MECP2 mutations in Malaysian Rett syndrome patients

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ABSTRACT

__Introduction:__ Rett syndrome (RS) is a severe neurodevelopmental disorder characterised by normal neurological development followed by progressive developmental regression. The X-linked dominant inheritance of RS has been mapped to the gene that encodes the methyl-CpG-binding protein-2 (*MECP2*) at Xq28. In the present study, denaturing high-performance liquid chromatography (DHPLC) was used to detect mutations in the *MECP2* gene in 20 Malaysian RS patients.

__Methods:__ Polymerase chain reaction (PCR) was carried out to amplify the *MECP2* coding exons 2, 3, and 4 in a total of eight reactions (exons 2, 3a, 3b, 4a, 4b, 4c, 4d and 4e). Subsequently, PCR products were analysed by DHPLC.

__Results:__ Mutations in the *MECP2* gene were detected in 13 of the 20 (65 percent) RS patients. 11 patients had mutations in exons 3b and 4a and six patients had mutations in exon 4c. These mutations were mainly concentrated in the methyl-CpG-binding domain and the transcriptional-repression domain.

__Conclusion:__ Through the use of post-PCR high-performance liquid chromatography, 65 percent of 20 RS patients were found to have mutation(s) in the *MECP2*.

Keywords: denaturing high-performance liquid chromatography, *MECP2* mutation, methyl-CpG-binding domain, mutation analysis, Rett syndrome, transcriptional-repression domain

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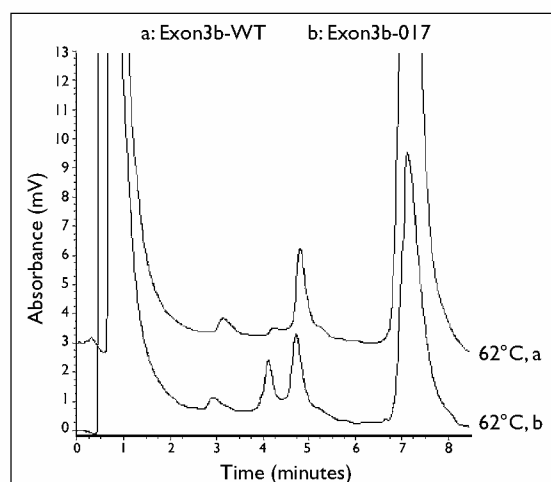
Fig. 1 Chromatograms of patient sample 017 from exon 3b after mutation detection analysis at 62°C. WT: wild type.
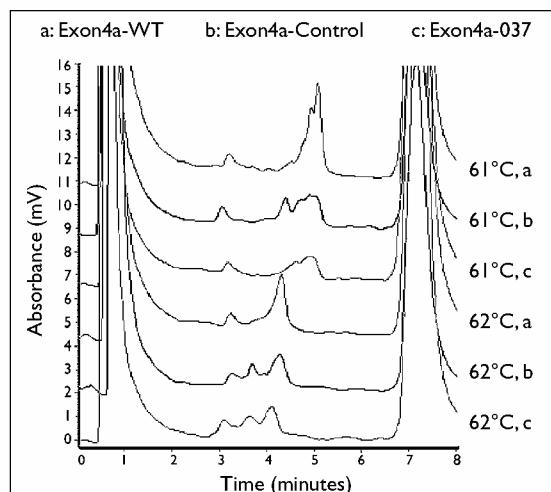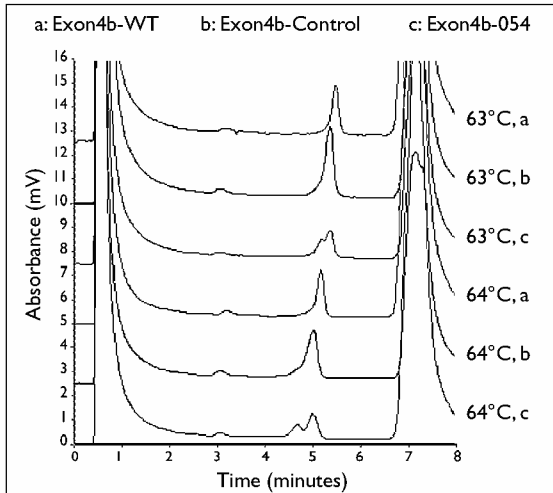

Fig. 2 Chromatograms of patient sample 037 from exon 4a after repetition of mutation detection analysis at 61°C and 62°C. WT: wild type; Control: positive control (467insC).

INTRODUCTION

Rett syndrome (RS) is a severe neurodevelopmental disorder first described by Andreas Rett in 1966.[1] In its classical form, RS is characterised by normal neurological development until 6–18 months of age, followed by a progressive developmental regression with clinical abnormalities, such as progressive encephalopathy, autistic behaviour and stereotyped hand movements. RS occurs almost exclusively in females and it affects approximately 1:10,000 to 1:15,000 females worldwide.[2] Early diagnosis of RS helps clinicians to manage their patients better and provide genetic counselling. In addition, due to its diverse presentation, many children with RS are misdiagnosed based on clinical features alone.

Fig. 3 Chromatograms of patient sample 054 from exon 4b after repetition of mutation detection analysis at 63°C and 64°C. WT: wild type; Control: positive control (R255X).

Fig. 4 Algorithm shows the molecular diagnosis of RS.

Previous studies have identified missense, nonsense, frameshift mutations in the coding region of methyl-CpG-binding protein-2 (*MECP2*) in 80% of patients with RS. Most of the mutations lie within the methyl-CpG-binding domain (MBD) or transcriptional repression domain (TRD). A number of deletions have also been identified. DNA sequence is the gold standard for the identification of point mutations, but methods, such as denaturing high-performance liquid chromatography (DHPLC), provide a screening prior to DNA sequencing. DHPLC relies on the principle of heteroduplex analysis by ion-pair reverse-phase liquid chromatography under partially denaturing conditions.[3] The aim of this study was to use DHPLC to detect mutations in the *MECP2* gene in Malaysian RS patients.

METHODS

Blood samples (~ 4.0 ml of peripheral blood) from 15 female and five male RS patients were collected from the Kuala Lumpur Hospital, Penang Hospital, Selayang Hospital and University of Malaya Medical Centre. The patients were reviewed by paediatric neurologists and clinical geneticists, and the diagnosis of RS was based on clinical features and disease progression.[4] Ethics approval and informed consent were obtained for the study. Classical cases of RS were defined as those fulfilling all five major diagnostic criteria, or four out of the five major and two out of the six minor (supportive) diagnostic criteria. The major diagnostic criteria include an apparently normal prenatal and perinatal period with an apparently normal psychomotor development through the first six months of life. Head circumference is normal at birth with subsequent deceleration of head growth between five months and four years of age. Between ages six and 30
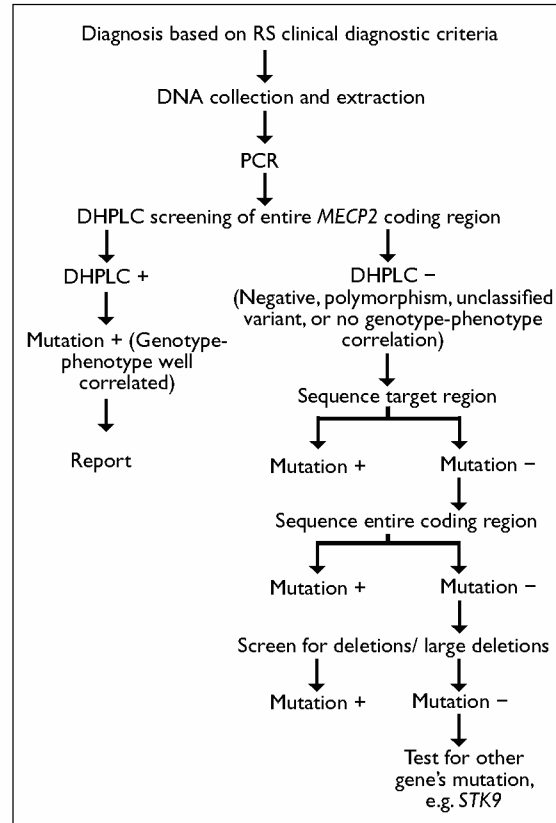
months temporally, there is reduction or loss of acquired purposeful hand skills associated with communication dysfunction and social withdrawal. In addition, there is development of severely-impaired expressive and receptive language and the presence of apparent severe psychomotor retardation. The hallmark of RS is the stereotypic hand movements after purposeful hand skills are lost. Another prominent feature is the appearance of gait apraxia and truncal apraxia/apraxia between ages one and four years. The minor (supportive) diagnostic criteria are: growth retardation, vasomotor autonomic dysfunction and atrophy of the feet, abnormalities of the electroencephalogram, scoliosis, ventilatory irregularities, and the presence of shortened fourth metacarpal or metatarsal bones.

Variant RS is diagnosed by fulfilling at least three of the six main criteria and at least five of the 11 supportive criteria. The main criteria are: the absence or reduction of hand skills, reduction or loss of speech (including babble), hand stereotypies, reduction or loss of communication skills, deceleration of head growth from early childhood, and regression followed by recovery of interaction. Supportive criteria include breathing irregularities, air swallowing or abdominal bloating, bruxism, abnormal locomotion, scoliosis or kyphosis, lower limb amyotrophy, cold/discoloured feet (usually hypotrophic), sleep

Table I. PCR reaction mix for each exon.

PCR mix	Volume (µL)				
	Exon 2, 4b, 4d and 4e	Exon 3a and 4a (55°C, 40 ×)*	Exon 3b	Exon 4a (54.9°C, 35 ×)*	Exon 4c
Sterile MiliQ water	16.0	16.5	16.8	16.3	17.3
10 × *Pfu* buffer	2.5	2.5	2.5	2.5	2.5
25 mM MgSO₄	1.5	1.0	0.7	1.5	1.5
10 mM dNTP mix	0.5	0.5	0.5	0.5	0.5
Forward primer	1.5	1.5	1.5	1.5	1.0
Reverse primer	1.5	1.5	1.5	1.5	1.0
Pfu polymerase	0.5	0.5	0.5	0.2	0.2
DNA template	1.0	1.0	1.0	1.0	1.0
Total volume	25.0	25.0	25.0	25.0	25.0

* Annealing temperature and number of PCR cycles.

Table II. PCR cycles and annealing temperatures for each exon.

Exon	Annealing temp (°C)	Number of PCR cycles
2 and 4b	59.0	30
3a	60.0	40
3b	60.0	40
4a	54.9 / 55.0	35 / 40
4c	63.0	35 / 30
4d and 4e	57.0	30

disturbances including night time screaming, inexplicable episodes of laughing or screaming, apparently diminished pain sensitivity, and intense eye contact and/or eye pointing. All the above patients in this study had some features of RS. Out of 20 patients, six (30%) were classical RS patients and 14 (70%) were variant RS patients. Among the female patients, there were six classical RS (40%) and nine variant RS (60%) patients. All of the five male patients were variant RS patients.

Genomic DNA were isolated using the Genispin™ Blood DNA Kit purchased in BioSyn Tech, Kuala Lumpur, Malaysia.[5] Polymerase chain reaction (PCR) primers were synthesised to amplify *MECP2* coding exons 2, 3, and 4 in a total of eight reactions (exons 2, 3a, 3b, 4a, 4b, 4c, 4d and 4e).[6] The positive control DNA were kindly provided by the Wessex Regional Genetics Laboratory. The mutations carried out were: exon 2 (C→ G@1–75), exon 3a (108delAGAA), exon 3b (R106W), exon 4a (467insC), exon 4b (R255X), exon 4c (R270X), exon 4d (1164del44) and exon 4e (V481M). Exon 1 was just described when the project began.[7] The protocol applied was derived from the Wessex Regional Genetic Laboratory. During that time, PCR for exon 1 was not well described yet. Hence, a mutation analysis for exon 1 was not performed in this study.

PCR were carried out in 25-µL volumes containing 1.0 µL genomic DNA, 10 × *Pfu* buffer (200 mM Tris-HCl [pH 8.8 at 25°C], 100 mM (NH₄)₂SO₄, 100 mM KCl, 1% Triton X-100), 25 mM MgSO₄, 10 mM dNTP mix (dATP, dCTP, dGTP and dTTP), 100 mM forward and reverse primer, and 2.5u/ µL *Pfu* DNA polymerase. A different PCR reaction mix was applied for each exon (Table I). PCR conditions included an initial denaturation at 94°C for 15 min, followed by PCR cycles of denaturation (30 s at 94°C), 30 s of annealing step (Table II shows the different cycles and annealing temperatures applied for each exon), and 30 s at 72°C of extension followed by a final extension step at 72°C for 7 min. PCR products were separated on 1% agarose gel at 90 V for 45 min. For exon 3b, different samples had a different PCR reaction mix and conditions. Approximately-sized PCR products were obtained through optimisation.

The PCR reaction was scaled up to provide sufficient volume for DHPLC analysis. At the end of the PCR cycle, a hybridisation step was incorporated to encourage heteroduplex formation. DHPLC was performed using the WAVE™ DNA-fragment analysis system.[8] PCR products of exons 2, 3a, 3b, 4a, 4b, 4c, 4d and 4e were injected into the WAVE™ system to check for PCR quality and size under non-denaturing conditions. When the analysis showed that the PCR product sizes were synthesised correctly and carried no impurities, mutation detection analysis was then carried out using temperatures optimised (58°C and 61°C for exon 2; 59°C for exon 3a; 62°C for exon 3b; 61°C and 62°C for exon 4a; 63°C and 64°C for exons 4b and 4c; 64°C and 65°C for exon 4d; and 63°C for exon 4e) to produce the most prominent peak for a suspected mutation. The data analysis was based on visual inspection of the chromatograms, and comparisons with normal controls (sample 043). Positive controls were included in each run. Any extra peak(s), shoulder(s) or different retention times observed on a major peak from a patient's chromatogram compared to the wild type, and peak(s) from a patient's chromatogram being similar to the positive control, was suspected to have mutation.

Table III. Probable domain change location for each exon detected.

Exon	Positive control	Probable domain change location	Patient ID
2	C→G@1–75	Other coding sequence	028, 047
3a	108delAGAA	Other coding sequence	None detected
3b	R106W	MBD	017, 034, 047, 054
4a	467insC	MBD	018, 027, 028, 030, 037, 041, 050
4b	R255X	Inter-domain region and nuclear localisation signal	048, 054
4c	R270X	TRD and nuclear localisation signal	017, 027, 041, 048, 054, 055
4d	1164del44	Other coding sequence	None detected
4e	V481M	Other coding sequence and 3'UTR	None detected

RESULTS

In DNA extraction/PCR and agarose gel electrophoresis, distinct bands of PCR products were observed after agarose gel electrophoresis for exons 2, 3a, 3b, 4a, 4b, 4c, 4d and 4e with their respective appropriate sizes of 234 bp, 336 bp, 350 bp, 383 bp, 379 bp, 366 bp, 404 bp and 326 bp except for positive control of exon 3b despite numerous attempts. In DHPLC, of the 20 RS patients, 13 (65%) had mutations in *MECP2*, six of the 13 were found to have two mutations each and another patient had three mutations. 11 patients had mutations in exons 3b and 4a which corresponded to the MBD region, while six patients were found to carry mutations in exon 4c which corresponded to the TRD (Table III). Figs. 1–3 show chromatograms of patients with mutation.

DISCUSSION

DHPLC after PCR detected mutations in the *MECP2* gene in 13 of the 20 (65%) RS patients. Additionally, six of the 13 had two mutations each and another had three mutations. Such mutations could be pathogenic or mere sequence polymorphisms.[3] 12 female RS patients (60%) and one male RS patient (5%) were identified to have mutations. Of the 13 patients with mutations, five (38%) were classical RS patients and eight (62%) were variant RS patients. For female patients with mutations, five out of six (83%) were classical RS and seven out of nine (78%) were variant RS patients. One male patient with mutation was a variant RS patient (20%).

It has been reported that *MECP2* mutations were detected in 75% of sporadic and 45% of familial RS.[9-13] Nonsense and missense mutations were clustered in the MBD and the TRD, whereas frameshift mutations were located near the C terminus of the protein.[14] Nonsense mutations that affected both the MBD and TRD are the most pathogenic mutations.[2] In this study, 11 patients had mutations in exons 3b and 4a (Table III). Since the MBD region is located in these exons, it is suggested that these patients had mutations in the MBD. Clinical features of these 11 patients included global delay/regression, acquired microcephaly, autistic feature, severe learning difficulty, hand wringing/washing (stereotypic hand movements), epilepsy, episodic hyperventilation, small hands and feet, and speech delay. The TRD is located in exon 4c. Six patients were found to carry mutations in exon 4c, suggesting that they had mutations in the TRD (Table III). They were diagnosed to have clinical features similar to those of patients suspected to have mutations in the MBD in this study.

The rate of mutation detection is lower than expected due to several factors. Only 20 patient samples were obtained for this study. Furthermore, a number of patients were classified with variant RS. More patients who fulfilled the diagnostic criteria of RS may give a higher mutation yield in all the exons known to be involved in RS. Other than that, there might be some mutations in exon 1, but mutation analysis for exon 1 was not performed. It is also possible that this method is unable to detect large deletions. Research done by Schollen et al, who used Southern blot analysis,[15] and Laccone et al, who used quantitative PCR,[16] identified large-scale deletions in three of nine and 15 of 171 patients with classic RS, respectively. Multiplex ligation-dependent probe amplification has also been used for the detection of large deletions.[17] As such, the identification of large-scale deletions not detected by standard PCR methodology provides an explanation, in part, for the 15%–20% of females with classic RS for whom a mutation had not been detected previously.[18]

It could be a false negative or a true negative when a clinically-diagnosed patient does not have a mutation. Buyse et al described one patient who initially was negative by DHPLC analysis, but direct sequencing of the complete *MECP2* coding region of this patient revealed a missense mutation.[3] This caused a false negative rate of 1.2%. Other studies involving DHPLC have reported a sensitivity and specificity of 100%.[19,20] It is possible that the false negative rate in RS studies using DHPLC may be due to an erroneous clinical classification of RS which may include other conditions that mimic RS. In addition, there is a wide variety of clinical presentation. The same mutation can give rise to different phenotypes in different patients. There are many types of mutation

detection or scanning methods, such as DHPLC, single-strand conformational polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE), confirmation sensitive gel electrophoresis (CSGE) and two dimensional gene scanning (TDGS). Most methods only do well with regard to specificity.[21] We used DHPLC in this study because it had been reported to match direct sequencing in sensitivity, lack any bias for mutations, be highly automated and offer high sample throughput.[22]

PCR primers were synthesised to amplify *MECP2* coding exons 2, 3, and 4 using a total of eight reactions (exons 2, 3a, 3b, 4a, 4b, 4c, 4d and 4e). This is because DHPLC declines in sensitivity with fragments of more than 400 base pairs associated with the appearance of broader and less characteristic chromatograms both for the normal and the mutated alleles.[3,14] Sequence analysis is the gold standard for the identification of point mutations or deletion/insertion mutations that involved a few bases. Nonetheless, direct sequencing is both costly and labour-intensive. We propose a strategy that may be possible to omit the direct sequencing and rely on DHPLC in the future once the genotype-phenotype correlation is well established. Sequencing will only be carried out for DHPLC negative samples that could be polymorphism, an unclassified variant or samples with no genotype-phenotype correlation. The overall algorithm of the molecular diagnosis of RS is shown in Fig. 4. In this study, variations in chromatogram patterns detected by DHPLC suggested the presence of *MECP2* mutations in 13 out of 20 (65%) RS patients. Further study with a larger well-defined sample size and DNA sequencing would be useful to differentiate whether the mutations found are pathogenic or polymorphic.

ACKNOWLEDGEMENTS

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