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Research article

Mutation analysis of the MECP2 gene in Romanian females with Rett syndrome

Analiza mutațiilor genei MECP2 la pacientele cu sindrom Rett din România

Simona Dumitriu¹, Enriko Klootwijk³, Naomi Issler^{3,4}, Horia Stanescu^{2,3}, Robert Kleta^{2,3,4,5}, Maria Puiu^{1*}

1. University of Medicine and Pharmacy Victor Babes, Timisoara

2. Neuroscience, Physiology and Pharmacology, University College London, London, UK

3. Centre for Nephrology, University College London, Royal Free Hospital, London, UK

4. Institute of Child Health, University College London, London, UK

5. Great Ormond Street Hospital for Children, London, UK

Abstract

The discovery of the methyl-CpG binding protein 2 (MECP2) gene located on Xq28 as being involved in Rett syndrome (RTT) has been followed by a broad spectrum of phenotypes being associated with mutations in MECP2. The distribution of MECP2 mutations has been studied in many populations, but only recently in Romania. We started the first local study searching for MECP2 mutations using PCR (polymerase chain reaction) - based methods and Sanger sequencing. We have investigated 9 patients, all girls, 7 with classical RTT and 2 with atypical RTT from the Western part of Romania. Mutation screening revealed 3 different mutations present in 4 patients and 5 nonpathogenic genetic variations. One of the frameshift mutations has not been previously described: c.225delG (p.P75fs). The detection rate for missense and frameshift mutations was 44 %. In this study we focused on a practical approach necessary for the molecular geneticist in the process of screening RTT patients for MECP2 mutations. The aim is to extend this protocol for screening MECP2 mutations in all cases of MECP2-related Disorders, to offer prenatal diagnosis, and subsequently complete it with techniques which can detect large rearrangements of this gene.

Keywords: MECP2 mutation, Rett syndrome, sequencing, protocol

Rezumat

Descoperirea genei methyl-CpG binding protein 2 (MECP2) situate pe cromozomul Xq28 ca fiind implicata in producerea Sindromului Rett (RTT) a fost urmata de asocierea unui spectru de fenotipuri cu mutatiile acestei gene. Distributia mutatiilor MECP2 a fost studiata in diverse populatii, doar recent acest studiu fiind ini-

*Corresponding author: Maria Puiu, University of Medicine and Pharmacy Victor Babes, E. Murgu Square, No.1, Timisoara
Phone: 0040745138917, E-mail: maria_puiu@umft.ro

tiat in Romania. Am initiat primul studiu localizat pentru identificarea mutatiilor genei *MECP2* utilizand PCR si secventiere Sanger. Am investigat 9 paciente, din zona de vest a tarii, 7 prezentand forma clasica de RTT si 2 prezentand forme atipice, provenite. Screeningul mutatiilor a relevat 3 mutatii diferite, prezente la 4 paciente si 5 variatii genetice nonpatogenice. Una dintre mutatiile detectate, deletia unei nucleotide in pozitia 225 (c.225delG) nu a mai fost descrisa anterior. Rata de detectie a mutatiilor in studiul nostru a fost de 44%. In aceast studiu ne-am centrat pe o abordare practica necesara geneticianului molecular in cadrul procesului de screening al mutatiilor genei *MECP2* la pacientii cu RTT. Dezideratul nostru este de a extinde protocolul de screening al mutatiilor *MECP2* la cazurile de tulburari asociate genei *MECP2*, de a oferi diagnostic prenatal, si de a-l completa cu alte tehnici care pot detecta rearanjamente ale genei *MECP2*.

Cuvinte cheie: mutatii, *MECP2*, sindrom Rett, protocol.

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Introduction

Rett syndrome (RTT) (OMIM 312750) is an X-linked dominant neurodevelopmental disorder (1) and a common cause of mental retardation in females with a worldwide prevalence of 1 in 10,000~15,000 (2). The clinical manifestations in the *classical* form of RTT are characterized by cognitive deterioration after a period of apparently normal development of 6-18 months, with loss of acquired skills such as language and hand usage and the development of autistic features, stereotypical hand wringing movements, gait ataxia and autonomic symptoms (3). The RTT population has proved to be a lot more heterogeneous phenotypically than originally thought. Atypical RTT variants are commonly observed alongside typical variants. The individuals with *atypical* RTT generally show less severe symptoms than those with classic RTT. A large spectrum of varieties has been clinically recognised with variable levels of impairment of hands use and gait motor control, less severe than classic RTT, also associated with retained, although atypical and telegraphic language, and milder mental retardation. Multiple cases have been categorized within three variants: the preserved speech variant/ Zappella variant (4), the congenital variant with delayed early development (5), and the early-onset seizures variant (6).

MECP2 mutations have been demonstrated to be the primary cause of RTT. *De novo*

heterozygous mutations have been identified in 70–90% of the sporadic cases of RTT (7).

MECP2 mutations were implicated not only in the pathogenesis of classical RTT, but also in a large proportion of the cases of atypical RTT variants. A majority of the female cases with the preserved speech variant also have a mutations in *MECP2* (8, 9). In the congenital variant with delayed early development a mutation has been found in a fraction of 20-40% (7).

MECP2 is a four-exon gene located at the terminal end of the long arm of the X chromosome (Xq28), encoding a protein composed of 486 amino acids. *MECP2* participates in transcriptional repression preventing unscheduled transcription of other genes by binding to methylated CG dinucleotides in some genes promoters (7).

MECP2 has two major functional domains: methyl-CpG-binding domain (MBD) and a transcriptional repression domain (TRD) (Figure 1). MBD is an 85-amino acids domain (amino acids 78–162) through which *MECP2* binds preferentially to methylated DNA at CpG sites (10). TRD is a 104 amino acid transcriptional repression domain (amino acids 207–310) which realizes the role of *MECP2* in silencing of downstream genes by its interaction with the transcriptional repressor Sin3A, which in turn recruits HDACs (11). Two other motifs with specific functions have been described in addition to MBD and TRD. The first, lying

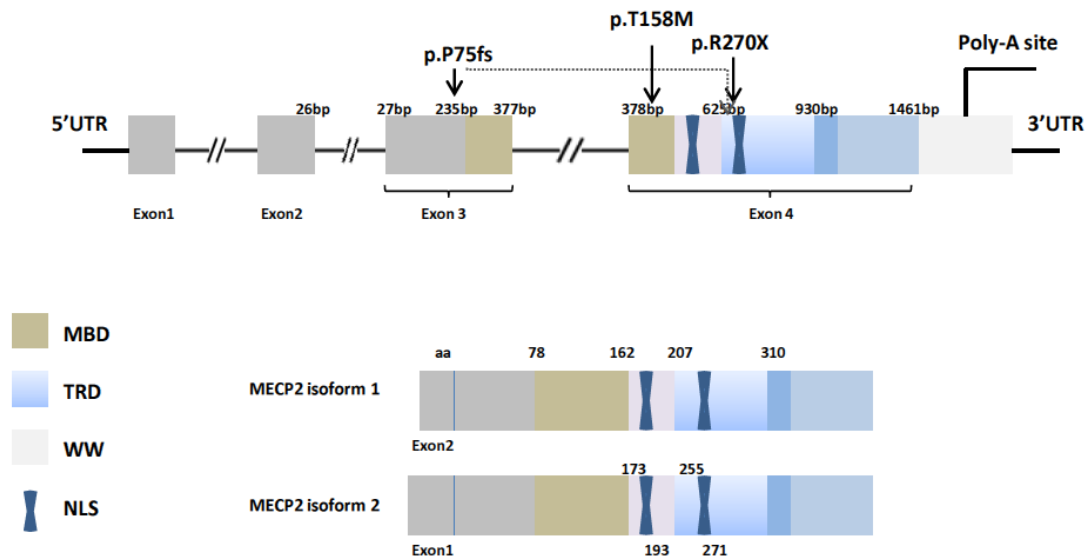


Figure 1. MECP2 splicing variants. The two main protein isoforms of 486 amino acids and 498 amino acids are produced by alternative splicing of the MECP2 transcript, differing in their N-terminal regions, which are encoded by exon 2 of the gene in the case of isoform 1 and exon 1 in isoform 2. The positions of the mutations identified in our female patients are indicated by arrows. MBD, methyl-CpG binding domain; NLS, nuclear localization signal; poly-A, polyadenylation; TRD, transcriptional repression domain.

within the TRD (amino acids 255-271), is the nuclear localization signal, which is sufficient for transportation in the nucleus (12). The last 63 amino acids, the carboxy-terminal segment of the gene, facilitates the binding of MECP2 to both naked DNA and the nucleosome DNA and increases protein stability (13).

Since the first report of the mutations in methyl-CpG binding protein 2 genes in patients with RTT, over 2000 mutations of MECP2 have been identified in patients with classical and atypical RTT. Missense mutations identified in MECP2 in patients with RTT were found in the functional domains, MBD and TRD, but also in the C-terminus region. *In vitro* studies have demonstrated that many missense mutations within the MBD have a significant impact on the affinity of MECP2 for methylated DNA (14). This function is retained if the mutation affects TRD, but the ability of MECP2 to repress transcription is impaired (15).

Patients and methods

Patients

We investigated 9 DNA samples from sporadic cases of female patients with an age median of 4.2 years, referred to the Genetic Department for clinical and molecular diagnosis. The recruitment criteria for these cases followed the RettSearch Consortium Guidelines for diagnostic criteria and nomenclature for both classical and variants form (3). Written informed consent was obtained. Personal and familial mental disabilities histories were obtained from parents and participating relatives.

Molecular analysis

We isolated total genomic DNA from peripheral blood using standard protocols: the WizardTM Genomic DNA purification kit (Promega Madison, WI, USA). DNA concentration was estimated using the NanoDrop[®] ND-1000 Spectrophotometer.

Table 1. Sequencing primer sequences for *MECP2*

Exon 1 ¹ (17)	F	5'- GCACTCGGTGCATCTGTGGACAGAG -3'
	R	5'- CATCCGCCAGCCGTGTCGTCCGAC -3'
Exon 2(1)	F	5'- TGTGTTTATCTTCAAAATGT-3',
	R	5'- GTTATGTCTTTAGTCTTTGG-3',
Exon 3	F	5'- CTGGGGCCTTGCATGTGGTG -3'
	R	5'- GGTCATTTCAAGCACACCTG-3'
Exon 4	F	5'- CGAGTGAGTGGCTTTGGTGA -3'
	R	5'- ACAGATCGGATAGAAGACTC-3'
	F	5'- CCACCCAGGTCATGGTGATC -3'
	R	5'- TGAGTGGTGGTGATGGTGGT-3'
	F	5'- GCAGGAGACCGTACTCCCCATC -3'
	R	5'- GCTCTCCCTCCCCTCGGTGT-3'
	F	5'- GCAGGAGACCGTACTCCCCATC -3'
	R	5'- GCTCTCCCTCCCCTCGGTGT-3'
	F	5'- GGAGAAGATGCCAGAGGAG -3'
	R	5'- CGGTAAGAAAAACATCCCCA -3'

Exon 1 being situated in a GC-rich region of the gene, the chosen length of the oligonucleotides was 25 nt.

The entire coding sequence of the gene, including exon/intron boundaries, was analyzed using PCR amplification and dideoxy Sanger sequencing.

We designed the following primer pairs using the available genomic sequence (NG_007107.1) (16) of the *MECP2* locus to amplify the coding exons and the 3'UTR. The primer sequences are presented in *Table 1*.

PCR was performed with a mix containing 1.5 units Taq DNA polymerase, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, 0.5 μM of forward and 0.5 μM reverse primer, 0.2 mM dNTP and stabilizers. ReadyMixTaq PCR Reaction Mix with MgCl₂ (Sigma-Aldrich Biotechnology LP) was used for exons 2, 3 and 4. For the amplification of exon 1 and of the adjacent intronic regions the optimization of the PCR protocol implied the use of the same reaction volume and a GC-rich solution with the end concentration of 2 units of FastStart Taq DNA polymerase, 2 mM MgCl₂, 1 μM of forward and 1 μM reverse primer, 0.2 mM dNTP, 10 mM Tris-HCl, 50 mM KCl, dNTP and stabilizers provided by FastStart Taq DNA polymerase dNTPack® (Roche Applied Science).

In order to generate sufficient amounts of the desired product we optimized the cycling conditions additional to the optimization of the PCR mix adapted to the different regions of the gene. PCR cycling protocols are listed in *Table 2*.

Amplicons were verified by gel electrophoresis. DNA fragments were recovered with the High Pure PCR Product Purification Kit following instructions (Roche Applied Science).

For sequencing, the amplified products were denatured at 96°C for 20 sec, annealed at 50°C for 20 sec and extended at 60°C 4 min for 30 cycles, using the same primers pairs as for the PCR reaction. After the sequencing reactions, the mixture of fragments was separated by capillary gel electrophoresis at 450–500 V on polyacrylamide gel, the fluorophore's wavelength being detected by the spectrograph. We have used Beckman Coulter CEQ™ 8000 Genetic Analysis System® (Beckman Coulter, Inc.) All of the products were sequenced in forward and reverse reactions. The sequence analysis was performed using a CEQ8000 capillary sequencer (Beckman Coulter, Inc.).

All the mutations and sequence variants are described according to the HUGO approved nomenclature (17) in *Table 3*. The reference se-

Table 2. PCR cycling conditions for *MECP2*

		Exon 1	Exon 2	Exon 3	Exon 4
Denaturation		94°C for 7 min	95°C for 5 min	94°C for 4 min	94°C for 4 min
35 cycles	Denaturation	95°C for 1 min	95°C for 1 min	94°C for 1 min	94°C for 1 min
	Annealing	65 °C for 1 min	50°C for 1 min	64.6 °C for 1 min	55-60 °C for 1 min
	Elongation	72 °C for 1 min	72 °C for 1 min	72 °C for 1 min	72 °C for 1 min
Extension		72 °C for 2 min	72 °C for 2 min	72 °C for 2 min	72 °C for 2 min

Table 3. Summary of results for *MECP2* sequencing

Patient	Genetic modification			Involved functional domain	Age	RTT type
1	c.808C>T (26)	p.R270X	Frameshift	TRD, NLS, WW	5y	Classic
2	c.1562G>A		3'UTR	-	3y	Congenital variant
3	c.377+58C>T		Intronic variant	-	7y	Classic
4	c.225delG	p.P75fs	Frameshift	TRD, NLS, WW	8y	Preserved speech variant
5	c.473C>T (27)	p.T158M	Missense	MBD	4y	Classic RTT
6	c.378-88C>G		Intronic variant	-	4y	Classic RTT
7	c.473C>T (28)	p.T158M	Missense	MBD	16y	Classical RTT
	c.602C>T (29)	p.A201V	SNP rs61748381			
8	WT	-	-	-	3y	Classic RTT
9	WT	-	-	-	3y	Classical RTT

New mutations are presented in bold.

MBD: methyl-CpG-binding domain, TRD: translation repression domain, NLS: nuclear localization signal, WW domain binding region, SNP: single nucleotide polymorphism

quence used for *MECP2* was NG_007107.1 for the coding sequence (16).

Results and discussion

When interpreting Sanger sequencing electropherograms of *MECP2*, the occurrence of two overlapping peaks of fluorescence signals suggests heterozygosity in the concerned patient at a given position. The derived forward and reverse sequences were aligned to the reference sequence version (GRCh37/hg19) using the BLAT tool of the UCSC Human Genome Browser (18). Known variants were identified based on UCSC's dbSNP137 variation & repeat track (19).

We have analyzed the samples from Romanian RTT patients for mutations in the coding region of the *MECP2* gene. All patients

but two showed classic symptoms of RTT, according to the accepted clinical criteria (3).

Two atypical RTT variants were also described: one preserved speech variant (Zappella variant) in patient 4 and one congenital variant in patient 2.

In two of the patients in our study we identified a missense mutation replacing a C with a T at nucleotide 473 (c.473C>T, p.T158M) (Figure 2A). T158M is one of the most frequently described mutations in *classical* RTT, being identified in almost 10% of the cases in the literature. Replacement of threonine, a hydrophobic amino acid, with a polar amino acid, methionine is affecting the C-terminal stretch of MBD (Figure 1).

The change of the secondary structure observed in this case is less important than the one observed for the other mutant forms of *MECP2*. It

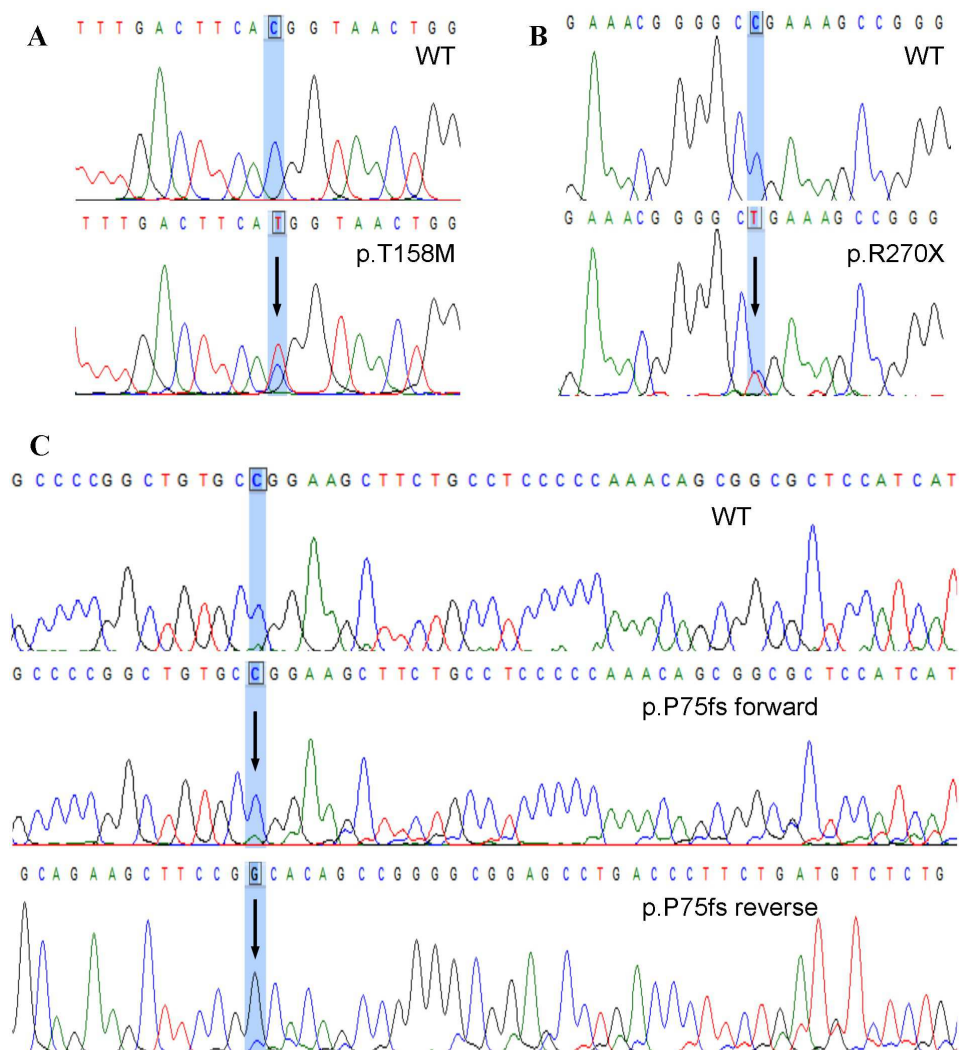


Figure 2. A,B: Original sequencing traces showing heterozygous mutations in *MECP2* (lower traces) and of healthy controls (upper traces). Mutations indicated by arrows. **C:** the heterozygous deletion occurring on one allele throws the alignment of the two allele sequences; the two sequences, prior the deletion being in alignment (WT), changed from one sequence to two overlapping sequences both in the forward and reverse sequencing. The position of the deleted nucleotide indicated by arrows. WT-wild type

has been suggested that the hydrophobic residues placed in the hairpin loop play a role in stabilizing the orientation of the hairpin relative to the rest of the molecule (20). Mutant T158M-MBD was proven to have only a two-fold decrease of its selectivity for methylated DNA compared with the wild-type *MECP2* (14).

The impact of this mutation has been studied by comparison with the *Mecp2* T158A/y variant in a *Mecp2* mouse model, which showed that another T158 mutation, threonine to alanine change, causes reduction in the affinity of *Mecp2* for methylated DNA and decreases *Mecp2* protein expression *in vivo*.

The same effect on *Mecp2* protein levels was described for T158M and confirmed using fibroblasts obtained from a female RTT patient carrying *MECP2* T158M mutation. These data revealed two consequences of the T158M mutation: impairing *MECP2* binding to DNA and decreasing *MECP2* protein stability (21).

Another mutation identified in one patient is a previously described frameshift deletion (22) occurring in a (CCACC) rich region, c.808C>T (Figure 2B). Given the interest raised by the severe clinical impact of this mutation, studies have shown that R270X mouse exhibited also the severe early-onset disease, confirming the crucial importance of the R270 region for *Mecp2* function.

MECP2 has three AT-hook-like domains in a 250 amino acid stretch, the second one containing amino acids 265–272, affected by R270X (Figure 1) (23). The role of the AT-hook-like domains is to bind to AT-rich DNA regions. This mutation leads to a loss of function of AT-hook-like 2 domain.

MECP2 first binds methylated DNA through MBD and second, for an efficient binding to the A/T-rich motif adjacent to methylated CpG dinucleotides, requires AT-hook-like domains (24) in order to realize chromatin packing. Truncated *MeCP2*-R270X leads to a loss of function of the AT-hook-like 2 domain causing the loss of *MECP2* ability to maintain chromatin order and to act as transcription repressor. The effect of the mutations in this region on chromatin organization impacts the normal synaptic function and neuronal response (25).

Sequence analysis for patient 4 identified a heterozygous frameshift mutation: deletion of 1 bp at position 225: c.225delG (p.P75fs) (Figure 2C). Since no other variant was identified in the patient besides this deletion we hypothesized that this G deletion was also pathogenic. The novel mutation c.225delG described here, causes a frameshift and a premature stop at the 224 codon after 48 amino-acids. If the mRNA would be translated, the resulting protein would lack functional domains TRD, NLS and WW.

To our knowledge, data published until now show that patients with mutations in the N-terminal regions of *MECP2* display a milder phenotype, usually associated with the preserved speech variant. Patients presenting this variant have better function overall. The clinical phenotype of these girls presents less dominating hand stereotypes, with a later age of onset, better use preserved speech and better gait control. The autonomic symptoms are also less common (26). This mutation affecting an amino-acid from the N-terminal region of *MECP2*, inducing a stop codon in TRD after 48 amino-acids may explain the atypical phenotype in our patient, with the presence of a telegraphic expressive language and retained ability to walk.

We detected variations located on non-coding regions of *MECP2*. Novel identified polymorphisms, c.377+58C>T, c.378-78C>T and c.378-84C>G affect the +58 nucleotide of the 3' splicing site of the exon 3, and the – 78 and – 84 nucleotides in the 5' splicing site of exon 4, respectively, their potential impact on the protein level being unknown. In the same patients a pathogenic variant has not been identified in the coding sequence of *MECP2*. The implication of these polymorphisms for the phenotypic variability or susceptibility for RTT in this population is to be analyzed further.

The selection of patients according to strict clinical criteria are reported to lead to a high likelihood of finding a mutation in the *MECP2* coding region, nearly to 90% when applied to large series (27). In smaller groups this is situated in a broad interval between 35 and 77% of cases (27). In our study, we report the identification of mutations in the coding sequence of *MECP2* in 44% of the investigated cases. Given that RTT diagnosis and categorization of the form in concordance with the criteria is still difficult in stages previously to the stabilization phase, we consider that our study reported a positive correlation between molecular diagnosis and clinical diagnosis in an early phase.

Given that the screening for *MECP2* mutations using sequencing of the coding re-

gion and the flanking area of the gene did not report mutations in some of the patients with classical RTT and with the atypical congenital variant we consider that a search for large genomic rearrangements of *MECP2* will be the next recommended step for molecular diagnosis in these patients. Literature data have previously described that large rearrangements of the *MECP2* gene were identified in around 37% of classic and 7 % of atypical RTT patients which previously tested negative for a mutation in the coding region (28).

The results of our study reflect the fact that precise definition of the phenotype increases the likelihood of obtaining a higher yield by genotyping. Our protocol aims to adapt a complex, time-consuming and expensive protocol for the sequencing of the entire *MECP2* gene, by creating the advantage of quick and less expensive diagnosis.

Given that at least 85% of the mutations discovered in RTT patients were found to affect exon 3 and exon 4 of the gene, no mutation being found to affect exon 2, we recommend that the sequencing protocol should cover the two main functional domains of *MECP2*, by sequencing exons 3 and 4, and subsequently, if the genotyping results do not correlate to the phenotype to continue with exons 1 and 2.

Our data provide a contribution towards understanding the Romanian population genetic characteristics and genotype phenotype correlation at the level of *MECP2* gene. Research should be further developed in order to elucidate a mutation spectrum at the population level and connect it with existing databases within the Eastern European or Western regions.

Acknowledgements

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Electronic Database Information

Accession numbers and URLs for the data presented herein are as follows:

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for RTT [MIM 312750] and MeCP2 [MIM 300005]).

NCBI reference sequence for *MECP2*, <http://www.ncbi.nlm.nih.gov/gene/4204> (NG_007107.2).

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