patients after sequencing of the entire coding region of *MECP2* gene.

Fig. 1. Multiplex allele-specific PCR of RTT patient samples. L1: PCR amplification with wild-type primer of P2 patient, L2: PCR amplification with mutant primer of P2 patient, L3: PCR amplification with wild-type primer of P1 patient, L4: PCR amplification with mutant primer of P1 patient, M: 100 bp DNA ladder, L5: PCR amplification with mutant primer of mutation-negative control, L6: PCR amplification with wild primer of mutation-negative control, L7: PCR amplification with wild primer of P9, L8: PCR amplification with mutant primer of P9. L2: Patient P1 amplified only 141 bp fragment with mutant primer confirming presence of p.R133C mutation. Four bands seen in the other lanes with wild primers indicated absence of mutation targeted for allele-specific PCR. Absence of any amplification in L2, L5, L7 indicated no mutation in the four hotspots targeted for allele-specific PCR.

To examine the relationship between specific mutation and clinical presentation, we used five major clinical features: onset of regression, deceleration of head growth, psychomotor development, hand stereotypies, and seizures. When the five clinical features were analyzed separately (Table 1), the cases with mutations showed an association with the feature of deceleration of head growth compared to that of the other eight cases without mutation. In the case P1 with mutation, the HC was 49 cm detected at 7 years of age. Her height was 125 cm and the weight was 20 kg (less than 10th percentile). On physical examination, she was hypoactive and hypotonic with stereotypic hand movements like hand wringing, mouthing, and bruxism. Upon clinical examination, she was found to have good eye contact unlike the other

Fig. 2. DNA sequence chromatogram showing the presence of mutations (A) p.R133C (C > T) mutation in exon 4 of *MeCP2* gene of P1 patient. Since the mutation is heterozygous, two peaks at location indicated by an arrow correspond to two nucleotides that are C (wild type) and T (mutant). (B) c.806delG mutation in exon 4 of P9 patient. The heterozygous deletion is indicated by an arrow. Due to the heterozygous single-base deletion of G in one of the alleles, a frameshift in the reading frame of bases is seen subsequent to this deletion site. (C) p.C339S mutation in exon 4 in P3 patient. Since the mutation is heterozygous, two peaks at location indicated by an arrow correspond to two nucleotides that are G (wild type) and C (mutant).

cases. For the other mutation-positive patient P3, she presented as global developmental delay, she does not use her hand, HC being 43 cm. HC of the other mutation-positive patient P9 was found to be 45.5 cm (below 10th percentile) at the time of presentation. On physical examination, she also was found to be hypotonic with no obvious dysmorphic features.

Fig. 3. Schematic representation of strategy to amplify the exon 3 and 4 of *MECP2*. Exon 4 was amplified in two parts with an overlap fragment of 468 bp.

DISCUSSION

As already stated, the disease-causing mutations in *MECP2* gene have been reported in 80% cases with classical RTT (10, 11). The observed *MECP2* mutations were caused by C > T transitions at CpG dinucleotides. In the present study, we adopted a strategy of allele-specific PCR and direct sequencing to identify mutations in all four exons. We identified three mutations in heterozygous state confirming the clinically observed phenotype of this dominant syndrome. Two of these mutations (p.R133C and c.806delG) were found in the functional domain, thereby, probably critically affecting the function of MeCP2 protein.

The clinical symptoms with respect to the detected mutations were variable. The mutation p.R133C is part of eight hotspots reported in European population of classical cases (12). These eight hotspots that cause changes in amino acids are p.R106W, p.R133C, and p.T158M (located in the MBD); p.R168X (located between the MBD and TRD), and p.R270X (located in the NLS) as well as p.R255X, p.R294X, and p.R306C (located in the TRD). But interestingly, Leonard et al. (2003) reported that the phenotype of a patient with p.R133C mutation is milder with better ambulation and hand use and found mostly in atypical cases (13). Unlike Leonard et al. (2003), our patient with p.R133C mutation met all the essential criteria of classic RTT except delayed onset of regression. Therefore, this patient falls under the category of atypical RTT. Patient P9 revealed c.806Gdel in *MECP2* gene and she fulfilled all obligatory criteria of Classical RTT. One of the atypical Rett patients, patient 3 showed a novel variant p.C339S, functional implication of which needs to be determined. Rest of the seven patients did not have any mutations in *MECP2* gene.

This report suggests that the hotspot screening along with c.806delG is a useful strategy for preliminary confirmation of clinical findings in Indian patients with classic RTT.

With more researches on the correlation between hotspot *MECP2* mutations among patients of various racial/ethnic origins with neurological disorders that share similar clinical manifestations with RTT, we might obtain a better understanding of the relationship between *MECP2* mutations and the atypical RTT-like neurological diseases (14).

In our preliminary analysis, we failed to detect *MECP2* mutations in the other patients who were clinically classified as atypical cases. These cases need to be analyzed for mutations of other candidate genes reported earlier viz CDKL5 (15, 16) and NTNG1 (17), which have been found in phenotypes overlapping significantly with RTT. We suggest that screening for *MECP2* mutations is not fully informative in atypical cases and requires screening of additional candidate genes. However, *MECP2* screening is indicated in the early stages of developmental arrest or developmental plateau before regression sets in. This will be particularly beneficial in cases of girls with unexplained epileptic encephalopathy or autistic features before full-blown classic RTT is evident.

Our findings strongly indicate that primarily exon 4 of *MECP2* gene should be sequenced first. If negative, other exons may have to be screened in specific cases. The molecular-diagnosis approach described in this study can be a basis for further development in clinical use.

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