MUTATION IN BRIEF

Large Genomic Rearrangements in *MECP2*

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In 1999, mutations in the X-linked gene methyl-CpG-binding protein 2 (*MECP2*) were first reported in patients with Rett syndrome (RTT). The *MECP2* gene is located at Xq28 and consists of 4 exons. About 80-90 % of the classic RTT patients harbor mutations in the coding region of *MECP2*, while the molecular cause is unknown in the remaining 10-20 %. Several groups have searched for large rearrangements within the *MECP2* and the results indicate that a fraction of *MECP2*-negative RTT cases has large deletions of the *MECP2*. In this study we have used the Multiplex Ligation-dependent Probe Amplification (MLPA) technique to screen 45 RTT patients, who have previously been tested negative for mutations in the coding region of *MECP2*. The *MECP2*-MLPA is a semi-quantitative multiplex PCR approach. It determines the relative number of copies of each *MECP2* exon. With this approach we detected seven RTT patients with genomic deletions and further characterized the deletions using real time quantitative PCR (qPCR) and long-range PCR. The seven patients were given a severity score and their X chromosome inactivation profiles were determined in order to identify a possible genotype-phenotype correlation. The results from this study indicate that large deletions in *MECP2* cause classic RTT. © 2005 Wiley-Liss, Inc.

KEY WORDS: Rett syndrome; RTT; MECP2; deletion; MLPA

INTRODUCTION

Rett syndrome (RTT; MIM# 312750) is an X-linked dominant neurodevelopmental disorder almost exclusively diagnosed in females. The prevalence is about 1 in 10,000-15,000 female births (Hagberg et al., 1983). Patients with classic RTT make some progress until 6-18 months of life. They then suffer a gradual reduction of acquired purposeful hand use and speech, accompanied by deceleration of head growth and temporary loss of contact, manual and gait dyspraxia, seizures and respiratory dysfunction. Repetitive, stereotyped hand movements appear during the neurological regression and are, in combination with the reduction of function, a hallmark of the disease. In 1999, mutations in the X-linked gene methyl-CpG-binding protein 2 (*MECP2*; MIM# 300005) were first reported in RTT patients (Amir et al., 1999). MeCP2 is an abundantly expressed protein acting as a global transcription repressor (Nan et al., 1997). *MECP2* is located at Xq28 and undergoes X chromosome inactivation

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(XCI) (D'Esposito et al., 1996). The 76 kb *MECP2* comprises of 4 exons. Two main features characterize the genomic structure: a large intron 2 (60 kb) and an 8.5 kb highly conserved 3´UTR (Reichwald et al., 2000).

About 80-90 % of the classic RTT patients harbor mutations in the coding region of *MECP2*, while the molecular cause is unknown in the remaining 10-20 % (Naidu et al., 2003; Neul and Zoghbi 2004).

Several groups have searched for large rearrangements within the *MECP2*. Among these, Schollen et al. have identified three patients with large deletions by Southern blotting (Schollen et al., 2003). Recently, Laccone et al. reported 15 patients with *MECP2* deletions using a qPCR strategy and Erlandson et al. detected three deletions in their patient group using the Multiplex Ligation-dependent Probe Amplification (MLPA) technique (Erlandson et al., 2003; Laccone et al., 2004).

In this study we have used the MLPA technique to screen 45 RTT patients. All patients have been tested negative for mutation in all four exons of *MECP*2. This includes exon 1, in which a mutation has been discovered recently (Mnatzakanian et al., 2004). The *MECP2*-MLPA is a semi-quantitative multiplex PCR approach. It determines the relative number of copies of each *MECP2* exon. With this approach we detected seven RTT patients with genomic deletions and further characterized the deletions using real time qPCR and long-range PCR. The seven patients were profiled according to the Kerr et al. recommendations and their X chromosome inactivation patterns were determined in order to identify a possible genotype-phenotype correlation (Kerr et al., 2001).

METHODS

Patients

Forty-five RTT patients were investigated with the MLPA approach.

Group 1A: 11 Danish RTT patients, (6 classic, 5 non-classic), who have been clinically evaluated by the author JBN.

Group 1B: 12 Norwegian RTT patients, (9 classic and 3 non-classic), clinically investigated by the author OS.

Group 1C: 5 English RTT patients, (4 classic and 1 non-classic), clinically investigated by the author AK.

Group 2: 17 patients referred by other clinicians to the Department of Clinical Genetics, University Hospital, Rigshospitalet, Copenhagen, Denmark. They have not been clinically examined for RTT by the authors, but a suspicion of the diagnosis RTT has been maintained.

DNA Analysis

The *MECP2* –MLPA test kit is developed and manufactured by MRC-Holland, Amsterdam, The Netherlands, and the analysis was carried out as described (Schouten et al., 2002). In short, 100 ng genomic DNA was denatured at 98°C and hybridized with *MECP2*-MLPA probe mix at 60°C overnight. Next, heat-stable Ligase-65 was added and ligation was performed at 54°C for 15 min. PCR primers, dNTP and polymerase were added and PCR was carried out for 33 cycles (30 s at 95°C, 30 s at 60°C and 60 s at 72°C). The probe amplification products were analyzed on an ABI model 310, using Genescan-ROX 500 standards and Genescan software. Alterations were suspected if a relative peak area of a probe target sequence deviated more than 30% from the female control.

In order to identify the extent of the deletions we undertook a real time PCR approach. We designed primers/amplicons with the use of Primer Express Software upstream and downstream of the exons in question. The amplicons were scattered with intervals of 10 kb and then with 5 kb and 2 kb. SYBR Green PCR master mix was used for the PCR according to the manufacturer's instructions. The numbers of MECP2 region copies were measured relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH; MIM# 138400) on an ABI Prism 7000 sequence detection system. Two normal individuals were included. Each assay was run in duplicate and evaluated with a comparative method validated by Applied Biosystems with the formula $2^{-\Delta\Delta Ct}$. By applying the *GAPDH* Ct values, we ensured that nearly exact amounts of reference and patient DNA were compared. When a deletion was defined, the upstream and downstream PCR primers from the amplicons flanking the deletion were used for long range PCR (Eppendorf). The PCR products were sequenced on an ABI model 310. (All primer sequences were designed from clones AF030876 and NP_002037. They are available on request.)

XCI was assessed in peripheral leukocytes using the androgen receptor gene as target (Allen et al., 1992). A skewed XCI was defined as a pattern with > 80 % of the inactivated chromosomes being either maternal or paternal.

RESULTS

In group 1A we detected 4 classic patients with deletion of both exons 3 and 4, in group 1B one classic patient with a deletion of exons 1 and 2, and in group 1C one classic patient with a deletion covering exons 3 and 4 and one classic patient harboring a deletion encompassing part of exon 4 (Fig. 1 and Table 1). In group 2 no evidence for large rearrangements was found.

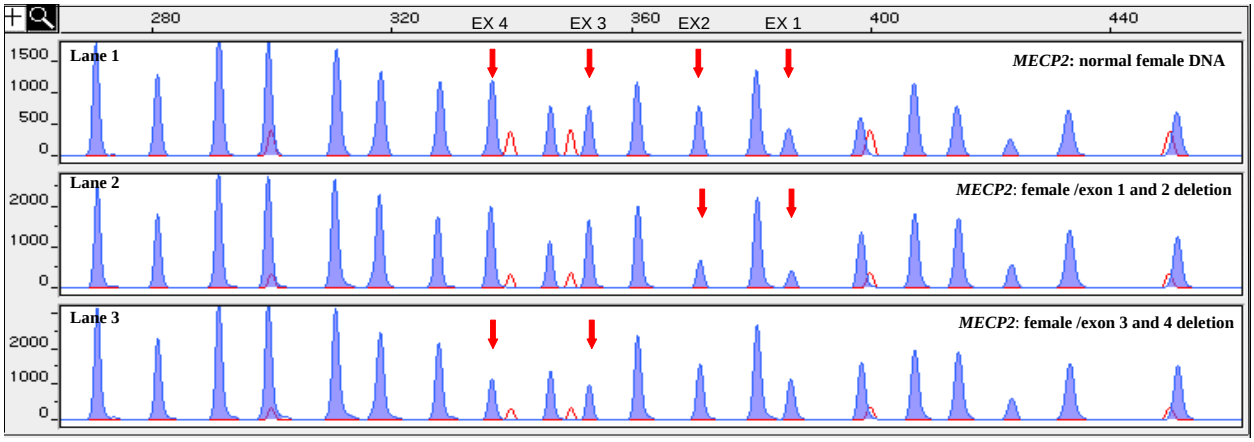

Figure 1. Detection of *MECP2* exon alterations by MLPA. Probe mix P015 contains 24 probes, 4 probes recognize the *MECP2* exons, indicated by arrows in lane 1. Lanes 2 and 3 illustrate the presumed deletions as the area of the peaks from patient DNA is approximately half that of the controls. A detailed description of the P015 probe mix can be found on www.mrc-holland.com.

Table 1: Peak Area of Each Probe Signal Was Divided by the Total Peak Area of All 24 Probe Signals

MECP2 Probe	Probe Size (nt.)	Female controls N = 25 Relative Area	Patient Id 266 Relative Area fraction	Patient Id 33 Relative Area fraction	Patient Id 56 Relative Area fraction	Patient Id 66 Relative Area fraction	Patient Id 80 Relative Area fraction	Patient Id 308 Relative Area fraction	Patient Id 306 Relative Area fraction
Exon 1	337	0.94 ± 0.10	**0.58**	1.24	0.99	0.99	0.86	1.25	1.25
Exon 2	355	1.04 ± 0.07	**0.52**	1.06	0.97	0.98	1.08	1.05	1.00
Exon 3	373	1.01 ± 0.06	1.06	**0.62**	**0.52**	**0.52**	**0.53**	**0.56**	1.06
Exon 4	391	1.03 ± 0.06	1.18	**0.53**	**0.62**	**0.62**	**0.55**	**0.53**	**0.56**

The resulting relative peak area of each probe signal was divided by the relative peak area obtained by a female DNA control. The presence of two copies of a probe target sequence should therefore result in a relative area equal to 1.00, whereas the presence of 1 copy probe target sequence should result in a 0.50 relative area.

The seven patients with deletions have been clinically evaluated according to the criteria of Kerr et al. (2001). The severity score with regard to muscle tone, scoliosis, epilepsy, feeding difficulty and ability to walk alone varied from 4 to 10 in two 6-year-old girls to 6 in a 28-year-old woman (Table 2).

Using a real time PCR approach we were able to locate the seven deletions within a region of 4 kb. In three cases the exact extent of the deletions was determined by amplification and sequencing of the junction fragments.

We identified a 19785 bp deletion in patient Id 33 (c.27-12530_6533del). The deletion begins 12521 bp upstream from exon 3 and spans half of *MECP2* 3´-UTR in exon 4. Sequence analysis of patient Id 66 revealed a

50531 bp deletion and an insertion of 14 bp (c.26+25171_10015+5324delins14). The deletion overlaps 34464 bp of intron 2, continues through exons 3 and 4 and ends 3387 bp into the Interleukin-1 receptor-associated kinase 1 gene (*IRAK1*; MIN # 300283). Neither of the patient's parents harbor the insert. In patient Id 306 we determined the 5´ breakpoint within the DPR (Deletion Prone Region) in exon 4 (Laccone et al., 2004). The deletion (c.1032_10015+5919del) comprises 14913 bp, locating the 3´breakpoint 3979 bp into *IRAK1*. Several attempts were made to determine the exact breakpoints in the last 4 cases. We were able to narrow the location of the deletions down to within 4 kb ranges. Several PCR approaches failed. The findings are summarized in Fig. 2 and Table 2.

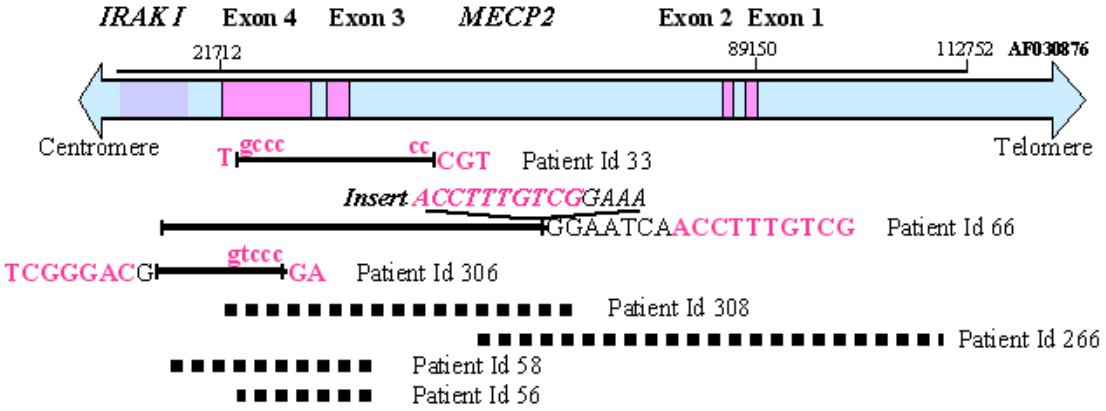

Figure 2. Schematic representation of the location of the deletions and the wild type sequences that could be the causes to rearrangements.

Table 2: Characterization of Seven Genomic Deletions in *MECP2*

Patient Id	Exons del	Size of del	Breakpoint position of del	Clinical severity score	Age of scoring, yr	Actual age, yr	XCI
33	3-4	19785 bp	Breakpoint positions c.27-12530_6533del	8	18	34	Random
66	3-4	50531 bp	Breakpoint positions c.26+25171_10015+5324delins14	4	6	6	Random
306	4	14913 bp	Breakpoint positions c.1032_10015+5919del	10	27	Dead	Random
266	1-2	Estimated 80 kb	c.1-?_26+?	6	28	28	10:90
308	3-4	Estimated 55.7 kb	c.27-?_10015+?	10	13	14	Random
58	3-4	Estimated 32.5 kb	c.27-?_10015+?	10	6	7	Random
56	3-4	Estimated 15 kb	c.27-?_10015-?	8	14	14	Random

The nomenclature of the mutations relates to the mRNA sequence XM_048395.1. Position 1 corresponds to the A of the initiation codon ATG in exon 2. The genomic sequence of the intronic breakpoints is the AF030876. This genomic clone is oriented from centromere to telomere direction. The seven patients were clinically evaluated according to guidelines of Kerr et al.(Kerr et al., 2001), by the authors JBN, OS, and AK.

DISCUSSION

Using the MLPA approach we detected 7 patients with large deletions in the *MECP2* gene. All the deletions were found in the subgroup of 19 patients with classic RTT among the 28 well-characterized patients belonging to groups 1A-C examined by one of the authors. The detection rate among the classic patients who screened negative with the conventional mutation detection methods was 37 % (7/19). In group 2 clinical investigation of the patients by the authors was not possible and we did not find any deletions or duplications that could confirm the RTT diagnosis. Re-evaluation of the diagnoses of the patients in group 2 is being considered

Our results from the real time PCR approach revealed the seven deletions, which span from 15 kb to approximately 80 kb. Together the deletions cover the whole *MECP2* and in 3 cases also a part of the *IRAK1* gene, at least the first 7 exons. These three patients do not display additional clinical features.

Analysis of the sequences surrounding the breakpoints in patient Id 33 indicates that the rearrangement might be mediated by homologous recombination. A sequence of five nucleotides (TGCCC) covering the 5´breakpoint is identical but oppositely oriented to the wild type sequence overlapping the 3´breakpoint. In patient Id 66, 10 bp of the 14 bp insertion (ACCTTTGTCG) is present in the wild type sequence close to the 5´breakpoint. Kutsche et al. have presented a possible molecular mechanism that would lead to this type of mutation (Kutsche et al., 2002). In patient Id 306 the 5´ breakpoint was located in the DPR in exon 4. The wild type sequence that covers the patient's 5´ breakpoint in the DPR, is an inverted repeat 1 bp following the patient's 3´breakpoint. Laccone et al. have reported 8/11 cases and Schollen et al. reported 2/3 cases with a breakpoint in the DPR (Schollen et al., 2003; Laccone et al., 2004). Our investigation showed only 1/7 cases with a breakpoint located in the DPR region.

In the last four cases we were only able to estimate the deletions within a 4 kb range. Different attempts were made, but due to the high degree of sequence homology and the fact that the template was diploid DNA, PCR products from the mutated alleles could not be generated.

An important issue is whether a genotype-phenotype correlation can be established for the large deletions in *MECP2*. We anticipate that no *MECP2* mRNA would be generated from the mutated allele carrying the deletions spanning exons 1 and 2. Furthermore, no functional MeCP2 protein, without its methyl-CpG binding domain (MBD) and the transcriptional repression domain (TRD), would be expected from the alleles harboring the exons 3 and 4 deletions. All seven patients were diagnosed with classic RTT. Patient Id 266 with a deletion of exons 1 and 2 was the only one that showed a skewed XCI profile. The range of the clinical severity score with regard to muscle tone, scoliosis, epilepsy, feeding difficulty and ability to walk alone for the seven patients is 4 to 10, which is the maximal score for severity. From these results we cannot establish any specific correlation between genotype and phenotype. The phenotype cannot be judged alone by the scoring figure, but this can give an idea of how severely the patients are affected.

In all previously reported cases with sufficient clinical information, patients with large deletions in *MECP2* meet the criteria for classic RTT (Bourdon et al., 2001; Yaron et al., 2002; Erlandson et al., 2003; Schollen et al., 2003; Laccone et al., 2004). It is appealing to speculate that these severe mutations cause classic RTT and tissue specific patterns of XCI account for the detailed phenotype seen among these classic RTT patients. However, a larger study of clinically well-characterized patients, which also includes atypical RTT cases, is required to test this hypothesis.

Routine DNA testing of *MECP2* mutations mainly involves DHPLC, DGGE or direct sequence analysis of the coding region of *MECP2* and immediate flanking intronic regions. These methods do not detect larger genomic rearrangements, which could be present in a significant proportion of RTT patients. Especially for patients with classic RTT where sequencing fails to demonstrate a mutation a search for large genomic rearrangements seems promising. The MLPA approach is a simple and fast technique that easily reveals such rearrangements and could straightforwardly be implemented in the routine molecular screening protocol.

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