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ORIGINAL ARTICLE

MECP2 mutations in Czech patients with Rett syndrome and Rett-like phenotypes: novel mutations, genotype–phenotype correlations and validation of high-resolution melting analysis for mutation scanning

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Rett syndrome (RTT) is an X-linked neurodevelopmental disorder characterized by developmental regression with loss of motor, communication and social skills, onset of stereotypic hand movements and often seizures. RTT is primarily caused by *de novo* mutations in the methyl-CpG-binding protein 2 gene (*MECP2*). We established a high-resolution melting (HRM) technique for mutation scanning of the *MECP2* gene and performed analyses in Czech patients with RTT, autism spectrum conditions and intellectual disability with Rett-like features. In the cases with confirmed *MECP2* mutations, we determined X-chromosome inactivation (XCI), examined the relationships between genotype and clinical severity and evaluated the modifying influence of XCI. Our results demonstrate that HRM analysis is a reliable method for the detection of point mutations, small deletions and duplications in the *MECP2* gene. We identified 29 pathogenic mutations in 75 girls, including four novel mutations: c.155_1189del1035;909_932inv;insC, c.573delC, c.857_858dupAA and c.1163_1200del38. Skewed XCI (ratio >75%) was found in 19.3% of the girls, but no gross divergence in clinical severity was observed. Our findings confirm a high mutation frequency in classic RTT (92%) and a correlation between the *MECP2* mutation type and clinical severity. We also demonstrate limitations of XCI in explaining all of the phenotypic differences in RTT.
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INTRODUCTION

Rett syndrome (RTT; MIM no. 312750) is a severe X-linked neurological disorder that is one of the leading genetic causes of intellectual disability in females. The prevalence of RTT is ~1:10 000–15 000 female births worldwide.[1] The classic form of RTT is characterized by apparently normal psychomotor development throughout the first 6–18 months of life, followed by developmental stagnation and regression. During the regression phase, patients partially or completely lose acquired motor and communication skills. They also develop distinctive stereotypic hand movements. Some communication and social skills may be recovered during the next phase, known as the pseudostationary stage. Seizures, breathing abnormalities when awake (i.e., episodes of hyperventilation and breath holding), peripheral vasomotor disturbances and scoliosis are additional common symptoms. Motor deterioration tends to progress with the patient's age. There are also individuals with atypical or variant RTT who manifest several but not all typical RTT features.[2,3] RTT is mostly caused by *de novo* mutations in the methyl-CpG-binding protein 2 (*MECP2*) gene, located on Xq28. The *MECP2* gene comprises four exons and encodes the ubiquitously expressed methyl-CpG-binding protein 2 (MeCP2).[4] The protein contains five main domains: (1) the N-terminal domain (NTD); (2) a methyl-CpG-binding domain (MBD), which specifically binds to methylated DNA; (3) an interdomain (ID); (4) a transcriptional repression domain (TRD), which interacts with a number of transcriptional corepressors and other protein factors and contains a nuclear localization signal (NLS); and (5) the C-terminal domain (CTD). There are two isoforms of the protein, MeCP2_e1 and MeCP2_e2, which differ in their N termini and relative expression levels in various tissues. MeCP2_e1 is the predominant isoform in the brain and MeCP2_e2 is more abundant in the placenta, liver and skeletal muscles.[5,6] MeCP2 is a multifunctional protein that is especially, but not exclusively, involved in the regulation of gene expression and chromatin remodeling.[7–11]

More than 1000 different mutations have been reported in the *MECP2* gene, as listed in The Human Gene Mutation Database[12] and several locus-specific databases, such as RettBASE.[13] These mutations account for up to 95–97% of classic[14] and ~50–70% of atypical RTT cases.[15] Moreover, *MECP2* mutations have been less frequently associated with other neurodevelopmental conditions, such as autism,

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Angelman syndrome-like phenotype, mild-to-severe intellectual disability and other behavioral and psychiatric conditions.[16–19]

There is considerable variability in the severity of clinical features among RTT patients, which is caused primarily by the type and location of the *MECP2* mutation.[14,20–23] In addition to the causative mutations, X-chromosome inactivation (XCI) is another important factor influencing the severity of clinical manifestations. Skewed XCI favoring a normal *MECP2* allele has been reported in asymptomatic carrier mothers and sisters with mild clinical expression in the rare familial cases of RTT.[24,25]

In this study, we summarize the spectrum of *MECP2* mutations in Czech patients with RTT and Rett-like features. This is the first study referring specifically to a Czech population as the previous study by Zahorakova *et al.*[26] included data from patients of a more general Slavic origin. We introduce high-resolution melting (HRM) analysis as a reliable screening technique for presequencing analysis of the *MECP2* gene and report four novel mutations. We evaluate the correlation between genotype and phenotype as well as the possible contribution of XCI to the severity of clinical symptoms.

MATERIALS AND METHODS

Samples

Patients (aged 2–17 years) were assessed by pediatric neurologists, pediatricians and clinical geneticists. Clinical documentation and questionnaires (based on the questionnaire by Bebbington *et al.*[20]) were used for evaluation of clinical severity. RTT patients were diagnosed according to the current diagnostic criteria by Neul *et al.*[2] Patient samples (peripheral blood or isolated DNA) were acquired from departments of medical genetics, child neurology and pediatrics in the Czech Republic. Among 487 patients (463 girls, 24 boys), 63 girls exhibited a classic RTT phenotype; 18 girls showed an atypical RTT phenotype; 47 patients (including 3 boys) were diagnosed with child autism; and 359 patients (including 21 boys) presented developmental delay and intellectual disability with some RTT features. Genomic DNA was extracted using the salting out procedure.[27] DNA extracted from other tissues, such as buccal swabs and hair follicles was used to validate the HRM analysis.

The study was approved by the Committee of Medical Ethics of the First Faculty of Medicine of Charles University in Prague, and written informed consent was obtained from the parents of all patients.

HRM analysis

Exons 2, 3 and 4, including flanking noncoding regions, were amplified using the primers and PCR conditions summarized in Table 1. The primers were designed to generate amplicons of 200–400 bp. Amplification reactions were carried out in a total volume of 10 µl containing 1x Plain Combi PP Master Mix (Top-Bio, Prague, Czech Republic), 2% dimethyl sulfoxide, 200 nM each primer, 1x LCGreen Plus+ Melting Dye (Biofire Defence, Salt Lake City, UT, USA) and 20 ng of genomic DNA. The cycling profile was 94 °C/90 s, followed by 40 cycles of 94 °C/30 s, 55–64 °C/30 s and 72 °C/30 s, with a final extension at 72 °C/5 min. Each PCR product was overlaid with 15 µl of mineral oil in a 96-well plate and centrifuged briefly at 2000 *g*. The samples were subsequently denatured at 95 °C/30 s, reannealed at 25 °C/30 s and transferred to a LightScanner instrument (Biofire Defence). Then, the samples were gradually denatured by increasing the temperature (from 65 to 98 °C at the rate of 0.1 °C/s). The resultant melting curves were analyzed using the original LightScanner software v.1.5 (Biofire Defence). The melting curves were normalized using the default curve shift adjustment (0.05). The patients' samples were then compared with wild-type control samples. The analysis was carried out with the "auto group" option at high sensitivity. Amplicons with several melting domains were normalized and analyzed in several steps, first evaluating the whole amplicon and then each domain separately. The male samples were melted two times: after the first HRM analysis, each male sample on the plate was spiked with a wild-type control sample and analyzed as described above.

DNA sequencing

Exon 1 and amplicons with aberrant HRM profiles were prepared for sequencing analysis using the primers and conditions summarized in Table 1. Amplification reactions (exons 2–4) were carried out in a total volume of 12.5 µl containing 1x PPP Master Mix (Top-Bio), 200 nM each primer and 20 ng of genomic DNA. Exon 1 was amplified in a total volume of 12.5 µl containing 1x Combi PP Master Mix (Top-Bio), 200 nM each primer, 0.02 mM 7-deaza dGTP, 1 M betaine, 6% dimethyl sulfoxide and 20 ng of genomic DNA. The cycling profile was 94 °C/90 s, followed by 33 cycles of 94 °C/30 s, 55–64 °C/30 s and 72 °C/40 s, with a final extension at 72 °C/5 min. The PCR products were purified, then sequenced in both directions and analyzed on ABI PRISM 3100-Avant and 3500xL genetic analyzers (Applied Biosystems, Foster City, CA, USA). The acquired sequences were compared with the GenBank Reference sequence NG_007107.

Multiplex ligation-dependent probe amplification

Mutation-negative samples were tested for large deletions using the SALSA MLPA P015 probe mix (MRC-Holland, Amsterdam, The Netherlands) according to the manufacturer's instructions. Fragment analysis was performed on ABI PRISM 3100-Avant and 3500xL genetic analyzers (Applied Biosystems), and the MLPA data were analyzed using Coffalyser.Net software (MRC-Holland).

In silico analysis of novel sequence variants

DNA screening of the parents was performed to determine the *de novo* origin of the sequence variants. Pathogenicity was investigated using several prediction tools: SIFT (http://sift.jcvi.org/), PolyPhen2 (http://genetics.bwh.harvard.edu/

Table 1 Primers (5′ → 3′ direction) and PCR conditions for amplification of *MECP2* exons

Amplicon	*Length (bp)*	*Forward primer*	*Reverse primer*	T_a *(°C) HRM*	T_a *(°C) seq*
Exon 1	522	GCCGAAAGCAGCCAATCAACAGC	CGGGAGGGGGAGGGTAGAGAGGA	—	59
Exon 2	258	TTATCTTCAAAATGTCCCAA	GGCCAAACCAGGACATATAC	55	55
Exon 3a	389	AAGATCTGAGTGTATGATGG	GGTCATCATACATGGGTC	59	55
Exon 3b	285	GCAAAGCAGAGACATCAGAA	CCTGGGCACATACATTTTCCT	59	55
Exon 4a	362	AGAGCGTTGTCACCACCATC	TTTCTCCAGGACCCTTTTCA	59	59
Exon 4b	312	CTTCACGGTAACTGGGAGAG	TCACCATGACCTGGGTGGAT	64	59
Exon 4c	380	AACCACCTAAGAAGCCCAAA	CTGCACAGATCGGATAGAAGAC	64	59
Exon 4d	361	CGACCCTCAGGCCATTCCCA	GGGGGCCTTTGGGGACTCTG	64	59
Exon 4e	337	AGGAAGTGGTGAAGCCCCTG	CAGCCGTCGCTCTCCAGTGA	64	59
Exon 4f	204	ACCTCCACCTGAGCCCGAGA	CCCTCCCCTCGGTGTTTGT	64	59
Exon 4g	391	CCCAAGGAGCCAGCTAAGAC	GTCTTCAACCTGACTGTGCTT	64	59

Abbreviations: HRM, high-resolution melting; MECP2, methyl-CpG-binding protein 2; T_a, annealing temperature.

pph2/), Mutation Taster (http://www.mutationtaster.org/), SNPs&GO (http://snps.biofold.org/snps-and-go/snps-and-go.html), PredictSNP (http://loschmidt.chemi.muni.cz/predictsnp/), PMut (http://mmb.pcb.ub.es/PMut/) and MutPred (http://mutpred.mutdb.org/).

X-chromosome inactivation

The XCI pattern was determined using a modified *HUMARA* assay.[28] The forward primer was fluorescently labeled with 6-FAM, and the PCR products were resolved through capillary electrophoresis on a 3500xL genetic analyzer (Applied Biosystems). The data were analyzed using GeneMarker software (Softgenetics, State College, PA, USA), and the XCI ratio was calculated as described previously.[29] The XCI was considered to be skewed if the ratio between inactive and active alleles was higher than 75%. Parental origin of the inactivated X chromosome in patients with skewed XCI was assessed by the comparison of allele sizes of patients and parents. The given XCI ratios represent the ratio of inactive paternal to maternal alleles. Since more than 95% of *MECP2* mutations are of paternal origin,[30] the XCI ratio may also represent the ratio of cells with active wild-type *MECP2* to cells with active mutated *MECP2* in the majority of cases.

RESULTS

Mutation analysis of the *MECP2* gene

In this study, the *MECP2* gene was analyzed in 487 patients (463 girls, 24 boys). The analysis workflow was as follows: HRM of exons 2–4, followed by sequencing of exon 1 and amplicons with an abnormal HRM profile and, finally, MLPA.

We successfully optimized conditions for HRM analysis of exons 2–4. The sensitivity of the method was validated in 60 samples with a previously identified *MECP2* mutation or polymorphism (38 different genotypes).[26] All samples were correctly distinguished from wild-type control samples (100% sensitivity); that is, all fragments with sequence variants had abnormal melting profiles (Figure 1). Owing to the absence of a positive control sample with a sequence alteration in exon 2, the sensitivity of the method for this exon could not be determined. Specificity was assessed by evaluating 64 previously sequenced wild-type samples and also reached 100%. Optimal results (i.e., melting curves of wild-type samples arranged close together) were obtained regardless of the tissue from which the genomic DNA originated (e.g., blood, buccal swabs, hair follicles). Various methods of DNA extraction also did not significantly interfere with the HRM analysis, which was successfully tested on patient DNA samples that were extracted by and shipped from other departments. Minor curve variations observed for some samples could instead be attributed to suboptimal DNA quality (e.g., slightly fragmented DNA, A260/A280 ratio >2). All male samples were spiked with a wild-type control after the first HRM analysis and analyzed again. This step generates artificial heterozygosity if a hemizygous mutation is present in the original sample.[31] In our male patients, all sequence changes were distinguished in a hemizygous state. Most of the mutations and polymorphisms that we detected through HRM showed a unique melting profile. Only certain sequence variants exhibited very similar melting curves (e.g., c.1157_1197del41 and c.1157_1200del44 in amplicon 4e and c.1130G>A and c.1135G>A in fragment 4 g—see Figure 1), and thus the software placed them in the same group. Because we sequenced all of the HRM-positive samples, the correct genotype was determined in each patient. Exon 1 showed more widely distributed melting curves of wild-type amplicons than the other exons, probably because of its GC-rich and repetitive sequence and the requirement for several PCR additives. Hence, exon 1 was excluded from the HRM analysis and analyzed only through direct sequencing.

This study revealed 29 different pathogenic mutations in 75 patients, all of whom were girls (Table 2). Pathogenic mutations were present in 58 out of 63 classic RTT patients (92%). Among the 18 patients with atypical RTT, 10 carried a mutation (55.6%), while 1 mutation-positive case was confirmed among the 47 patients with autism (2.1%). In the group of 359 patients who showed developmental delay and intellectual disability with Rett-like features, pathogenic mutations were identified in 6 girls (1.7%). We identified 10 missense, 8 nonsense and 10 frameshift mutations as well as 1 large deletion. To date, we have not detected any splice-site mutations in the *MECP2* gene in Czech patients. The most common mutations in the Czech cohort are summarized in Table 3. The single girl with a confirmed *MECP2* mutation among autism patients carried the mutation p.Arg168*.

We report four novel frameshift mutations leading to a change in the amino-acid sequence and premature termination of the protein: c.155_1189del1035;909_932inv;insC (p.His52Argfs*16), c.573delC (p.Ser194Alafs*16), c.857_858dupAA (p.Ala287Lysfs*3) and c.1163_1200del38 (p.Pro388Glnfs*4). The mutation c.857_858dupAA was found in a girl with classic RTT, three other novel mutations were identified in atypical RTT patients. The novel mutation c.155_1189del1035;909_932inv;insC (p.His52Argfs*16) was initially detected as a large heterozygous deletion via the MLPA method. PCR amplification of the region covered by the MLPA probes with a normal signal produced two amplicons, corresponding to a longer wild-type allele and a shorter mutated allele. Subsequent sequencing of the PCR products revealed the precise molecular defect. We also identified 30 nonpathogenic variants (Table 2).

X-chromosome inactivation

XCI analysis was completed in 62 girls with a confirmed *MECP2* mutation (52 with classic RTT, 7 with atypical RTT, 2 with intellectual disability and Rett-like features and 1 with autism). The XCI results for each clinical group are summarized in Table 4. The majority of patients with a skewed XCI were clinically diagnosed as classic RTT patients. The only one Rett-like patient with the skewed XCI (82:18) carried the mutation c.Arg306Cys. Extreme skewing was present in two classic RTT patients harboring the mutations p.Arg106Gln (95:5) and p.Arg255* (98:2), but their clinical phenotypes were not significantly milder than in other classic RTT patients with the same mutations in our cohort. With regard to the relationship between XCI and clinical severity, we observed two results of relevance: (1) one classic RTT patient with the p.Arg168* mutation and the XCI ratio 87:13 exhibited a later onset of symptoms than other RTT patients with this mutation; characteristics of RTT occurred gradually after the age of 24 months, but the individual symptoms were not otherwise significantly milder. (2) One classic RTT patient with the p.Arg133Cys mutation and the XCI ratio 23:77 presented a slightly more severe phenotype with regard to absent speech, prominent stereotypic hand movements and poorly controlled epilepsy, which is not generally observed in RTT patients with this particular mutation. No other significant discrepancies between *MECP2* mutation and the severity of the phenotype, which could be explained by the skewed XCI, were found in the rest of the cohort.

DISCUSSION

Mutations in the *MECP2* gene are highly correlated with RTT, especially with its classic form, although they have also been detected in a small proportion of patients with other autism spectrum disorders and intellectual disabilities.[16–19] Analysis of this gene for routine diagnostic purposes is usually performed through a combination of DNA sequencing and copy number analysis. The broad spectrum

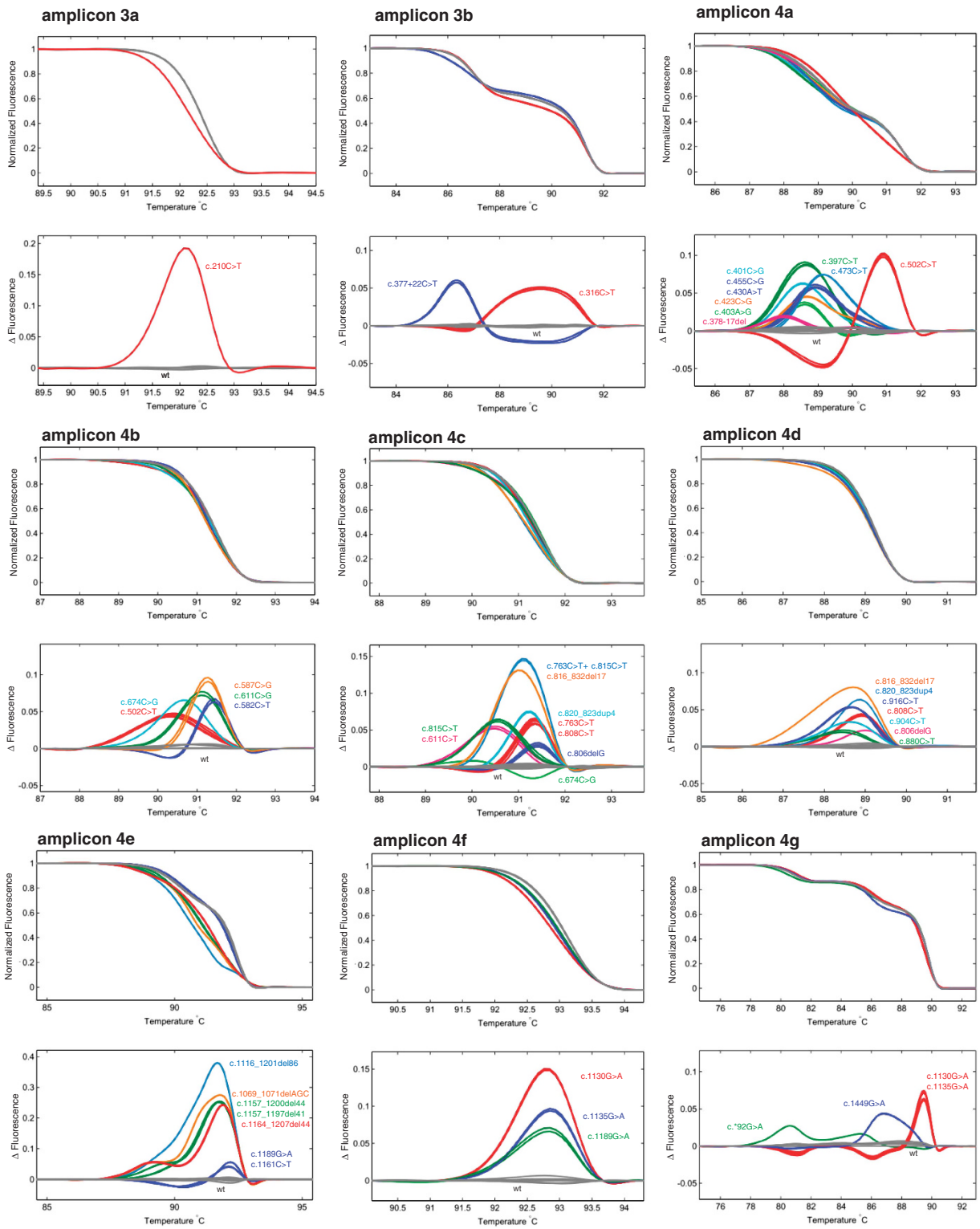

Figure 1 High-resolution melting (HRM) curves for methyl-CpG-binding protein 2 (*MECP2*) exons 3 and 4. For each amplicon, normalized melting curves (top) and difference plots (bottom) are shown. Groups of genotypes were generalized using the "auto group" option at high sensitivity. All control samples with known sequence variants were distinguished from wild-type (wt) control samples during the validation phase.

of *MECP2* mutations requires analysis of the whole coding sequence and flanking noncoding regions. Various mutation-scanning techniques, such as denaturing high-performance liquid chromatography, have often been applied to avoid unnecessary sequencing of mutation-negative samples.[32,33] HRM is a simple, fast, reliable and cost-effective method for genotyping and mutation screening. Since its discovery,[34] HRM has been widely used for the analysis of numerous genes. In this study, we introduce HRM as a highly suitable screening method for

Table 2 *MECP2* mutations and polymorphisms identified in Czech patients

Nucleotide change[a]	*Amino-acid change*	*Location*	*Cases*	*Comment*
Pathogenic mutations				
c.155_1189del1035; 909_932inv;insC	**p.His52Argfs*16**	**NTD**	**1**	**1x atypical RTT**
c.189_190delGA	p.Glu63Aspfs*27	NTD	1	1x classic RTT
c.316C>T	p.Arg106Trp	MBD	4	4x classic RTT
c.317G>A	p.Arg106Gln	MBD	1	1x classic RTT
c.323 T>A	p.Leu108His	MBD	1	1x classic RTT
c.392C>A	p.Ala131Asp	MBD	1	1x classic RTT
c.397C>T	p.Arg133Cys	MBD	5	3x classic RTT, 1x atypical RTT, 1x Rett-like features
c.423C>G	p.Tyr141*	MBD	1	1x classic RTT
c.430 A>T	p.Lys144*	MBD	1	1x classic RTT
c.455C>G	p.Pro152Arg	MBD	3	3x classic RTT
c.468C>G	p.Asp156Glu	MBD	1	1x classic RTT
c.473C>T	p.Thr158Met	MBD	12	11x classic RTT, 1x atypical RTT
c.502C>T	p.Arg168*	ID	3	2x classic RTT, 1x autism
c.573delC	**p.Ser194Alafs*16**	**ID**	**1**	**1x atypical RTT**
c.611C>G	p.Ser204*	ID	1	1x classic RTT
c.674C>G	p.Pro225Arg	TRD	1	1x Rett-like features
c.763C>T	p.Arg255*	TRD-NLS	9	9x classic RTT
c.806delG	p.Gly269Alafs*20	TRD-NLS	1	1x atypical RTT
c.808C>T	p.Arg270*	TRD-NLS	4	3x classic RTT, 1x Rett-like features
c.816_832del17	p.Gly273Argfs*52	TRD	1	1x classic RTT
c.820_823dupAGTG	p.Val275Glufs*57	TRD	1	1x classic RTT
c.857_858dupAA	**p.Ala287Lysfs*3**	**TRD**	**1**	**1x classic RTT**
c.880C>T	p.Arg294*	TRD	3	2x classic RTT, 1x Rett-like features
c.916C>T	p.Arg306Cys	TRD	7	5x classic RTT, 1x atypical RTT, 1x Rett-like features
c.1063_1236del174; 1189_1231_inv43	p.Ser355Cysfs*37	CTD	1	1x classic RTT
c.1116_1201del86	p.His372Glnfs*4	CTD	1	1x classic RTT
c.1157_1197del41	p.Leu386Hisfs*5	CTD	2	1x classic RTT, 1x atypical RTT
c.1163_1200del38	**p.Pro388Glnfs*4**	**CTD**	**1**	**1x atypical RTT**
c.1164_1207del44	p.Pro389*	CTD	5	2x classic RTT, 2x atypical RTT, 1x Rett-like features
Nonpathogenic variants				
c.−235G>T	—	5′-UTR	1	1x Rett-like features
c.−220delC[b]	—	5′-UTR	473	See note below
c.18_23dup6 (exon 1[a])	p.Ala7_Ala8dup	NTD	5	2x atypical RTT, 1x autism, 2x Rett-like features (1 boy)
c.45_47dupAGG (exon 1[a])	p.Gly16dup	NTD	1	1x Rett-like features
c.375C>A	p.Ile125=	MBD	1	1x Rett-like features
c.377+22C>G	—	Intron 3	1	1x Rett-like features
c.378−74C>T	—	Intron 3	3	1x classic RTT, 1x atypical RTT, 1x Rett-like features
c.378−65C>G	—	Intron 3	1	1x Rett-like features
c.378−17delT	—	Intron 3	3	1x autism, 2x Rett-like features
c.426C>T	p.Phe142=	MBD	1	1x classic RTT
c.582C>T	p.Ser194=	ID	2	1x autism, 1x Rett-like features
c.587C>G	p.Thr196Ser	ID	2	1x classic RTT, 1x Rett-like features
c.683C>G	p.Thr228Ser	TRD	2	1x classic RTT, 1x Rett-like features
c.686C>T	p.Ser229Leu	TRD	1	1x classic RTT
c.815C>T	p.Pro272Leu	TRD	1	1x classic RTT
c.819G>T	p.Gly273=	TRD	1	1x Rett-like features
c.834C>T	p.Ala278=	TRD	1	1x atypical RTT
c.897C>T	p.Thr299=	TRD	2	2x Rett-like features
c.996C>T	p.Ser332=	CTD	1	1x Rett-like features
c.1035 A>G	p.Lys345=	CTD	1	1x Rett-like features
c.1133C>G	p.Ala378Gly	CTD	1	1x Rett-like features
c.1161C>T	p.Pro387=	CTD	1	1x Rett-like features
c.1168_1173del6	p.Pro390_Pro391del	CTD	1	1x Rett-like features
c.1189G>A	p.Glu397Lys	CTD	2	2x Rett-like features
c.1197C>T	p.Pro399=	CTD	1	1x Rett-like features
c.1330G>A	p.Ala444Thr	CTD	4	1x classic RTT, 3x Rett-like features
c.1449G>A	p.Glu483=	CTD	1	1x Rett-like features
c.*9G>A	—	3′-UTR	1	1x Rett-like features
c.*92C>T	—	3′-UTR	2	1x classic RTT, 1x Rett-like features
c.*156G>T	—	3′-UTR	1	1x Rett-like features

Abbreviations: CTD, C-terminal domain; ID, interdomain; MBD, methyl-CpG-binding domain; MECP2, methyl-CpG-binding protein 2; NTD, N-terminal domain; RTT, Rett syndrome; TRD, transcriptional repression domain; UTR, untranslated region.
Novel mutations are highlighted in bold.
[a]Variants are numbered according to the reference sequence NM_004992. Variants located in exon 1 and the 5′-UTR are numbered according to the reference sequence NM_001110792.
[b]The reference sequence NG_007107 does not contain the deletion c.−220delC (there are two cytosines at positions c.−220 and c.−221 in the reference sequence). c.−220delC is more frequent than the wild-type allele, and it was observed in the majority of our patients: 465 homozygous, 3 heterozygous girls and 5 hemizygous boys.

presequencing analysis of exons 2–4 of the *MECP2* gene. The method shows excellent sensitivity and specificity, with little influence of the origin of the DNA and the extraction method. It is especially successful in detecting heterozygous variants, which makes the *MECP2* gene a perfect candidate for testing. Application of the HRM prevents the need to sequence all the exons of the gene when looking for novel mutation. The HRM workflow does not require any processing, reagent addition or separation of PCR products; therefore, there is a minimal chance of sample interchange or contamination. Unlike other screening techniques, the HRM method is non-destructible and PCR products with abnormal melting profiles can be directly processed for sequencing.

In the present study, we identified 29 different pathogenic mutations in 75 female patients. The highest mutation frequency was confirmed in classic RTT patients (92%), among whom pathogenic mutations were identified in 58 out of 63 girls. In accordance with other reports, *MECP2* mutations were less frequent in atypical RTT cases (55.6%) and even rarer in patients with child autism (2.1%) and intellectual disability with Rett-like features (1.7%). Some patients from the last group were too young at the time of examination to be designated with a definitive diagnosis of RTT according to revised diagnostic criteria.[2] A future follow-up clinical evaluation might show that at least some of these patients exhibit the classic RTT phenotype. Mutation-negative patients may carry pathogenic mutations in the regulatory regions of the *MECP2* gene. In atypical RTT patients and patients with Rett-like features, mutations in *CDKL5*,[35–37] *FOXG1*[38,39] and other genes, as demonstrated by exome sequencing studies,[17,40] have been identified.

Four novel mutations were detected: c.155_1189del1035;909_932inv;insC (p.His52Argfs*16), c.573delC (p.Ser194Alafs*16), c.857_858dupAA (p.Ala287Lysfs*3) and c.1163_1200del38 (p.Pro388Glnfs*4). In the girl with the c.155_1189del1035;909_932inv;insC (p.His52Argfs*16) mutation, developmental delay occurred soon after birth. She was diagnosed with atypical RTT. There was no apparent period of normal development followed by a regression phase, which is typical of classic RTT. At the age of 3 years, this patient was unable to sit independently and she never walked. Purposeful hand use was very poorly developed and was hampered by dominating stereotypic hand movements. She never talked and exhibited poor eye contact. Her first seizure occurred at the age of 2 months, and epilepsy has been pharmacologically compensated. The patient has had no history of breathing difficulties thus far. The rather severe phenotype observed in this patient is presumably primarily caused by the *MECP2* mutation. The XCI ratio in blood was 75:25, which may imply that the mutated *MECP2* is inactive in most blood cells. However, if the ratio in the brain is skewed in a way of preferential inactivation of wild-type *MECP2*, it might contribute to more severe manifestation of the disease in the patient.

The second novel mutation, c.573delC (p.Ser194Alafs*16), was identified in an 18-month-old atypical RTT girl with developmental delay and microcephaly manifesting since birth. She is unable to sit independently and has not learned to walk. Her purposeful hand use has deteriorated, and stereotypic hand movements occurred beginning at the age of 10 months. The patient uses no meaningful words, but her nonverbal communication and social skills improved after regression period. Breathing abnormalities were present from birth but ceased gradually, and the patient has had no history of seizures thus far. The mutation is located between the MBD and TRD and results in the creation of premature stop codon. Owing to earlier translation termination, mutated MeCP2 lacks the important TRD and CTD domains, and, more importantly, the NLS, which could explain the severe phenotype. Mutations truncating the MeCP2 protein before or within the TRD mostly lead to more severe impairment than C-terminal truncations.[14,20,23]

The third novel mutation, c.857_858dupAA (p.Ala287Lysfs*3), was identified in a girl with acquired microcephaly and a mild developmental delay manifesting since birth. The regression period with loss of acquired speech and motor functions (e.g., independent sitting and walking, purposeful hand use) occurred at the age of 15 months. Stereotypic hand movements have been present since the age of 2 years and later epileptic seizures and breathing disturbances developed. Her eye contact and social skills improved gradually and the girl fulfills the diagnostic criteria for classic RTT. The mutation causes premature translation termination in the TRD, and presumably impairs interactions of the mutated MeCP2 protein with other cofactors.

The girl carrying the last novel mutation, c.1163_1200del38 (p.Pro388Glnfs*4), shows a milder atypical RTT phenotype (Zappella variant), with a normal head circumference, and regression onset occurred at the age of 3 years. At the age of 4.5 years, she was able to walk independently and use simple words. Her purposeful hand use has been partially conserved, and according to her parents, stereotypic hand movements occur only when she is agitated or nervous. The patient exhibits some breathing difficulties, but no epileptic seizures. The mutation is located in the CTD, where numerous small deletions occur, accounting for ~10% of *MECP2* mutations altogether. C-terminal deletions generally tend to yield a milder phenotype with a preserved ability to walk and use some words, as observed in our patient.[14,23]

The clinical severity varies among patients with RTT and can be primarily attributed to the type and location of the *MECP2* mutation. Several general trends have been confirmed for the most common mutations.[14,20,22,23] Regarding the less frequent mutations, determination of genotype–phenotype correlations is often difficult owing to the limited number of patients with the same mutation. A bias caused by

Table 3 The most common *MECP2* mutations among Czech patients with RTT and Rett-like features

	Classic RTT	*Atypical RTT*	*Rett-like features*
p.Thr158Met	11 (17.5%)	1 (5.6%)	0 (0%)
p.Arg255*	9 (14.3%)	0 (0%)	0 (0%)
p.Arg306Cys	5 (7.9%)	1 (5.6%)	1 (0.3%)
p.Arg133Cys	3 (5.2%)	1 (5.6%)	1 (0.3%)
p.Arg106Trp	4 (4.8%)	0 (0%)	0 (0%)
p.Arg270*	3 (4.8%)	0 (0%)	1 (0.3%)
p.Pro389*	2 (3.2%)	2 (11.1%)	1 (0.3%)

Abbreviations: MECP2, methyl-CpG-binding protein 2; RTT, Rett syndrome.
Number of cases and calculated frequencies for common mutations in each clinical group are presented. The mutations are arranged according to their frequency among classic RTT patients.

Table 4 Results of X inactivation in 62 patients with confirmed *MECP2* mutation

	Not informative	*Random XCI*	*Skewed XCI*	*Extremely skewed XCI*
Classic RTT (52 patients)	8 (15.4%)	33 (63.5%)	9 (17.3%)	2 (3.8%)
Atypical RTT (7 patients)	2 (28.6%)	5 (71.4%)	0 (0%)	0 (0%)
Rett-like features (2 patients)	1 (50%)	0 (0%)	1 (50%)	0 (0%)
Autism (1 patient)	1 (100%)	0 (0%)	0 (0%)	0 (0%)

Abbreviations: MECP2, methyl-CpG-binding protein 2; RTT, Rett syndrome; XCI, X-chromosome inactivation.
Not informative are the samples homozygous for *HUMARA* locus. The XCI was considered to be skewed if the ratio between active and inactive alleles was higher than 75% and extremely skewed if the ratio was higher than 95%.

individual differences that often present in RTT patients hampers the ability to confirm such associations statistically in small patient cohorts. These variations are likely caused by other factors, such as XCI and other genetic and epigenetic modulators. Additionally, incomplete manifestation of all stages of RTT in very young patients requires repeated clinical follow-up evaluations of later-occurring symptoms. We attempted to perform a genotype–phenotype evaluation in our cohort, although the groups of patients with the same mutation were relatively small.

The most common *MECP2* mutation, p.Thr158Met, was present in 12 patients (11 classic RTT, 17.5%; 1 atypical RTT, 5.6%). The mutation is located in the MBD and is often present in classic RTT cases with an intermediate severity of clinical symptoms.[22] Despite the subtle variability in the severity of individual symptoms, all but one of the girls could be described as displaying classic RTT. The only patient with different clinical severity was a girl, who showed a more severe atypical RTT phenotype, with delayed psychomotor development from birth, developmental arrest at the age of 3 months and no speech or purposeful hand use. The mean age at the onset of regression in the rest of the patients with p.Thr158Met was 17.5 ± 2.5 months.

The p.Arg133Cys mutation generally tends to be associated with a milder phenotype and is often present in patients with the preserved speech (Zappella) variant of RTT.[41] These patients usually exhibit a normal head circumference, delayed onset of regression, and better hand use, ambulation and language skills. The p.Arg133Cys mutation preferentially impairs binding to 5-hydroxymethylcytosine rather than 5-methylcytosine, which allows the MBD to function partially and therefore inflicts a less severe phenotype.[42] A similar trend was observed in only two out of five Czech girls with this mutation, only one of whom presented preserved speech. This girl was diagnosed with atypical RTT (Zapella variant). The other girl with milder overall phenotype did not fulfill the diagnostic criteria for RTT and remaining three girls with p.Arg133Cys were diagnosed with classic RTT.

Other missense mutations located in the MBD (p.Arg106Trp, p.Arg106Gln, p.Leu108His, p.Ala131Asp, p.Pro152Arg, p.Asp156Glu) conferred a moderate-to-severe classic RTT phenotype in our cohort. These mutations likely interfere with MeCP2 binding to methylated DNA and consequently affect transcriptional regulation. Our observations are also in accordance with previous studies that have shown more severe clinical features in patients with the p.Arg106Trp and p.Pro152Arg mutations.[22,43]

Girls with early truncations located upstream of or within the TRD (p.His52Argfs*16, p.Glu63Aspfs*27, p.Tyr141*, p.Lys144*, p.Arg168*, p.Ser194Alafs*16, p.Ser204*) and those with mutations located in the NLS region (p.Arg255*, p.Arg270*) were the most severely affected in our cohort. These mutations abolish the ability of MeCP2 to relocate to the nucleus, which explains their marked impact on the clinical phenotype. The average age at regression onset among 18 classic RTT patients with early truncations was 16 ± 4.1 months, and many patients were non-ambulant and showed poor hand use, dominant stereotypic hand movements and severely affected communication skills. Two girls with above-mentioned novel mutations p.His52Argfs*16 and p.Ser194Alafs*16 presented severe atypical RTT phenotypes. One girl had a severe intellectual disability and did not fulfill the diagnostic criteria for RTT. She carried the mutation c. Arg270*. However, we also observed two exceptions: (1) the mutation p.Arg168*, which is one of the most severe, was present in a girl with a clinical diagnosis of atypical autism and a considerably milder phenotype than the other Czech girls with p.Arg168*. This finding contributes to the growing number of "non-Rett" cases with *MECP2* mutations and links MeCP2 to other autism spectrum disorders. (2) The mutation p.Gly269Alafs*20, which is situated in the NLS region, was present in a girl with a very mild atypical (Zappella) variant of RTT. Recently, Das *et al.*[43] described two patients with this mutation, one of whom exhibited a milder phenotype, whereas the other exhibited a severe phenotype. These differences in phenotypic expression in our patients could not be explained by a skewed XCI; therefore, involvement of other factors is plausible.

Frameshift mutations p.Gly273Argfs*52, p.Val275Glufs*57 and p.Ala287Lysfs*3 were associated with a classic RTT phenotype of intermediate severity. These mutations are located in the TRD after the NLS. The transport of the MeCP2 protein to the nucleus and its ability to bind DNA should be unaffected owing to the presence of an intact MBD and NLS, but its interactions with corepressor complexes are abolished.

The mutation p.Arg294* belongs to the eight most common *MECP2* mutations, but it was not especially frequent in our patients, although it was recurrent (2 classic RTT girls, 3.2%; 1 girl with intellectual disability and Rett-like features, 0.3%). This mutation is located at the end of the TRD, explaining its milder phenotypic outcome, which is similar to that of p.Arg133Cys and C-terminal truncations.[14,20,22] In our cohort, p.Arg294* and C-terminal truncations were also associated with less severe phenotypes, especially in terms of delayed regression (26.6 ± 6.4 months), a later onset of stereotypic hand movements and better ambulation and hand use. Two girls with C-terminal deletions showed a skewed XCI, but their phenotype (both girls were diagnosed with classic RTT) was not significantly different from the rest of the group.

Girls with the p.Arg306Cys mutation presented varying levels of severity regarding the overall phenotype and individual characteristics. The mutation was the third most common *MECP2* mutation among Czech patients; it was identified in five classic RTT girls (7.9%), one atypical RTT girl (5.6%) and one girl with intellectual disability and Rett-like features (0.3%). This mutation interferes with the transcriptional regulation properties of MeCP2 via inhibition of the activity-dependent phosphorylation of Thr308 and prevention of binding of the corepressor complex N-CoR/SMRT.[44,45] The assessment of this mutation in genotype–phenotype analyses is equivocal. Some studies show it to induce a more severe phenotype, with an absence of speech and affected behavioral characteristics,[14,23] whereas others tend towards the opposite conclusion.[20,46]

The inactivation of one of the X chromosomes renders females mosaic for two cell populations. This phenomenon is of a special importance in X-linked dominant disorders. A milder phenotype can be expected if skewing favors expression from an X chromosome with a normal allele. Several girls in our cohort (9 classic RTT, 17.3%; 1 Rett-like features, 50%) exhibited skewed XCI in the blood, and we evaluated the possible association between the XCI pattern and clinical severity. Only two classic RTT girls presented with a different phenotype than could be theoretically expected according to the causative *MECP2* mutation. We found extremely skewed XCI ($\geqslant$95%) in two other classic RTT girls, but their phenotypes did not display significant deviations in clinical severity. Our results show that there are limitations of XCI in explaining the different phenotypic manifestations of RTT. The most widely used *HUMARA* assay[28] cannot be applied if the patient is homozygous at the locus, which occurs in ~10–20% of cases. Analyses of other loci may be applicable in such cases.[47] Another element is that the XCI ratio determined in the blood may not be the same as in the brain, and other factors, such as modifier genes,[48–50] should be considered.

In conclusion, our report highlights the wide spectrum of *MECP2* mutations associated with RTT and Rett-like phenotypes, and the novel mutations described herein provide an additional contribution to the diverse group of known molecular defects in the *MECP2* gene. HRM analysis of the *MECP2* gene, as described in this study, represents a reliable, cost-effective and time-saving alternative to other screening methods that are currently used for *MECP2* mutation scanning before sequencing. The determination of genotype–phenotype correlations and the contribution of XCI to the severity of clinical symptoms can serve as the basis for further developments in clinical management and counseling. However, the existence of individual cases with differences in clinical severity emphasizes the need to proceed with caution when predicting the future progression of clinical manifestations in addition to providing the basis for further investigations of additional modifiers.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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