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Multiplex Ligation-Dependent Probe Amplification (MLPA) Detects Large Deletions in the *MECP2* Gene of Swedish Rett Syndrome Patients

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ABSTRACT

Mutations in the methyl-CpG-binding protein-2 (*MECP2*) gene on Xq28 have been found to be a cause of Rett syndrome (RS). In a previous mutation screening, we found *MECP2* mutations in 81% of Swedish classical Rett women. In this study, we have analyzed 22 patients for *MECP2* deletions using multiplex-ligation-dependent probe amplification (MLPA). Clinically, 11 of the patients who were classical Rett women, 3 were *forme fruste*, 1 was congenital RS, and 7 were Rett variants. As inclusion criteria, we used DNA from patients in whom previous sequencing results showed no mutations in coding portions of the *MECP2* gene. MLPA is a method based on multiplex PCR. In one PCR, as many as 40 probes are amplified with the same primers. The specificity of the amplification products is determined by the site-specific hybridization of each probe construct, prior to amplification. Each PCR product has a unique length, which makes it possible to identify it by size separation. In 3 of 11 (27%) classical Rett women, we detected large deletions in *MECP2* using MLPA. All these patients had deletions covering two exons; in 2 cases the deletion involved exons 3 and 4 and, in one case, exons 1 and 2 were missing. In the *forme fruste*, congenital and Rett-variant patients, we found no large deletions. We have found that MLPA is useful when it comes to finding large deletions compromising whole exons in *MECP2*. Used as a complementary method to DNA sequencing, it revealed new *MECP2* mutations in classical RS patients.

INTRODUCTION

RETT SYNDROME (RS; MIM #312750) is a neurodevelopmental disease that almost exclusively affects females. It is characterized by normal development for the first 6–18 months, followed by developmental regression with the loss of acquired skills. The patients first lose purposeful hand movements and an interest in the surrounding world, along with speech. They develop apraxia with characteristic stereotypical hand-wringing movements that resemble hand washing, autistic behaviour, and learning disabilities. With increasing age, they can also develop additional complex neurologic findings. The prevalence of the disease is at least 1:10,000 in western Sweden (Hagberg and Hagberg, 1987). RS is caused by mutations in the *MECP2* gene (methyl CpG binding protein 2-gene) located on Xq28 (Amir *et al*., 1999). The gene product MeCP2 binds to single methylated CpG, deacetylases, and cofactor Sin3A and, thereby, contributes to the repression of other genes (Nan *et al*., 1993).

So far, a wide variety of mutation types have been reported. Missense mutations are the most frequently observed, but nonsense mutations and small deletions/insertions are also common (Wan *et al*., 1999; Cheadle *et al*., 2000; Huppke *et al*., 2000; Hoffbuhr *et al*., 2001; Weaving *et al*., 2003). This is consistent with our results from a mutation screening of *MECP2* in Swedish classical RS females (Erlandson *et al*., 2001).

Multiplex ligation-dependent probe amplification (MLPA) is a mutation screening method based on multiplex PCR (Schouten *et al*., 2002). The specificity of the amplification products is determined by the site-specific hybridization of each probe construct. The PCR is conducted with as many as 40 different amplicons, all amplified in one reaction, with the same PCR primers. The amplification products each have a unique length, which makes it possible to identify them by size sepa-

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ration. In the analysis, the signal strengths from the probes are represented by corresponding peak areas. Peak areas are compared with those of a control individual (after normalization) one by one. We used an Excel file to calculate the results. Because of the control individual and control probes in each run, and the normalization calculations, we were able to compare the results from each probe quantitatively.

Until recently, no suitable screening method for detecting whole-exon deletions was available. Fluorescence *in situ* hybridization (FISH) has been used for this purpose, with limited success (Bourdon *et al*., 2001; Nielsen *et al*., 2001). We have found that MLPA (Schouten *et al*., 2002) is useful when it comes to finding large deletions comprising whole exons of *MECP2*. Used as a complement to DNA sequencing, it makes it possible to define a substantial number of *MECP2* mutations in RS patients.

MATERIALS AND METHODS

Patients

In all, we screened 22 patients for *MECP2* deletions with MLPA. The inclusion criteria were previous DNA sequencing results without mutations in the *MECP2* gene detected. Clinically, 11 of the patients were classical Rett women, 3 were mild and borderline (*forme fruste*), one was congenital RS, and 7 were Rett variants (atypical).

DNA preparation

Genomic DNA was extracted from fibroblasts or blood samples anticoagulated with EDTA, using phenol extraction or DNA Isolation kit (PUREGENE, Gentra systems).

PCR conditions

The PCR prior to DNA sequencing of the *MECP2* gene was carried out as described previously (Erlandson *et al*., 2001). For some of the samples, we used five amplicons for PCR and DNA sequencing of the coding parts of the *MECP2* gene (primer sequences are available upon request). In these cases, we had one amplicon for exons 2 and 3 and, for exon 4, we used three overlapping amplicons.

DNA sequencing

The coding exons of *MECP2* were sequenced on an ABI 3100, ABI 377, or ABI 310 (Applied Biosystems) automatic sequencer. The PCR fragments were purified using an ExoSAP-IT (USB) or QIAquick PCR Purification Kit (QIAGEN) and sequenced in the forward and reverse directions using Big-Dye Terminator chemistry (Perkin-Elmer).

MLPA

We used probe mix P015 (MRC Holland) for the MLPA, which was performed in thermal cyclers with heated lids (PTC-100 (Multi) or Gene Amp PCR System 2700 (ABI)). P015 included 21 probes (Table 1 and Fig. 1), 10 of which were derived from the X chromosome (six specific for testing *MECP2*) and the remaining 11 from other chromosomes in the genome.

Table 1. Excel Analysis Results from Patient 3

Probe name[a]	*Chromosome*	*Type*	*Ratio (case/ctrl)*[b]
Y	Y	Control	1.00
8q	8	Control	1.11
Xq28	X	Control	1.13
6q22	6	Control	1.23
Xq22	X	Control	1.10
12	12	Control	1.12
LICAM[c]	X	Specific	1.00
6p21	6	Control	0.87
MeCP2 ex4	X	Specific	***0.52***
18q21	18	Control	1.00
MeCP2 ex3	X	Specific	***0.53***
20q13	20	Control	1.15
MeCP2ex2	X	Specific	1.02
5q31	5	Control	1.01
MeCP2ex1	X	Specific	1.03
15q25	15	Control	1.07
Xq25	X	Control	1.07
17q12	17	Control	0.95
Xq27.3	X	Control	0.94
17p13	17	Control	1.00
SYBL1	X	Specific	0.94

Exons 3 and 4 are deleted (marked in bold italic) and the ratios were approximately half of the expected.

[a]Probe names to the left are taken from the P015 kit (MRC Holland), and correspond to the location of the hybridization site.

[b]The expected ratio was 1.00, but there was some normal variation, which was seen in, for example, probe 6p21.

[c]Shaded rows indicate *MECP2*-specific probes.

A total of 100 ng of genomic DNA for each sample was diluted with TE buffer to 5 μl and denatured at 98°C for 5 min. SALSA probe mix (1.5 μl) and 1.5 μl of MLPA buffer for each sample were added. After incubation at 95°C for 1 min, the probes were hybridized for 16–20 hr at 60°C. The temperature was then lowered to 54°C, and 32 μl of ligase-65 mix was added to each sample. The probes were ligated for 15 min of incubation at 54°C and the ligase was deactivated by heat (98°C for 5 min). PCR reaction was performed in a 50-μl volume: 10 μl of ligase reaction was pipetted onto another 96-well plate and each sample was mixed with 4 μl of 10× SALSA PCR buffer and 26 μl of dH_2O. The plate was then heated to 60°C and 10 μl of polymerase mix (with SALSA FAM PCR primer-dNTP mix) was added. PCR conditions were 95°C for 30 sec, 60°C for 30 sec, 72°C for 60 sec, and repeated in another 32 cycles. Extension was carried out at 72°C for 20 min. Next, 1 μl of PCR product was mixed with 9.4 μl of formic acid and 0.6 μl of ROX labeled internal size standard (ROX-500, ABI). Genotyping was performed on an ABI-3100 automated sequencer: Polymer POP-6 in 36-cm capillaries, run temperature 60°C, and cap fill volume 184 steps. For the prerun, the voltage was 15 kV for 180 sec; the injection voltage was 3.0 kV for 10 sec for the run. The run voltage was 15 kV for 1,500 sec. The data delay time was 1 sec.

Aberrant results (±30% of expected normal value in data analysis) were independently rerun in a second MLPA reaction for confirmation.

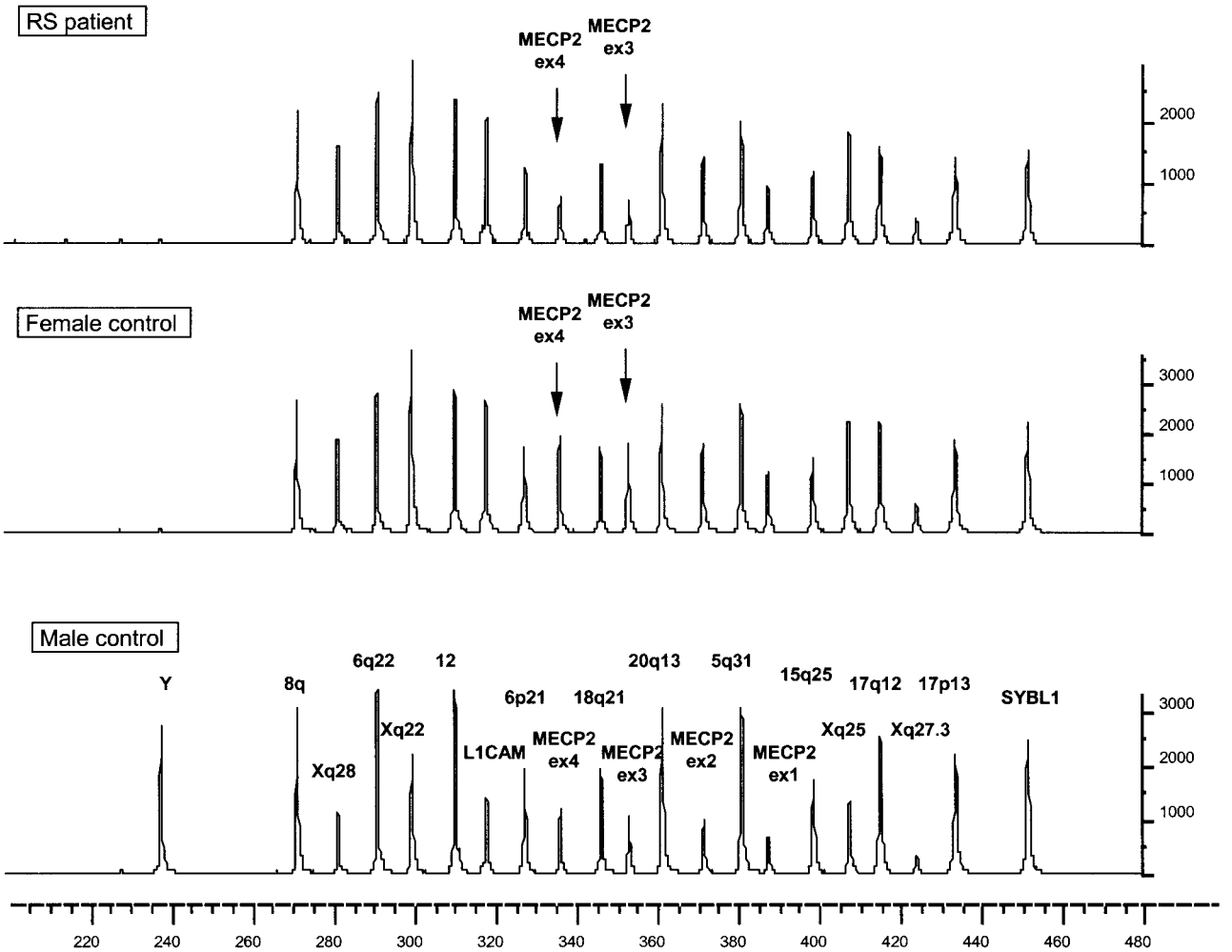

FIG. 1. Fragment analysis of the P015 kit with 21 probes. This patient (no. 3) was deleted over exons 3 and 4 (indicated by arrows). The probes differed slightly in signal strength, and the length of the probes had a negative effect on the amplitudes. One of the probes was derived from the Y chromosome and was therefore only detected in the male control. The variations in the amplitudes in the male control were due to probes located in the X chromosome. If a male was used as a control, we had to consider the possibility that the expected ratio (patient: control) for probes in the X chromosome (apart from the pseudo-autosomal region) was 2.00.

Data analysis

Analyses were performed with Genescan 3.7 (Fig. 1). Results from Genescan were copied to Excel files (Windows), where the final results were calculated (Table 1). We divided each peak area by the sum of all the peak areas for that sample. Each individual probe-relative area was then compared with the corresponding probe-relative area obtained by a control sample, thus creating a ratio (case/control) for the final results (Schouten *et al*., 2002).

RESULTS

MLPA results

We detected large deletions in *MECP2* using MLPA in 3 of 11 (27%) classical RS women, who were previously DNA sequenced (across the coding parts of the gene) with normal results. One of the atypical patients had a missense mutation E397K, previously described as a normal variant (Amir and Zoghbi, 2000). All of the patients with deletions had two exons missing; one case with deletion of exons 1 and 2, and 2 cases with deletion of exons 3 and 4 (Table 2). In this small series, the *forme fruste* RS, congenital RS, or other Rett-variant patients did not display any large deletions in *MECP2*.

TABLE 2. THE PATIENTS WITH DELETIONS DETECTED BY MLPA

Patient number	*MLPA result*	*Diagnosis*
1	Exon 1 and 2 deleted	Classical Rett
2	Exon 3 and 4 deleted	Classical Rett
3	Exon 3 and 4 deleted	Classical Rett

The ratios (case/control) of the deleted exons were approximately half the values of the control peaks, thereby, indicating the loss of one allele. All of the patients with positive or aberrant results were rerun in a second MLPA reaction for confirmation.

DISCUSSION

Previous reports state that approximately 60–80% of classical RS women have mutations in the *MECP2* gene which are detectable by DNA sequencing (Huppke *et al.*, 2000; Hoffbuhr *et al.*, 2001; Weaving *et al.*, 2003). This leaves about 20–40% of the patients without any molecular explanation for the diagnosis. In our study, MLPA detected large deletions in 27% of the sequence-negative group. This supports the theory that RS is a monogenic disease. We have found that MLPA, as a complement to DNA sequencing, is a useful tool for the detection of whole-exon deletions.

The 3 patients in which *MECP2* deletions were found each had two exons missing; one case with the deletion of exons 1 and 2, and 2 cases without exons 3 and 4 (Table 2). Clinically, the 2 patients with the deletion of exons 3 and 4 were at different ends of the spectrum of classical RS phenotypes. Patient 3 was one of the most severely affected patients in the Swedish RS registry, in contrast to patient 2, who had a very mild RS profile. In spite of this, both patients had the same exons missing. To account for this, we speculate that tissue specific levels of X-chromosome inactivation (XCI) might play a role in modifying the phenotypic presentation of these patients.

No deletions were found in our small series of patients with nonclassical forms of RS—atypical ($n = 7$), *forme fruste* ($n = 3$), or congenital RS patients ($n = 1$)—but we cannot exclude the possibility that whole-exon deletions might have been detected if a larger number of patients with these phenotypes had been analyzed.

The MLPA P015 kit comprises six specific probes for *MECP2*: one for each exon, one probe for the *L1CAM* (L1 cell adhesion molecule) gene upstream, and one probe for the *SYBL1* (synaptobrevin-like 1) gene downstream of *MECP2*. As a result, the analysis provides information about deletions including the flanking chromosomal regions. In none of the deletions we identified were sequences from *L1CAM* or *SYBL1* included.

With MLPA, there is a small risk of false positives as a result of small intragenic deletions (for example, the probe site itself could be deleted with the rest of the exon intact) or missense mutations at the 3′ end of the probe hybridization sites. Because DNA sequencing analysis was used prior to MLPA, we were able to avoid these potential sources of errors. Furthermore, we found that the probes differed in signal strength and that the amplitude, therefore, also varied (Fig. 1). We felt it was important to run positive controls (for all samples) in independent duplicate reactions.

In conclusion, in 3 classical RS patients, previously classified as *MECP2* mutation negative, we were able to identify large deletions in *MECP2* with MLPA. Therefore, it is likely that this method will prove useful in identifying deletions in previously analyzed RS patients, who were labeled as mutation-negative after DNA sequencing. One might start with *MECP2* mutation-negative familial RS, because at least one report notes that DNA sequencing identifies fewer *MECP2* mutations in familial cases of classical RS than in sporadic RS cases (Xiang *et al.*, 2000).

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