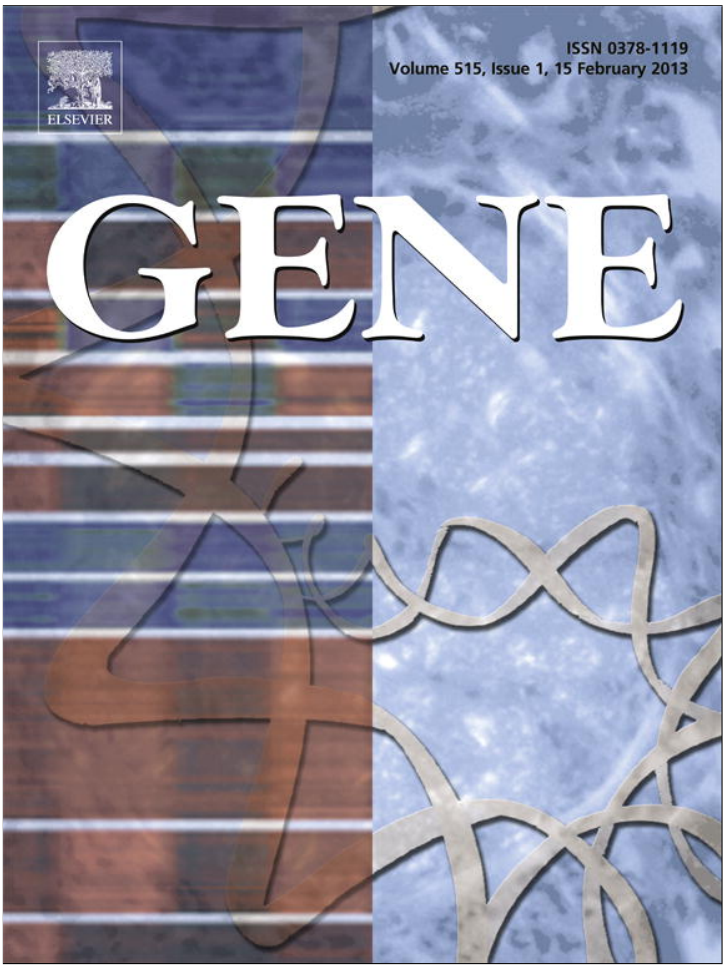

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Spectrum of MECP2 gene mutations in a cohort of Indian patients with Rett syndrome: Report of two novel mutations

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ABSTRACT

Rett syndrome (RTT) is an X-linked neurodevelopmental disorder, primarily affecting females and characterized by developmental regression, epilepsy, stereotypical hand movements, and motor abnormalities. Its prevalence is about 1 in 10,000 female births. Rett syndrome is caused by mutations within methyl CpG-binding protein 2 (MECP2) gene. Over 270 individual nucleotide changes which cause pathogenic mutations have been reported. However, eight most commonly occurring missense and nonsense mutations account for almost 70% of all patients. We screened 90 individuals with Rett syndrome phenotype. A total of 19 different MECP2 mutations and polymorphisms were identified in 27 patients. Of the 19 mutations, we identified 7 (37%) frameshift, 6 (31%) nonsense, 14 (74%) missense mutations and one duplication (5%). The most frequent pathogenic changes were: missense p.T158M (11%), p.R133C (7.4%), and p.R306C (7.4%) and nonsense p.R168X (11%), p.R255X (7.4%) mutations. We have identified two novel mutations namely p.385-388delPLPP present in atypical patients and p.Glu290AlafsX38 present in a classical patient of Rett syndrome. Sequence homology for p.385-388delPLPP mutation revealed that these 4 amino acids were conserved across mammalian species. This indicated the importance of these 4 amino acids in structure and function of the protein. A novel variant p.T479T has also been identified in a patient with atypical Rett syndrome.

A total of 62 (69%) patients remained without molecular genetics diagnosis that necessitates further search for mutations in other genes like CDKL5 and FOXG1 that are known to cause Rett phenotype. The majority of mutations are detected in exon 4 and only one mutation was present in exon 3. Therefore, our study suggests the need for screening exon 4 of *MECP2* as first line of diagnosis in these patients.

1. Introduction

Rett syndrome (RTT, OMIM#312750) is a progressive neurodevelopmental disorder with X linked dominant inheritance. It is characterized by normal early development of milestones followed by rapid regression in intelligence, language and motor skills and appearance of autistic features and stereotypic hand movements at a varying period of development. Other associated features are seizures, hyperventilation, apnea, scoliosis, growth retardation and gait dyspraxia (Kerr et al., 2001). This disorder occurs almost exclusively in females with a prevalence of about 1 in 10,000–15,000 female live births and a penetrance of almost 100% (Laourvick et al., 2006).

Rett syndrome is classified into classical and atypical based on the clinical presentation, although there is a lot of overlapping symptoms between the two. In 1999, Amir et al. (1999), identified the involvement of methyl-CpG binding protein 2 (*MECP2*) gene mutations in these cases.

MECP2 gene encodes for a broadly expressed nuclear MeCP2 protein which is thought to be involved in neuronal development and differentiation (Setoguchi et al., 2006). It regulates gene expression through CpG binding and acts as a transcriptional repressor (Galvao and Thomas, 2005). The gene is located on Xq28, has four exons of a combined length of 1775 bp that encodes 486 amino acids, most frequently encoded by exons 2, 3 and 4. However, Mnatzakanian et al. (2004) identified the previously unknown isoform of MeCP2 called MeCP2B (MECP2_E1 or MECP2α) encoded by exons 1, 3 and 4, and skipping exon 2. The first isoform was known as MECP2A (also known as MECP2_E2 or MECP2β). Although both forms are highly expressed in the brain, they differ in translation efficiency and are expressed at different relative amounts in various tissues, with MECP2B being more prevalent in the brain, thymus and lung, and during neuronal differentiation (Bienvenu and Chelly, 2006). The fourth exon of the *MECP2* gene is the largest one, containing a long (>8.5 kb) 3′ untranslated region,

Abbreviations: RTT, Rett syndrome; MECP2, methyl CpG-binding protein 2; MBD, methyl-CpG binding domain; TRD, transcriptional repression domain; NLS, nuclear localization signal; HDAC, histone deacetylase; PCR, polymerase chain reaction; XCI, X chromosome inactivation; CDKL5, cyclin-dependent kinase-like 5; FOXG1, forkhead box G1; DNA, deoxyribonucleic acid; DSM IV, Diagnostic and Statistical Manual of Mental Disorders (Fourth Edition).

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with several polyadenylation sites that enable the generation of multiple transcripts of different length.

The MeCP2 protein contains four functional domains: (i) a methyl-CpG binding domain (MBD) of 85 amino acids (amino acids 78–162) that binds to methylated CpG islands with high affinity, (ii) a central transcriptional repression domain (TRD) of 104 amino acids (amino acids 207–310) that interacts with transcriptional co-repressor Sin3A, which recruits histone deacetylase (HDAC) (Amir et al., 2000), (iii) a nuclear localization signal (NLS) within the TRD (amino acids 255–271) which may be responsible for the transport of MeCP2 into the nucleus (Nan et al., 1997) and (iv) C terminal domain which facilitates the binding of MeCP2 to DNA and contains a WW domain (amino acids 359–430) that is predicted to be involved in protein–protein interactions (Buschdorf and Stratling, 2004). Interactions between this transcription repressor complex and chromatin bound MeCP2 lead to the deacetylation of core histones H3 and H4 by histone deacetylases resulting in compaction of the chromatin, making it inaccessible to components of the transcriptional machinery. In addition, MeCP2 can perform histone deacetylase-independent transcriptional repression (Yu et al., 2000).

Majority of *MECP2* mutations are located within the either MBD or TRD, encoded by exons 3 and 4 in patients with classical and atypical Rett syndrome. This is useful in molecular diagnosis within the first year of life in suspected patient, 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 which cause pathogenic mutations have been described. However eight common mutations, which arise at CpG hotspots in *MECP2* result in the loss of function due to truncated, unstable or abnormally folded proteins. Moreover, large rearrangements that involve *MECP2*, including deletions, were reported in a significant proportion of patients with Rett syndrome. Altogether, *MECP2* abnormalities might account for more than 95% of sporadic cases of classical Rett syndrome in females.

Variability in the phenotype in Rett syndrome is often seen due to the type of mutation and X-chromosome inactivation. The *MECP2* mutations in Rett syndrome result from a de novo mutation, though inheritance of the disease mutation can occur due to an affected mother with mild symptoms caused by favorably skewed X-inactivation, or a parent with a germ-line mosaicism. The *MECP2* gene undergoes X inactivation. In one study, it was shown that of the 34 patients with classical RTT, 91% had random XCI (Amir et al., 2000). Non-random XCI was associated with milder phenotypes; preferential inactivation of the X chromosome with mutated *MECP2* gene protects against the deleterious effects of the mutations. Female carriers of RTT causing mutations but asymptomatic and/or who suffer from only mild learning disability, had a non-random XCI (Amir et al., 2000).

There are only a few reports about 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 their family. This finding implied the importance of genetic counseling and prenatal diagnosis to the family. 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. There are some isolated case reports of mutations in *MECP2* gene in patients with Rett syndrome, but no report on spectrum of mutations present in Indian patients of Rett syndrome is available. In this report we attempt to demonstrate the spectrum of *MECP2* mutations in patients with Rett syndrome from the Indian populace. Since genotype–phenotype correlation is complicated in Rett syndrome due to the phenomenon of X-chromosome inactivation, this study is being followed by an elaborate study on pattern of X chromosome inactivation to demonstrate the genotype–phenotype correlation.

2. Materials and methods

2.1. Patients

All patients screened in this study were sporadic. A total of 90 patients of Rett syndrome were analyzed in this study. The children who were subdivided into classical and atypical Rett disorders were diagnosed according to criteria of the Diagnostic and Statistical Manual of Mental Disorders (Fourth Edition) (DSM IV).

2.2. Genomic DNA isolation & PCR amplification

Whole blood samples from above patients were collected in EDTA tubes. Genomic DNA was isolated from 2.0 ml of blood collected from the above patients using QIAmp DNA extraction kit (Qiagen, GmBH, Germany). After isolation, the integrity was checked by running an 0.8% agarose gel electrophoresis.

PCR amplification was performed in 50 μl of 10 mM Tris HCl (pH 8.3) containing 50 mM 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-Fermantas, MD). All the four exons were amplified separately using specific primers designed from the wild type *MECP2* sequence. PCR was cycled 35 times; each cycle consisted of denaturation for 1 min at 94 °C, annealing for 45 s 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).

2.3. Sequencing and sequence analysis

The PCR products were gel purified using QIAquick Gel extraction kit (QIAGEN, GmBH, Germany) according to manufacturer's protocol. The gel purified PCR products were sequenced using gene specific primers on ABI PRISM 3130xl version 3.1 DNA Sequencer (Applied Biosystems, Foster City, CA, USA). The sequences were analyzed for the presence of mutations using Lasergen program (DNASTAR, Inc., Madison, USA). Multiple sequence alignment was carried out using MegAlign program of DNASTAR and WebLogo was created using online software (http://weblogo.berkeley.edu/ WebLogo: A Sequence Logo Generator). Ab initio structure modeling of MeCP2 protein was carried out using two popular algorithms freely available online namely I-Tasser (Roy et al., 2010) and Bhageerath (Jayaram et al., 2012).

2.4. X chromosome inactivation (XCI)

XCI was determined using a method described by Allen et al. (1992). Genomic DNA was predigested with the methylation-sensitive enzymes H*pa*II (New England Biolabs, Hitchin, Hertfordshire, UK). The triplet repeat at the HUMARA locus was then amplified by polymerase chain reaction (PCR) using fluorescent primers, and analyzed using an ABI 3130xl automated sequencer (Applied Biosystems, Foster City, California, USA). Allele peak areas were compared for H*pa*II digested and undigested DNA to determine the degree of XCI. H*pa*II predigestion will prevent PCR amplification of the HUMARA allele on the unmethylated (active) X chromosome; this allows the identification of the allele that is present on the more active X chromosome.

3. Results

This study consisted of 90 patients with features of classical and atypical Rett syndromes. All patients were Indian girls except for one affected male child who is suspected of having Rett syndrome in which mutation in *MECP2* gene was not detected. Patients were classified based on clinical symptoms according to DSMIV criteria.

Table 1
Types of hotspot mutation in *MECP2* gene identified along with its location at the protein level.

Sl. no	Hotspot mutation	Amino acid change	Region	Pathogenic/ polymorphism	No patients
1	c.397C>T	p.R133C	MBD	Pathogenic	2
2	c.916C>T	p.R306C	TRD	Pathogenic	2
3	c.502C>T	p.R255X	TRD-NLS	Pathogenic	2
4	c.473C>T	p.T158M	MBD	Pathogenic	3
5	c.316C>T	p.R106W	MBD	Pathogenic	1
6	c.502C>T	p.R168X	Inter-domain	Pathogenic	3
7	c.808C>T	p.R270X	TRD-NLS	Pathogenic	1

PCR amplification of exons 3 and 4 was initially carried out using gene specific primers, as majority of mutations are known to be present in exons 3 and 4 which encode MBD and TRD domains. Primers for exon 3 amplified a fragment of 596 bp. The exon 4 was amplified in two fragments. One primer pair for exon 4 amplified a fragment of 841 bp and the other pair amplified a fragment of 932 bp with an overlapping fragment of 468 bp. All the PCR fragments were gel purified for sequencing. Upon sequencing, we have identified hotspot mutations in 14 patients (Table 1) as well as other mutations (Table 2) in 13 patients. Those who were negative from mutations in exons 3 and 4 were screened from mutation in exons 1 and 2 of *MECP2* gene and no mutations were detected in exons 1 and 2. A total of 62 patients were found to be negative for mutations in *MECP2* gene.

A total of 19 different *MECP2* sequence variants were identified in 27 patients. Of the 19 mutations, we identified 7 missense mutations in 11 patients, 3 nonsense mutations in 6 patients, 3 frameshift mutations in 4 patients and large C-terminal deletion in 3 patients. The most frequent pathogenic changes were: missense p.T158M (11%), p.R133C (7.4%), p.R306C (7.4%) and nonsense p.R168X (11%), p.R255X (7.4%) mutations. Besides these mutations, we identified two novel deletion mutations in two patients. The novel mutation c.1155-1166delCCTGCC CCCACC (p.385-388delPLPP) caused a deletion of 4 amino acid residue (Fig. 1) and it is present in the C-terminal domain of the protein in atypical patient. The other novel mutation c.[868insCACA; 869-880delAGTCTTCTATCC] (p.Glu290AlafsX38) caused frameshift (Fig. 2) due to which there is premature termination of the protein and it is present in a classical Rett patient. We also identified a novel variant (p.T479T) in a patient with atypical Rett syndrome (Fig. 3).

Three large deletions at C terminal end of the protein have been identified. The clinical presentations of these patients are variable. Out of these three, one patient with g.412833-413171del338 mutation showed atypical phenotype and other two with c.767-1175del409 and c.1101-1201del100 showed classical phenotype.

A total of 62 patients did not show any mutation in *MECP2* gene, out of which 15 were classical and 47 were atypical patients. This necessitates further search for mutations in other genes like CDKL5 and FOXG1 which are known to be responsible for the causation of Rett phenotype. The majority of mutations were detected in exon 4

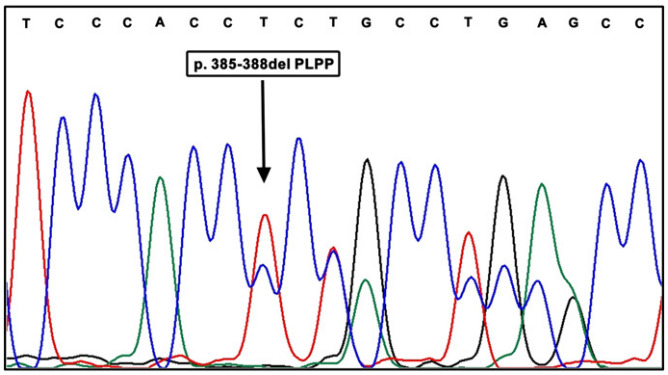

Fig. 1. Chromatogram of DNA sequence showing the presence of p.385-388delPLPP mutation in exon 4 of *MECP2* gene.

and only one mutation (p.R106W) was present in exon 3. Therefore, our study suggests the need for screening exon 4 of *MECP2* as a first line of molecular diagnosis.

3.1. Sequence and structure analysis

Multiple sequence alignment was carried out with its homologs retrieved from NCBI database and those include amino acid sequences of *Homo sapiens* (Accession No NP_004983), *Macaca mulatta* (AFJ71501), *Macaca fascicularis* (AF295597_1) *Pan troglodytes* (XP_521333), *Bos taurus* (NP_001193481), *Canis lupus familiaris* (XP_003435601), and *Oryctolagus cuniculus* (NP_001164841). The WebLogo was also created using online software (http://weblogo.berkeley.edu/ WebLogo: A Sequence Logo Generator). It was observed that the novel mutation p.385-388PLPPdel is well conserved among mammalian species (Figs. 4a and b).

We also tried to analyze the functional effect of novel mutation p.385-388PLPPdel using in-silico approach. However, the entire structure of MeCP2 protein has not yet been experimentally elucidated, only the structure of MBD is available. We tried to generate a theoretical model for the entire protein. There were no templates identified for the C-terminal region, hence a comparative homology modeling could not be carried out. De novo modeling was also attempted using two popular algorithms namely I-Tasser (Roy et al., 2010) and Bhageerath (Jayaram et al., 2012). Due to the absence of suitable templates, de novo modeling failed to predict a suitable structure (Supplementary Figs. 1a and b).

3.2. X chromosome inactivation status

XCI testing was completed in 78 of the 90 patients. After excluding the 12 who were homozygous, 10% (8 out of 78) were shown to have skewed XCI. The distributions of XCI are shown in Fig. 5a. Forty seven (60.2%) RTT patients had 50–59% of X inactivation, 24 (30.7%) had 60–79% of inactivation whereas 8 (10.2%) RTT patients had extremely skewed inactivation (Fig. 5b). We did not find any significant relation

Table 2
Types of mutations other than hotspots in *MECP2* gene identified along with its location at the protein level.

Sl. no	Mutation found	Amino acid change	Region	Pathogenic/polymorphism	No patients
1	c.806Gdel	p.G269*fs*	TRD-NLS	Pathogenic	2
2	c.710dupG	p.G238*fs*	TRD	Pathogenic	1
3	c.455C>G	p.P152R	MBD	Pathogenic	1
4	c.1437G>A	p.T479T	C-term	Novel variant	1
5	c.1189G>A	p.E397K	C-term	Polymorphism	1
6	c.602C>T	p.A201V	Interdomain	Polymorphism	1
7	c.582C>T	p.S194S	Interdomain	Polymorphism	1
8	Large deletion (g.412833-413171del338 and c.767-1175del409)	–	–	Pathogenic	2
9	C-terminal deletion (c.1101-1201del100)	–	–	Pathogenic	1
10	Novel mutation	(p.385-388delPLPP & p.Glu290AlafsX38)	–	Pathogenic	2

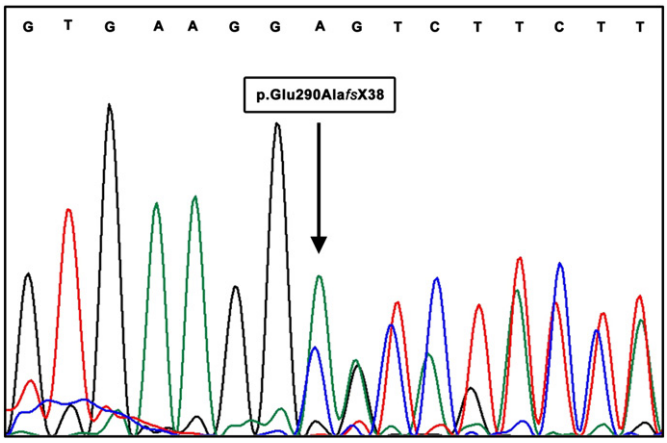

Fig. 2. Chromatogram of DNA sequence showing the presence of p.Glu290Ala*fs*X38 mutation in exon 4 of *MECP2* gene. There is a frameshift of DNA sequence from the point of mutation (indicated by an arrow) to 3′ end.

between mutation type and XCI, using logistic transformation of the percentage of the smaller allele present. The only XCI results of importance were associated with two individual mutations (p.R270X and p.R255X). Of which the p.R270X mutation positive patient had more skewing (where skewing was defined as greater than 80% of one X allele present) and associated with more severe phenotype. The p.R255X mutation positive patient had a milder phenotype. There were no significant relations found between any other mutations and phenotypes.

4. Discussion

Almost all mutations in *MECP2* occur de novo. The exceptions are familial cases where the mutation has been inherited from a healthy or mildly affected mother who has either a gonadal mosaicism or favorable XCI. About 67% of all *MECP2* mutations are caused by C>T transitions at 8 CpG dinucleotides (p.R106W, p.R133C, p.T158M, p.R168X, p.R255X, p.R270X, p.R294X, and p.R306C) and are located in the third and fourth exons. Although mutations are dispersed throughout the gene, a clustering of missense mutations occurs at 5′ of TRD, mostly in the MBD; they all involve evolutionarily conserved amino acids in functional domains of the protein. Nonsense, frameshift and splicing mutations appear distal to the MBD and result in premature termination of the protein and larger multinucleotide deletions occur in the C-terminal domain. Although deletions tend to affect the same region, entirely identical deletions are rare. It is hypothesized that the resultant truncated proteins still bind methylated DNA but cannot interact with the corepressor SIN 3A (Shahbazian and Zoghbi, 2001).

The clinical symptoms and their causes were slightly different in patients with different mutations. In MBD region, we have detected four

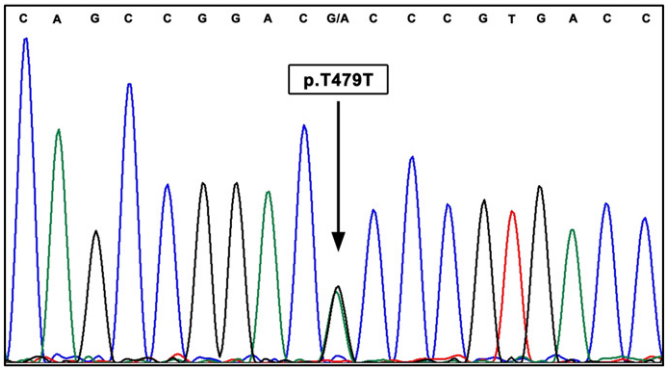

Fig. 3. Chromatogram showing the presence of p.T479T sequence variant in exon 4 of *MECP2* gene.

different types of mutations and the clinical severity varies among mutations. Similar to the finding of Leonard et al. (2003), p.R133C mutation positive patients showed a milder phenotype. These data showed that patients harboring the p.R133C mutation have better overall function. These girls showed improvements in learning to walk and also they remained ambulatory. In all patients with this mutation hand stereotypies were less dominating and had a later age of onset. Breathing and sleep disturbances were less common. Compared to p.R133C, mutation p.T158M present in the MBD domain revealed more severe phenotype. They had early onset of regression and head circumference was found to be below the 3rd percentile. Unlike Calvin et al. (2004), mutation p.T158M present in the MBD in our patient was found to be more pathogenic. The p.P152R mutation positive patient was severely handicapped and revealed classical phenotypes. The p.R106W mutation present in the exon 3 of *MECP2* gene revealed a severe phenotype. The head circumference was below 3rd percentile and age at onset of hand stereotypes was earlier. In-vitro studies have demonstrated that many missense mutations within the MBD (p.R106W, p.R133C and p.T158M) significantly reduce the affinity of MeCP2 for methylated DNA (Free et al., 2001; Yusufzai and Wolffe, 2000), therefore, these mutations may develop more severe phenotype.

In interdomain region, we identified one nonsense mutation (p.R168X) and two polymorphisms (p.A201V and p.S194S). Patients with the p.R168X mutation were more severe than the rest of the cohort in that the age at losing social interaction was earlier. The patients with two polymorphisms p.A201V and p.S194S revealed milder phenotypes. Two mutations p.R306C and c.710dupG present in the TRD region revealed milder phenotypes. There is a delay at onset of hand stereotypies and respiratory dysfunction. The milder phenotype for p.R306C was expected as Yusufzai and Wolffe (2000) showed that the p.R306C does not reduce repressive activity of MeCP2 in a cell transfection assay.

In TRD-NLS region, we have identified three mutations and the clinical severity varies among these mutations. For the p.R255X mutation, no significant differences in phenotype severity were identified in comparison to the rest of the cohort. However, phenotype associated with the p.R270X mutation, also in TRD-NLS region was clearly more severe than the rest of the cohort. They did not have early normal development and head circumference was <3rd percentile at the time of presentation. The age at onset of hand stereotypes and social interaction was much earlier in these patients. This finding was similar to Jian et al. (2005) who reported earlier that there is greater clinical severity among patients with mutations in the NLS region. We have identified a difference in clinical severity in patients with c.806delG present in the TRD-NLS region. One patient had severe phenotype with respect to age at onset of regression and hand stereotypies. She also has respiratory problem and epilepsy starting at the age of 1 year. While the other patient with this mutation showed a milder phenotype with respect to age at onset of regression and hand stereotypies. This patient's head circumference is 25th percentile at the time of presentation.

Three patients were identified with C-terminal deletions, two patients showed milder phenotype in terms of head circumference and age at onset of hand stereotypies. The other patient who showed a severe phenotype had a complex mutation. She had two mutations (p.T203M and c.1101-1201del100) and a polymorphism p.S357S. This patient was found to be severely affected, her head circumference was less than 3rd percentile, had earlier onset of regression, hand stereotypies and epilepsy along with respiratory dysfunction.

Patient with p.385-388delPLPP novel mutation showed more severe phenotype with earlier onset of regression, head circumference less than 3rd percentile and presence of epilepsy, although she did not have any respiratory problem. This mutation is present in the WW domain of the MeCP2 protein. As this domain is responsible for protein–protein interaction, it is expected that mutation in this domain can interfere in binding MeCP2 with its transcriptor repressor protein, thereby affecting the function of transcription repressor complex. We also observed that the p.385-388delPLPP region is well conserved

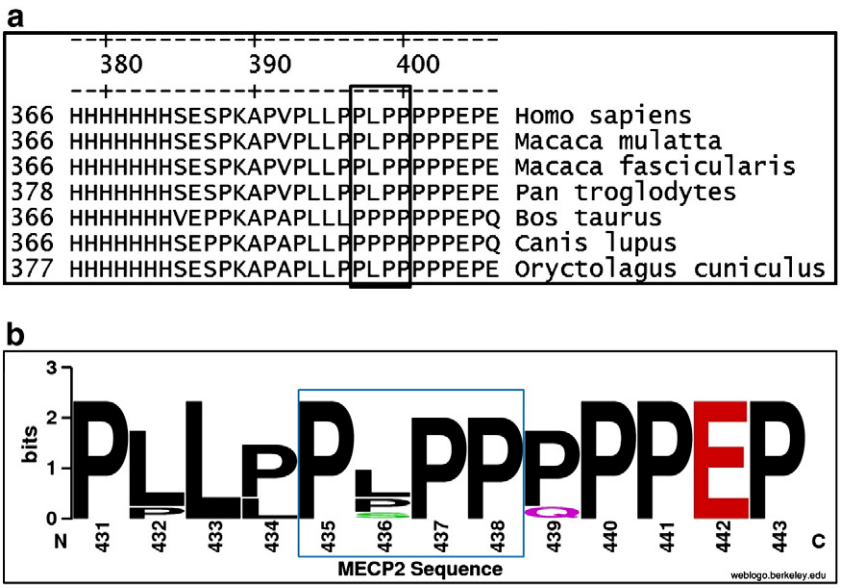

Fig. 4. Sequence conservation of p.385-388delPLPP mutation. (a) Multiple sequence alignment of the mutation indicated by box with homologous sequence from different species showing the extent of conservation of the mutation site. (b) WebLogo showing the conservation of the mutation indicated by box.

among mammalian species as depicted by alignment and WebLogo. The extent of conservation of this region defined the importance of its structure and function of the protein.

The patient with other novel mutation p.Glu290AlafsX38 had milder phenotypes. The age at onset of regression was much later viz 1 year and 6 months; head circumference was in 25th percentile.

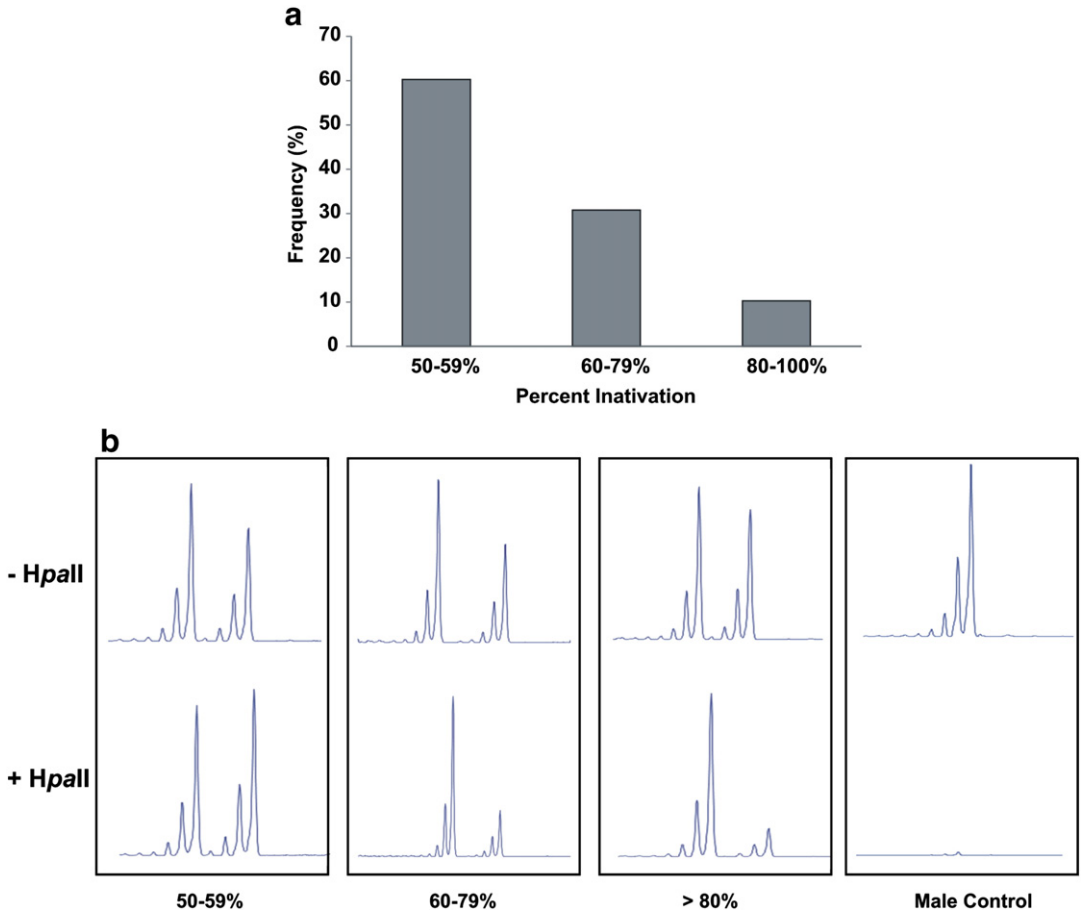

Fig. 5. Pattern of X chromosome inactivation (XCI) in blood sample of RTT patients. (a) Histogram showing the extent of XCI. Values on the graph represent the percentage inactivation of the allele of smaller CAG repeat size. Skewed inactivation is considered at least 80% inactivation of one allele. (b) Representative figure of two CAG repeat allele with percentage of inactivation. The top figure in each box represents the undigested DNA (−*Hpa*II) from female RTT patients and from a male control; the bottom figure of each box represents DNA digested with *Hpa*II prior to PCR (+*Hpa*II). The relative intensity of the two alleles after digestion represents the XCI pattern for each individual (expressed as ratio and normalized to the undigested samples). The bottom figure in male disappears, representing complete digestion of the unmethylated allele on the active X chromosome.

She had no hand stereotypes and epilepsy and was found to be hyperactive. The probable explanation for milder phenotype is that the truncated MeCP2 protein might still be targeted to the nucleus and be able to exert a partial effect on transcription repression. Therefore, the patient with this particular mutation showed milder phenotype.

The patient with novel variant (p.T479T) showed a milder phenotype. She has normal head circumference, age at onset of regression was 3 months. Multiple sequence alignment was carried out and found that this amino acid is well conserved across the species that indicates the importance of this residue (Supplementary Fig. 2).

4.1. Effect of X-chromosome inactivation

The inactivation of one of the X chromosomes occurs randomly in differentiating embryonal cells in females, resulting in cells that are mosaic with respect to which chromosome is active. The purpose is to equalize X-linked gene products between XX females and XY males. Non-random XCI was associated with milder phenotypes. Preferential inactivation of the X chromosome with mutated *MECP2* gene protects against the deleterious effects of mutations. Female carriers of RTT are causing mutations but asymptomatic or who suffer from only mild learning disability, had a non-random XCI (Zoghbi et al., 1990). We have demonstrated the skewing XCI associated with p.R270X mutation with severe phenotypes which is similar to the finding of Calvin et al. (2004). Another patient with p.R255X showed skewed inactivation, but still the patient has milder symptoms. In this case, there might be a favorable XCI where the mutant allele might be present in the inactive X chromosome. In all other mutations positive patients had a random X inactivation. Therefore, no significant relation between XCI and clinical severity was found.

In conclusion, we have confirmed that mutations in *MECP2* are responsible for RTT. Nineteen different mutations in *MECP2* have been detected in 27 of patients with RTT and seven common hotspot mutations were defined. Early diagnosis of RTT is now possible in patients with neurodevelopmental problems using mutation analysis of *MECP2* gene. Severity of clinical symptoms was associated with specific DNA mutations in some patients with RTT and it may therefore be more heterogeneous than reported previously.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gene.2012.11.024.

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