MUTATION IN BRIEF

Detection of Heterozygous Deletions and Duplications in the MECP2 Gene in Rett Syndrome by Robust Dosage PCR (RD-PCR)

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Fifty to eighty percent of Rett syndrome (RTT) cases have point mutations in the gene encoding methyl-CpG-binding protein-2 (*MECP2*). A fraction of *MECP2* negative classical RTT patients has large heterozygous deletions. Robust Dosage PCR (RD-PCR) assays were developed as a rapid, convenient and accurate method to detect large heterozygous deletions and duplications. A blinded analysis was performed for 65 RTT cases from Portugal by RD-PCR in the coding exons 2-4 of the *MECP2* gene. Neither the patients with point mutations nor the non-classical RTT patients without point mutation had a deletion or duplication. One of remaining eight female patients with classical RTT without point mutation had a heterozygous deletion. This is the first report of a deletion spanning the entire *MECP2* gene. The deletion was confirmed by southern blotting analysis and the deletion junction was localized 37kb upstream from exon 1 and 18kb downstream from exon 4. No duplications were detected. Our results suggest that RD-PCR is an accurate and convenient molecular diagnostic method.

KEY WORDS: Rett Syndrome; *MECP2*; RD-PCR; heterozygous deletion

INTRODUCTION

Fifty to eighty percent of Rett syndrome (RTT; MIM# 312750) cases have point mutations in the *MECP2* gene (Methyl-CpG-binding protein 2; MIM# 300005)(Amir et al., 1999; Miltenberger-Miltenyi., 2003). Southern blotting analysis, quantitative PCR and MLPA (Multiplex Ligation-Dependent probe amplification) have been used to detect large deletions and duplications in the *MECP2* gene of RTT patients without point mutation (Schollen et al., 2003; Erlandson et al., 2003; Ariani et al., 2004; Laccone et al., 2004). In those reports, 12 out of 59 (20.3%) classical RTT patients without point mutation have large deletions in *MECP2* gene.

Robust dosage PCR (RD-PCR) has been developed as a rapid, convenient and accurate method to detect heterozygous deletions and duplications (Liu et al., 2003). The accuracy and consistency of RD-PCR has previously been validated in multiple blinded analyzes with 100% accuracy (Liu et al., 2003; Nguyen et al., 2004). RD-PCR has the advantages of rapid optimization and validation of new assays, and inclusion of positive controls without the requirement of the heterozygous deletion. The enhanced RD-PCR protocol has the additional advantages of tolerance toward genomic DNA of variable quality and uniform and unbiased performance across

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regions of variable sequence context and GC content (Nguyen et al., 2004; Shi. et al., 2004).

In our study, we used, for the first time, RD-PCR to detect heterozygous deletions in the *MECP2* gene. A blinded analysis was performed for 65 RTT cases from Portugal. One of eight patients with classical RTT without point mutation had a heterozygous deletion spanning the entire coding sequence. The deletion was confirmed by southern blotting. This is the first biological analysis report on RD-PCR.

MATERIALS AND METHODS

Samples

The DNA was prepared from peripheral blood by Puregene DNA Isolation Kit (Gentra, Minneapolis, MN). The concentrations were measured by UV spectrophotometer at 260 nm and adjusted to a working concentration of 30 ng/μl in TE buffer. Forty-eight control samples were analyzed in a blinded validation analysis, in which gender was used as a surrogate for heterozygous deletions.

Sixty-five RTT patient samples from Portugal and two blinded male controls were screened in the study. Patients were diagnosed according to the RTT diagnostic criteria defined by Hagberg et al (Hagberg et al., 1985 and 2002), which includes psychomotor regression after a period of normal development, severe mental retardation, deceleration of head growth and loss of purposeful hand skills with appearance of stereotypical hand movements. 30 of the RTT samples were classical RTT patients, and 8 of these were negative *MECP2* mutation according to our previous work.

RD-PCR assay

Genomic DNA samples were incubated at 90^{o}C in TE buffer for 10 minutes to minimize RD-PCR bias (Shi *et al*., 2004). Four RD-PCR assays for the three coding exons of *MECP2* gene were designed (Table 1) according to Liu et al (Liu et al., 2003), except for the 5' universal tail of 5' ggccaagtgt- 3'. These assays were divided into two groups depending on whether the *ATM* or *FUT* gene was used as the autosomal control segment. Group I had two assays in exon2 and exon3 of the *MECP2* gene, exon 12 of the ataxia telangiectasia mutated (*ATM*) gene was the internal control. Group II had the other two assays in the coding part of exon 4 of the *MECP2* gene; the fucosyltransferase 2 (*FUT*) gene was the internal control.

Ten more RD-PCR assays were developed in the 3' and 5' flanking regions of the *MECP2* gene to localize the deletion junction. The primers were designed according to the genomic sequence from NT_025965 (GenBank accession number) (data not shown).

The PCR mixtures contained a total volume of 25μl: 1xExpand™ High Fidelity buffer#3 (Roche), 4.5 mM $MgCl_2$, 200 μM of each dNTP for Group I or 3.0 mM $MgCl_2$ and 150 μM dGTP/50 μM deaza-dGTP, 200 μM of each other dNTPs and 10% DMSO for Group II, 0.1-0.2 μM of each pair of primers, 1U Platinum *Taq* DNA polymerase (Invitrogen) and 1U platinum *Taq* DNA polymerase High Fidelity (Invitrogen), 0.5 μg of BSA, and 60 ng of genomic DNA. The cycling entailed denaturation at 94^{o}C for 15 sec, annealing at 55^{o}C for Group I or 65^{o}C for Group II for 30 sec, and elongation at 72^{o}C for 1 min for 23 cycles.

Quantitation

Twelve μl of PCR product was electrophoresed through a standard 2% agarose gel. Gels were stained in 0.2μg/ml Ethidium Bromide for 1 hour and scanned by Typhoon 9410 Imager (Amersham) with the following parameters: focal plane =+3 mm, laser wavelength= 532 nm, Green, emission filter =610 BP 30, photomultiplier voltage =600 V, pixel size =100μm and sensitivity =normal.

ImageQuant™ software was used to quantitate the PCR yield. Net signal of a product band was obtained by subtracting local background signal from total signal in arbitrary unit. The ratio of yields (ROY) is calculated by dividing the target net signal by the internal control net signal. For normalization, the ROY of the patient sample was divided by the average ROY of the normal females.

Table 1. List of Primer Pairs and PCR Segments

	3' sequence-specific region			Core PCR segment[c]			
	Name[a]	Sequence[b] (5'-3')	T_m (°C)	GC%	Region	Size	Tm (°C)	GC%
Group I	Assay 1							
	Target							
	MECP2-2(709463)D	5'TTTAGTCTTTGGGGTACTTTTA3'	45.4	32	Exon 2 of MECP2	492	74.1	38
	MECP2-2(709954)U	5'GGCTTGTGATAGTGTTGATTCT3'	47.8	41				
	Control[d]							
	ATM(38415)D	5'ATCCTGCAAGTTTACCTAAC3'	44.9	41	Exon 12 of ATM	418	75.2	41
	ATM(38829)U	5'GATCAGGGATATGTGAGTGT3'	46.4	43				
	Assay 2							
	Target							
	MECP2-3(649492)D	5'ACCTGGTCTCAGTGTTCATTGT3'	50.0	46	Exon 3 of MECP2	486	81.2	55
	MECP2-3(649977)U	5'CTTCAGGGAAGAAAAGTCAGAA3'	49.8	41				
Group II	Assay 3							
	Target							
	MECP2-4-1(647695)D	5'CTTTGTCAGAGCCCTACCCATA3'	52.7	50	Exon 4-1 of MECP2	447	83.5	61
	MECP2-4-1(648141)U	5'CCACCATCACCACCACTCAGAG3'	56.5	59				
	Control							
	FUT(502)D	5'TTCACCGGCTACCCCTGCTC3'	58.6	65	FUT2	504	83.5	61
	FUT(1006)U	5'GGAGTCGGGGAGGGTGTAAT3'	54.1	60				
	Assay 4							
	Target							
	MECP2-4-2(648547)D	5'CCCCCTGGCGAAGTTTGAAAAG3'	60.5	55	Exon 4-2 of MECP2	400	81.2	56
	MECP2-4-2(648946)U	5'CCACCATCCGCTCTGCCCTATC3'	61.4	64				

a. For example, MECP2-2(709463)D: MECP2=methyl CpG binding protein 2, Xq28, its sequence is from NT_025965.13 (GenBank accession number); 5' end of the 3' sequence-specific region of the primer begins at 709463; and D, downstream. The precise sizes and locations of the PCR fragment can be obtained from the information names. ATM=ataxia telangiectasia mutated, 11q22-q23, its sequence is from U82828; FUT=fucosyltransferase 2,19q13, its sequence is from D82933.

b. The sequence of the 3' sequence-specific region is shown. A 10-nucleotide universal tail (5' ggccaagtgt 3') is attached to the 5' end of each primer. Note that the control primers have been redesigned relative to previous report (Liu et al., 2003) to incorporate the 10-nucleotide universal tail.

c. The core PCR segment does not include the tails.

d. Exon 12 of ATM gene and FUT gene were internal controls of the Group I and Group II. They are listed in one assay in each group and left out in others.

Southern blotting analysis

Southern blot was performed using probes RTT2 (709610-709766, sequence is from NT_025965.13), RTT3 (649518-650043) and p(A)10 (639141-639564) that hybridized with exon 2, exon 3 and the end of the 3'UTR. Probes were generated by PCR from genomic DNA, purified from 1% agarose gel by QIAEX II (QIAGEN, Valencia, CA) and labeled with ^{32}P dCTP by Prime-It II Random primer (Stratagene, Cedar Creek, TX). The genomic DNA (8μg) of female control, male control and patient P3 was digested with *Hind III* and *Pst I* for probe RTT2, *Sac I* for probe RTT3 and *Hind III* and *Sac I* for probe p(A)10. Digested DNA fragments were separated in

a 1.5% agarose gel and blotted into a nylon membrane (Hybond H-N+; Amersham Pharmacia Biotech, Buckinghamshire, England). Hybridization was performed overnight at 65ºC and washings were carried out in a series of SSC/SDS solutions (0.1%SDS, 2%-0.1% SSC). Membranes were exposed to storage phosphor screen, scanned by Typhoon 9410 Imager (Amersham, Molecular Dynamics, Sunnyvale, CA). ImageQuant™ software was used to quantitate the signals.

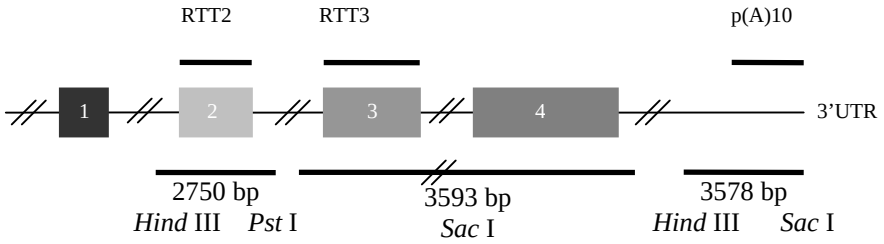


Figure 1. Schematic representation of the *MECP2* gene regions analysed by Southern blotting and localization of the probes used in the assay (figure is not to scale).

RESULTS AND DISCUSSION

Validation by blinded analysis with 100% accuracy

RD-PCR, a duplex PCR, amplifies an endogenous internal control and a target locus. The internal control has a known gene copy number per cell while the target has an unknown number per cell. The ROY was directly proportional to the ratio of the two input templates, so the copy number of the *MECP2* gene could be obtained according to the ROY and the known copy number of the internal control.

For validation of the four assays in the *MECP2* gene, a blinded analysis was performed with 48 blinded genomic DNA samples where either the sex status or the number of each status were unknown (Fig. 2A). The male sample was functionally equivalent to a RTT patient with large heterozygous deletion. All the males and females were determined with 100% accuracy. The standard deviations of ROY were around 0.04 in both male and female samples in each of the assay.

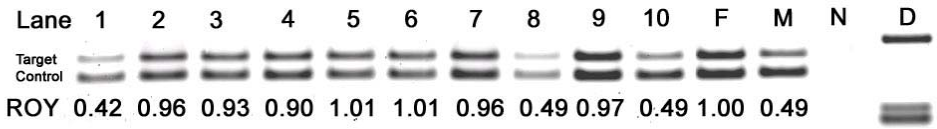
Large heterozygous deletion found in one patient

Exons 2, 3 and 4 of the *MECP2* gene were analyzed for deletions by four RD-PCR assays. ROYs of each assay were obtained and the copy numbers of the three coding exons 2-4 of the *MECP2* gene were determined in the 65 patient samples and two blinded male controls (Fig. 2B). All the 65 patient samples were previously scanned for point mutation in coding exons and immediate flanking intronic regions of *MECP2* gene by DOVAM-S (Detection of virtually all mutations-SSCP). The RD-PCR analysis was performed blinded to previous point mutation scanning. Only one of eight female patients with classical RTT without point mutation (P3) and two blinded male controls, P1 and P2 showed much lower ROY values than all the other female patients in all the four assays. ROYs of the female patient P3 were 0.44, 0.49, 0.51, and 0.52, indicating that the patient carried a large heterozygous deletion spanning the completely coding region of the *MECP2* gene. None of 22 patients with non-classical RTT with point mutations had a heterozygous deletion. No patient with duplication was observed.

Except for the three samples, P1, P2 and P3, the means and standard deviations of the ROYs were 1.00±.0.09, 1.02±0.08, 1.00±0.09 and 1.00±0.10 respectively; the ranges of ROYs were 0.83-1.22 for Assay 1, 0.83-1.21 for Assay 2, 0.83-1.21 for Assay 3 and 0.79-1.22 for Assay 4. The two blinded male controls were both detected as heterozygous deletions in every assay. All female patients without deletions or duplications had ROYs clearly distinguishable from the male controls and the patient with deletion. These strongly supported the accuracy of the RD-PCR assays.

Southern blotting analysis was used to confirm the deletion identified by RD-PCR method. Signal intensity of patient P3 was similar to that of the male control with probes RTT2, RTT3 and p(A)10 (Fig. 3), indicating only one copy of the *MECP2* gene in patient P3.

A



B

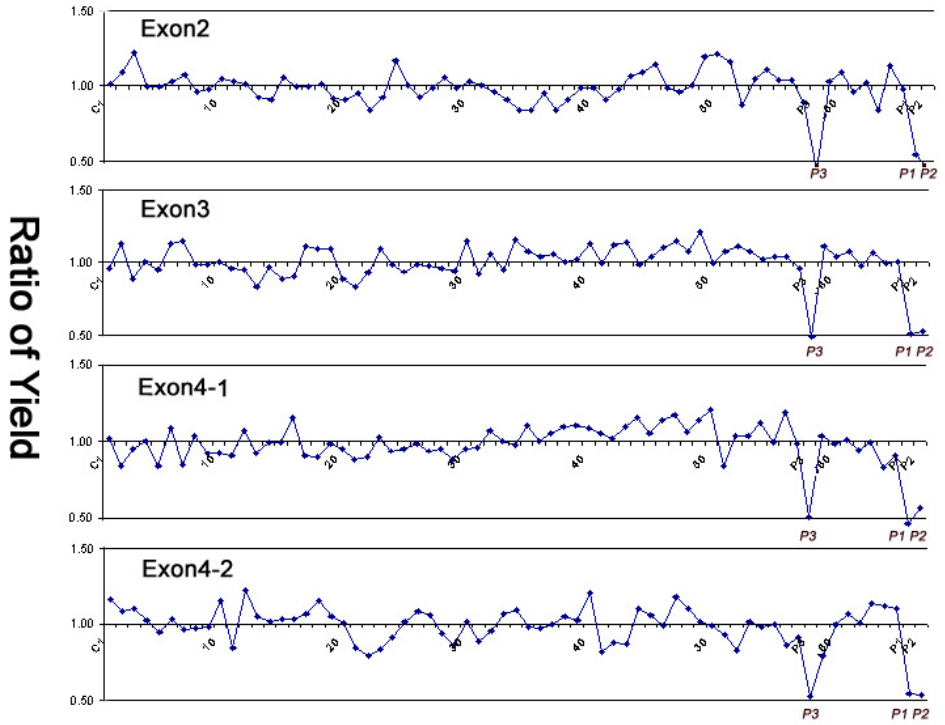


Figure 2. Analysis of copy number of the coding region of the *MECP2* gene. **A:** Blinded RD-PCR analysis for exon2. Lanes 1 to 10 are blinded normal control samples, where the gender is unknown. F is a normal female control, M is a normal male control, N is a negative control without DNA added. D is PhiX174 DNA/Hae III Markers, in which three bands of 603bp, 310bp, 281bp+217bp were shown. The ROY of each sample is indicated. **B:** ROY for four RD-PCR assays. Sixty-seven samples were tested for each assay. Y-axis is ROY, crossed with X-axis at 1.0. P1, P2, and P3 have much lower ROYs, indicating only one copy of the *MECP2* gene. P3 is the RTT patient with the deletion; P1 and P2 are male controls.

Characterization of the large deletion in the female patient P3

To localize the deletion junction, ten more RD-PCR assays were developed flanking the *MECP2* gene. The deletion junction was located within a region of 37.2kb upstream from 5' end of exon 1 and 18.1kb downstream from 3' end of exon 4 (Fig. 4). Long-distance PCR approaches were designed, but unfortunately failed, probably because the DNA was partially degraded.

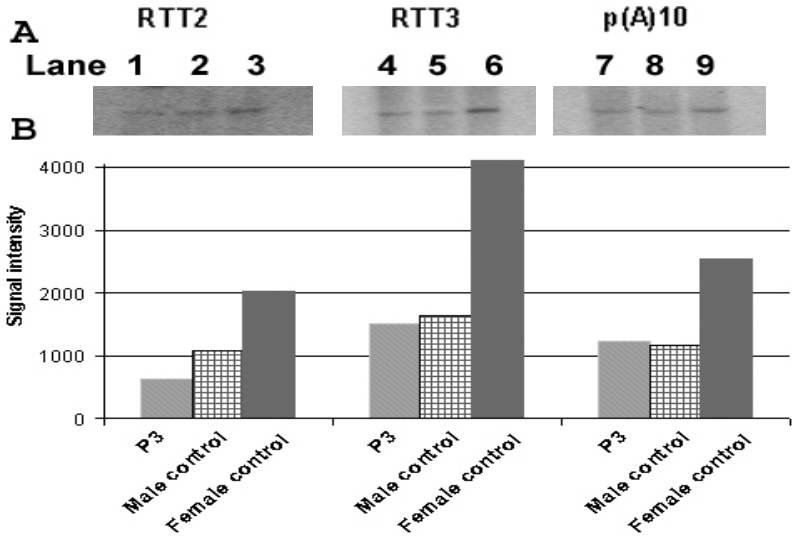


Figure 3. Southern blotting analysis. **A:** Images of southern blotting with probes RTT2, RTT3, and p(A)10. Lanes 1, 4, and 7 are patient P3; Lanes 2, 5, and 8 are male control; and lanes 3, 6, and 9 are female control. **B:** Quantitation of each individual. Signal intensity of patient P3 was similar to that of the male control, indicating only one copy of the *MECP2* gene in patient P3.

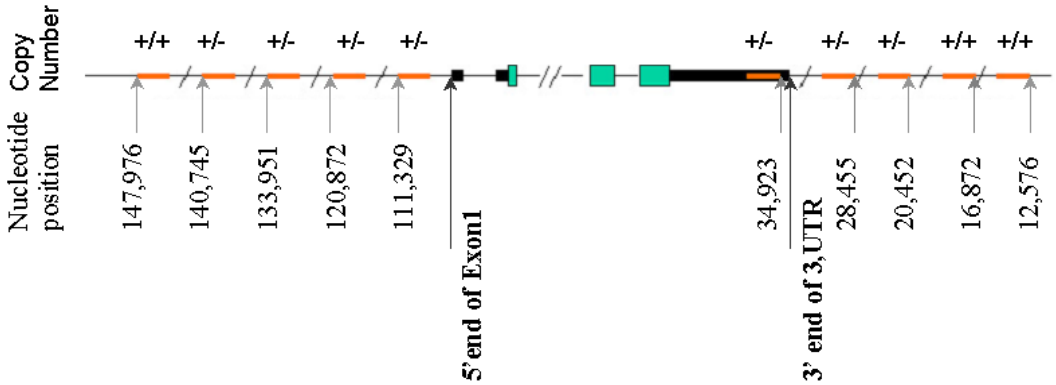


Figure 4. Localization of the deletion junction in the female patient P3. Ten RD-PCR assays were developed in the flanking region of the *MECP2* gene on X-chromosome. Primers were designed according to the genomic sequence from NT_025965 and the nucleotide positions are shown. +/+ indicates two gene copies at the test locus, while +/- indicates only one copy. The deletion junction was located within a region of 37kb upstream from 5' end of exon 1 and 18kb downstream from 3' end of exon 4 (3'UTR).

RD-PCR was used for detection of heterozygous deletions and duplications in the *MECP2* gene in RTT patients. One large deletion was identified in one of eight classical RTT patients without point mutations. The prevalence of *MECP2* gene heterozygous deletions detected by RD-PCR in our patients is 12.5% (1 out of 8), not significantly lower than the aggregate of the previous reports (12 out of 59) (Schollen et al., 2003; Erlandson et al., 2003; Ariani et al., 2004; Laccone et al., 2004). As illustrated by the ten additional dosage assays developed to characterize the deletion, rapid assay development and optimization are two important advantages of RD-PCR.

REFERENCES

Amir RE, Van dV, I, Wan M, Tran CQ, Francke U, Zoghbi HY. 1999. Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2. Nat Genet 23:185-188.

Ariani F, Mari F, Pescucci C, Longo I, Bruttini M, Meloni I, Hayek G, Rocchi R, Zappella M, Renieri A. 2004. Real-time quantitative PCR as a routine method for screening large rearrangements in Rett syndrome: Report of one case of MECP2 deletion and one case of MECP2 duplication. Hum Mutat 24:172-177.

Erlandson A, Samuelsson L, Hagberg B, Kyllerman M, Vujic M, Wahlstrom J. 2003. Multiplex ligation-dependent probe amplification (MLPA) detects large deletions in the MECP2 gene of Swedish Rett syndrome patients. Genet Test 7:329-332.

Hagberg B, Goutieres F, Hanefeld F, Rett A, Wilson J. 1985. Rett syndrome: criteria for inclusion and exclusion. Brain Dev 7:372-373.

Hagberg B, Hanefeld F, Percy A, Skjeldal O. 2002. An update on clinically applicable diagnostic criteria in Rett syndrome. Eur J Paediatr Neurol. 2002;6(5):293-7.

Laccone F, Junemann I, Whatley S, Morgan R, Butler R, Huppke P, Ravine D. 2004. Large deletions of the MECP2 gene detected by gene dosage analysis in patients with Rett syndrome. Hum Mutat 23:234-244.

Liu Q, Li X, Chen JS, Sommer SS. 2003. Robust dosage-PCR for detection of heterozygous chromosomal deletions. BioTechniques 34:558-6, 568.

Miltenberger-Miltenyi G, Laccone F. 2003. Mutations and polymorphisms in the human methyl CpG-binding protein MECP2. Hum Mutat 22:107-115.

Schollen E, Smeets E, Deflem E, Fryns JP, Matthijs G. 2003. Gross rearrangements in the MECP2 gene in three patients with rett syndrome: Implications for routine diagnosis of Rett syndrome. Hum Mutat 22:116-120.

Shi J, Nguyen V, Liu Q, and Sommer SS. 2004. Elimination of locus-specific inter-individual variation in quantitative PCR. BioTechniques 37:934-938

Nguyen V, Shi J, Liu Q, and Sommer SS. Robust Dosage (RD)-PCR protocol for the detection of heterozygous Deletions. BioTechniques 37: 360-364