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ORIGINAL ARTICLE

Mutation analysis of the *MECP2* gene in patients of Slavic origin with Rett syndrome: novel mutations and polymorphisms

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Abstract Rett syndrome (RTT), an X-linked dominant neurodevelopmental disorder in females, is caused mainly by de novo mutations in the methyl-CpG-binding protein 2 gene (*MECP2*). Here we report mutation analysis of the *MECP2* gene in 87 patients with RTT from the Czech and Slovak Republics, and Ukraine. The patients, all girls, with classical RTT were investigated for mutations using bi-directional DNA sequencing and conformation sensitive gel electrophoresis analysis of the coding sequence and exon/intron boundaries of the *MECP2* gene. Restriction fragment length polymorphism analysis was performed to confirm the mutations that cause the creation or abolition of the restriction site. Mutation-negative cases were subsequently examined by multiple ligation-dependent probe amplification (MLPA) to identify large deletions. Mutation screening revealed 31 different mutations in 68 patients and 12 non-pathogenic polymorphisms. Six mutations have not been previously published: two point mutations (323T>A, 904C>T), three deletions (189_190delGA, 816_832del17, 1069delAGC) and one deletion/inversion (1063_1236del174;1189_1231inv43). MLPA analysis revealed large deletions in two patients. The detection rate was 78.16%. Our results confirm the high frequency of *MECP2* mutations in females with RTT and provide data concerning the mutation heterogeneity in the Slavic population.

Keywords Rett syndrome · Mental retardation · *MECP2* gene · Mutation screening · MLPA

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Introduction

Rett syndrome (RTT; OMIM 312750), first described by the Austrian pediatrician Rett (1966), is one of the leading causes of mental retardation and developmental regression in girls. Its prevalence is estimated to be from 1:10,000 to 1:15,000 females worldwide with most cases being sporadic (Hagberg 1995). Patients with classical RTT show an apparently normal psychomotor development during the first 6–18 months of life. Thereafter, they enter a short period of developmental

stagnation followed by a rapid regression in language and motor development. Purposeful hand use is often lost and replaced by repetitive, stereotypic movements. Additional symptoms include acquired microcephaly, gait ataxia/apraxia, seizures, and episodic apnea and/or hyperpnea (Hagberg et al. 1983; Naidu 1997). The causative gene was mapped to Xq28 and identified as the *MECP2* gene (Amir et al. 1999). It encodes the methyl-CpG-binding protein 2 (MeCP2; OMIM 300005), which is involved in regulation oftranscription repression, epigenetic gene silencing (Amir et al. 1999; Van den Veyver and Zoghbi 2000), and RNA splicing (Young et al. 2004). Several different *MECP2* mutations have been identified in up to 90% of classical RTT patients worldwide. The mutation rate in atypical cases is much lower: 20–40% (Hoffbuhr et al. 2002; Laccone et al. 2004; Amir et al. 2005). We present a mutation analysis of the *MECP2* gene in RTT patients from the Czech Republic, Slovakia, and Ukraine. We tested 87 patients with classical RTT to ascertain the spectrum of disease-causing mutations of *MECP2* gene in the Slavic population.

Subjects and methods

Patients

Most of 87 unrelated sporadic female patients with a clinical diagnosis of RTT were diagnosed in neurological, genetic, or pediatric departments. Several patients were identified from Institutes of social work for mentally retarded children. All patients included in this study were diagnosed as having classical RTT, with psychomotor regression after a period of normal development, severe mental retardation, postnatal deceleration of head growth, loss of speech and purposeful hand use, and the appearance of stereotypic hand movements. The diagnosis was made according to diagnostic criteria defined by the Rett syndrome diagnostic criteria work group (Kerr et al. 2001).

Molecular analyses

Genomic DNA was extracted from peripheral blood samples anticoagulated with EDTA according to a standard protocol. Coding sequences of exons 1, 2, 3, and 4 with flanking exon/intron boundaries were amplified using following primer pairs: 1Fw 5′-tcaatc gcccctcagagca-3′, 1Rev 5′-cacgtcccgcccctgaccc-3′; 2Fw 5′-aaaaaggtcgtgcagctcaa-3′, 2Rev 5′-ggccaaaccaggaca tatac-3′; 3Fw 5′-tggcatgttctctgtgatactt-3′, 3Rev 5′-cctggg cacatacattttcct-3′; 4aFw 5′-tttgtcagagcgttgtcacc-3′, 4aRev 5′-ctgcacagatcggatagaagac-3′; 4bFw 5′-ggcaggaa gcgaaaagctgag-3′, 4bRev 5′-ctccctcccctcggtgtttg-3′, 4cFw 5′-ggagaagatgcccagaggag-3′, 4cRev 5′-gcactgatggcacc gaaaac-3′. The PCR amplification of exon 1 was carried out as published elsewhere (Evans et al. 2005), exons 2, 3, and 4 were amplified in a total volume of 25 µl including 1x Plain PP Master Mix [150 mM Tris–HCl, pH 8.8, 40 mM $(NH_4)_2SO_4$, 0.02% Tween 20, 5 mM $MgCl_2$, 400 µM dATP, 400 µM dCTP, 400 µM dGTP, 400 µM dTTP, 100 U/ml *Taq* DNA polymerase; Top-Bio, Prague, Czech Republic] and 0.4 mM of each primer. Thermal cycling conditions included an initial denaturation at 94°C for 2 min followed by 33 cycles of denaturation at 94°C for 30 s, annealing at 63°C for 30 s, and elongation at 72°C for 45 s, followed by final extension at 72°C for 10 min.

PCR products were purified from agarose gels using a QIAquick gel extraction kit (Qiagen, Hilden, Germany). Exon 2 was analysed by conformation sensitive gel electrophoresis (CSGE) as described (Bourdon et al. 2001). Exons 1, 3, and 4 were sequenced in both directions on automatic sequencer ABI PRISM 3100/3100-Avant Genetic analyzer (Applied Biosystems, Foster City, CA) using the ABI PRISM BigDye terminator v3.1 (Applied Biosystems). Whenever the identified mutation caused creation or loss of a restriction site, it was confirmed by restriction fragment length polymorphism (RFLP) analysis. To confirm del/inv mutations, the PCR fragment with the mutation was cloned into pCR4-TOPO vector (TOPO TA Cloning Kit for Sequencing, Invitrogen, Carlsbad, CA). Plasmid DNA was amplified in Top10 chemically competent *Escherichia coli* cells (Invitrogen), isolated using the QIAprep Spin Miniprep Kit (Qiagen), and sequenced. We used probe mix P015 (MRC Holland, Amsterdam, The Netherlands) for MLPA in all mutation-negative patients. MLPA was performed according to manufacturer's protocol (see Supplementary material). Genotyping was performed on an ABI PRISM 3100/3100-Avant Genetic analyzer (Applied Biosystems). Aberrant results were independently re-run in the second MLPA reaction for confirmation.

Ethics

The study was carried out in accordance with the Declaration of Helsinki of the World Medical Association and was approved by the Committee of Medical Ethics at the University Hospital. Informed consent was obtained from parents of all patients.

Results

The molecular analysis revealed 31 different mutations in 68 patients (78.16%), which is consistent with estimates reported elsewhere. Of the 68 patients with mutations, 34 had a missense mutation, 18 had a nonsense mutation, 13 carried a frame-shift mutation, 1 had an in frame deletion, and 2 had large-scale deletions detected by MLPA (Table 1). Three mutations were located in exon 3, the rest in exon 4. No sequence change was identified in exons 1 and 2. We identified six novel mutations (Fig. 1): two point mutations (323T>A, 904C>T), three deletions (189_190delGA, 816_832del17, 1069delAGC), and one deletion/inversion (1063_1236del174;1189_1231inv43).

We screened the parents and sisters of our patients for the mutations found in their daughters and sisters if their blood samples were available, but found none. The mutations were thus either de novo or due to germ-line mosaicism in the parents. Further sequencing analysis did not reveal any of newly described mutations in 200 normal chromosomes tested. Twelve non-pathogenic polymorphisms were identified in 14 patients, 4 of which are novel (Table 2).

Discussion

MECP2 is an X-linked gene encoding two almost identical isoforms of MeCP2 with alternative N-termini

Table 1 Mutations in the methyl-CpG-binding protein 2 gene (*MECP2*) detected in reported patients with Rett syndrome (RTT). *MBD* Methyl-CpG-binding domain, *TRD* transcription repression domain, *C-ter* C-terminal region, *MLPA* multiple ligation-dependent probe amplification

Exon	Nucleotide change[a]	Amino acid change	Domain of MeCP2	Recurrence (this study)	Reference
3	189_190delGA	E63fsX27	–	1	This study
3	316C>T	R106W	MBD	5 (4[b])	Amir et al. 1999
3	323T>A	L108H	MBD	1	This study
4	397C>T	R133C	MBD	6 (1[b])	Amir et al. 1999
4	401C>G	S134C	MBD	1	Cheadle et al. 2000
4	403A>G	K135E	MBD	1	Laccone et al. 2001
4	423C>G	Y141X	MBD	1 (1[b], 1[c])	De Bona et al. 2000
4	430A>T	K144X	MBD	1	Buyse et al. 2000
4	455C>G	P152R	MBD	1	Cheadle et al. 2000
4	473C>T	T158M	MBD	11 (10[b], 1[c])	Amir et al. 1999
4	502C>T	R168X	–	3 (4[b], 1[c])	Wan et al. 1999
4	611C>G	S204X	–	1 (1[b], 1[c])	Buyse et al. 2000
4	674C>G	P225R	TRD	1 (1[c])	Cheadle et al. 2000
4	750_751insC	G252fsX7	TRD	1	Zeev et al. 2002
4	763C>T	R255X	TRD	5	Amir et al. 1999
4	806delG	G269fsX20	TRD	2	Wan et al. 1999
4	808C>T	R270X	TRD	3 (1[b])	Cheadle et al. 2000
4	816_832del17	G273fsX52	TRD	1	This study
4	856_859del4	K286fsX2	TRD	1	Hoffbuhr et al. 2001
4	880C>T	R294X	TRD	4 (1[b], 1[c])	Cheadle et al. 2000
4	904C>T	P302S	TRD	1	This study
4	916C>T	R306C	TRD	5 (2[b], 1[c])	Wan et al. 1999
4	1063_1236del174;1189_1231inv43	S355fsX37	C-ter	1 (1[b], 1[c])	This study
4	1069delAGC	ΔS357	C-ter	1 (1[b])	This study
4	1116_1201del86	H372fsX4	C-ter	1 (1[b])	Amir and Zoghbi 2000
4	1157_1197del41	L386fsX5	C-ter	1 (1[b])	Cheadle et al. 2000
4	1157_1200del44	L386fsX4	C-ter	1	Huppke et al. 2000
4	1162C>T	P388S	C-ter	1	Conforti et al. 2003
4	1164_1207del44	P389fsX1	C-ter	3 (1[b], 1[c])	Buyse et al. 2000
Large deletions identified by MLPA					
3,4	Deletion of exon 3 and a part of exon 4			1	This study
4	Deletion of exon 4 near stop codon			1	This study

[a] Numbered according to GenBank NM_004992

[b] Number of parents tested for the *MECP2* mutation identified in proband

[c] Number of sisters tested for the *MECP2* mutation identified in proband

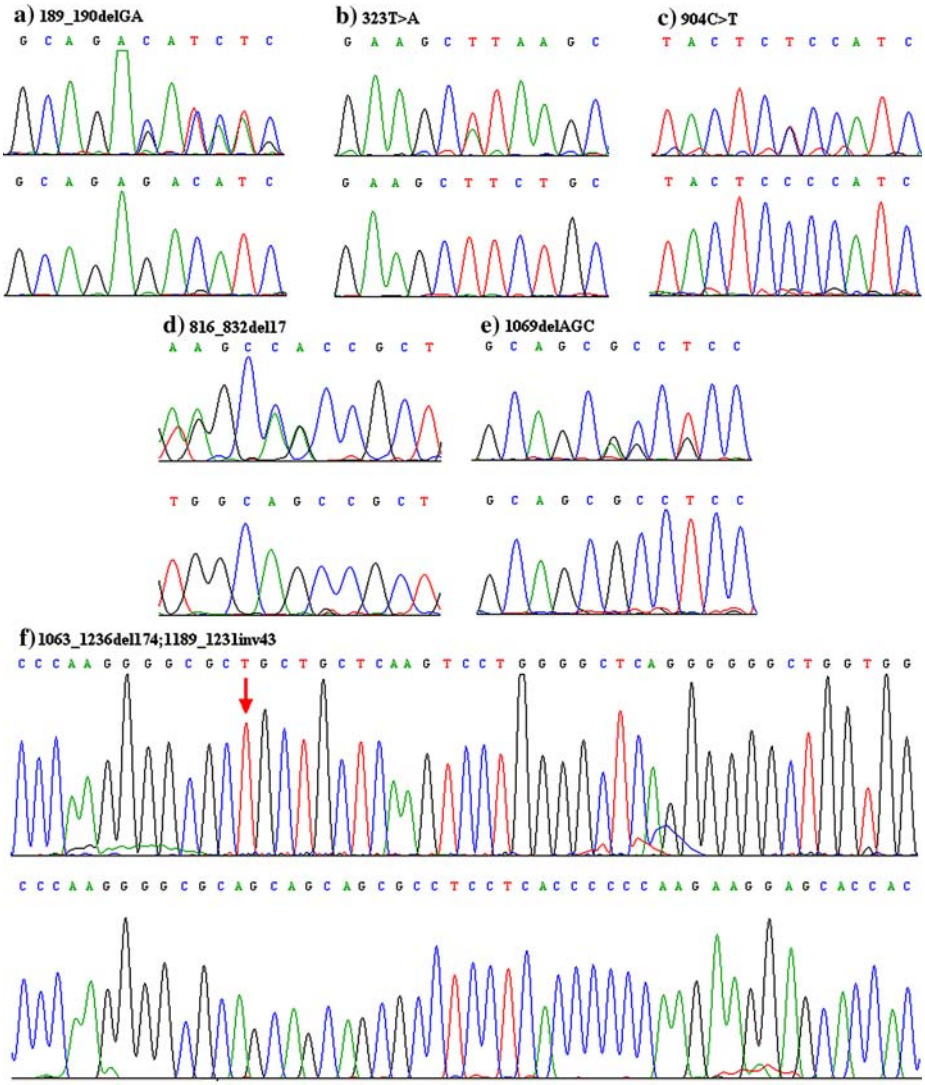

Fig. 1a–e Novel mutations in the methyl-CpG-binding protein 2 gene (*MECP2*) in sporadic Rett syndrome (RTT) patients. **a–e** *Top* patient, *bottom* healthy control. **f** Region containing del/inv mutation cloned into pCR4-TOPO vector. Each allele was sequenced separately. *Top* Allele with mutation (*arrow* indicates the first mutated base), *bottom* normal allele

Table 2 Non-pathogenic polymorphisms in the *MECP2* gene detected in reported patients with RTT

Exon/intron	Nucleotide change	Amino acid change	Domain of MeCP2	Recurrence (this study)	Parental origin	Reference
Intron 3	IVS3 + 22C>G	–	–	2	NA[a]	Couvert et al. 2001
Intron 3	IVS3–17delT	–	–	1	Paternal[b]	Erlandson et al. 2001
Exon 4	587C>G	T196S	–	1	Paternal	Shibayama et al. 2004
Exon 4	686C>T	S229L	TRD	1	NA	Cheadle et al. 2000
Exon 4	815C>T	P272L	TRD	1	Paternal	RettBASE[c]
Exon 4	819G>T	G273G	TRD	1	NA	RettBASE[c]
Exon 4	1161C>T	P387P	C-ter	1	NA	RettBASE[c]
Exon 4	1335G>A	T445T	C-ter	1	NA	RettBASE[c]
Exon 4	1553C>T	–	3′UTR	1	NA	This study
Exon 4	1789G>A	–	3′UTR	2	Paternal (both)	This study
Exon 4	1820G>C	–	3′UTR	1	NA	This study
Exon 4	1824G>C	–	3′UTR	1	NA	This study

[a] DNA from parents not available

[b] Polymorphism identified also in father's mother

[c] RettBASE: http://www.mecp2.chw.edu.au/

(Mnatzakanian et al. 2004). The MeCP2 protein has two major functional domains: the methyl-CpG-binding domain (MBD), which binds specifically to DNA methylated at CpGs; and the transcription repression domain (TRD), which mediates transcription repression through the recruitment of other proteins (Jones et al. 1998; Nan et al. 1998).

In this paper, we report the mutational analysis of the whole coding sequence of the *MECP2* gene. Altogether, we identified a heterogeneous spectrum of mutations, including several novel *MECP2* mutations and polymorphisms, in a high proportion (78.16%) of classical RTT patients. The transversion 323T>A in exon 3 leads to a substitution of a highly conserved lysine to histidine at position 108. The mutation is localized within the MBD and may affect methylation-specific binding of the protein to the DNA template. Missense mutations in the MBD most likely disrupt the integrity of the domain or interfere with MeCP2's ability to bind methylated DNA. The 904C>T transition causes the substitution of proline with serine at position 302, which lies within the TRD. This sequence change probably alters the ability of the protein to recruit co-repressor complexes and affects its function in the process of transcription repression. Mutations involving the TRD could interfere with the assembly of the transcriptional silencing complex, abolishing interactions with the Sin3A corepressor or histone deacetylase recruitment. Binding of truncated MeCP2 to methylated DNA could also provide steric hindrance to the transcription complex. MBD-containing mutant proteins without TRD might accomplish some degree of silencing, either by recruiting the silencing complex by a TRD-independent mechanism or by directly interfering with binding of transcription factors (Ballestar et al. 2000). The 189_190delGA deletion in exon 3 causes a frame-shift, with threonine at position 63 as the first affected amino acid. The mutation introduces a premature stop codon occurring 27 codons downstream (E63fsX27). Another frameshift mutation due to a 17-bp deletion (816_832del17) creates a stop codon after 51 missense amino acids in the TRD (G273fsX52). Deletion 1069delAGC leads to the removal of one serine without alteration of the coding frame (ΔS357). The deleted serine is a part of the SSSASS motif (deleted S357 underlined), which is conserved between human, rat and mouse. Its function is still not clear. A combined deletion/inversion mutation 1063_1236del;1189_1231inv43 was confirmed by cloning of the affected region of the *MECP2* gene into a vector and sequencing of each allele separately. The mutation alters the open reading frame, with codon 355 being the first affected, and leads to the creation of a premature stop codon 37 codons downstream (S355fsX37).

Most of the missense mutations were located in the MBD, while the nonsense mutations were more scattered, located from the end of MBD to the TRD. Deletions were located mostly in the C-terminal region. The eight most common mutations (316C>T, 397C>T, 473C>T, 502C>T, 763C>T, 808C>T, 880C>T, 916C>T), accounting for more than half of all mutations in probands with RTT, were located at CpGs, which would support the hypothesis that these motifs are mutation hot-spots, as suggested by, among others, Wan et al. (1999).

Novel polymorphisms 1553C>T, 1789G>A, 1820G>C, and 1824G>C are localized in the 3′UTR and do not affect the protein sequence. Unfortunately, we were not able to obtain DNA samples from the parents of these patients, hence the parental origin of the polymorphisms is unknown.

No pathogenic sequence variant was found in 19 patients, although they may have *MECP2* mutations that our methods could not reveal, e.g., mutations in the promotor region or intronic variations introducing novel splice sites. There have also been suggestions that RTT is a genetically heterogeneous disorder, and that other causative genes might exist. Several recent studies identified mutations in the *CDKL5* gene (OMIM 300203) encoding cyclin-dependent kinase-like 5 (formerly known as serine/threonine kinase 9; STK9) in patients with an atypical, early seizure variant of RTT (Tao et al. 2004; Weaving et al. 2004; Mari et al. 2005). CDKL5 is a nuclear protein whose expression in the nervous system overlaps with that of MeCP2 during neural maturation and synaptogenesis. Both proteins interact in vitro and in vivo but the role of CDKL5 in the pathological mechanisms of RTT is still not clear (Mari et al. 2005). A mutation screen of *CDKL5* should be considered in mutation-negative patients with early-seizure variant RTT. Moreover, one case of a *Netrin G1* mutation has been shown to be associated with a phenotype strongly overlapping that of RTT (Borg et al. 2005), but the causal link of this mutation with RTT, if any, remains to be established.

With the discovery of mutations in the *MECP2* gene, RTT became the first human disorder known to be caused by genetic defects in a component of the epigenetic silencing machinery. It is the first pervasive developmental disorder with a known genetic cause and is a prototype for the genetic, molecular, and neurobiological analysis of neurodevelopmental disorders. This study is the first report to document a mutation analysis of the *MECP2* gene in RTT patients of Slavic origin.

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