Original Article

Clinical profiles of four patients with Rett syndrome carrying a novel exon 1 mutation or genomic rearrangement in the *MECP2* gene

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Rett syndrome (RTT) is a neurodevelopmental disorder caused by mutations in the X-linked *MECP2* gene encoding methyl CpG binding protein 2 (MeCP2). Recently, a new isoform of MeCP2 including exon 1 was identified. This new isoform is more abundantly expressed in brain than the isoform including exons 2–4. Very little is known about the phenotypes associated with mutations in exon 1 of *MECP2* since only a limited number of RTT patients carrying such mutations have been identified so far. In this study, we screened a cohort of 20 girls with RTT for exon 1 mutations by sequencing and multiplex ligation-dependent probe amplification (MLPA). We identified one girl with a novel exon 1 mutation (c.30delCinsGA) by sequencing and three with genomic rearrangements by MLPA. Comparison of the phenotypes showed that the girls carrying a mutation or rearrangement encompassing exon 1 were more severely affected than the girls with rearrangements not affecting exon 1.

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Rett syndrome (RTT; MIM 312750) is a neurodevelopmental disorder with onset in early childhood that almost exclusively affects females. After a period of apparently normal development, affected girls present with developmental arrest, usually by 6–18 months of age. This short period of stagnation is followed by deterioration with regression of speech and motor skills, coincident with the onset of hand stereotypies, leading to loss of purposeful hand use, which is the hallmark of the disorder. Additional characteristics include delay in head growth, gait ataxia and apraxia, respiratory dysfunction, and seizures. Following this period of rapid deterioration, patients often stabilize and may recover some skills. Girls with RTT often survive into adulthood.

Rett syndrome is caused by mutations of the *MECP2* gene encoding methyl CpG binding protein 2 (MeCP2) (1). This gene is located on the long arm of the X chromosome (Xq28) and is subject to X-inactivation. It consists of four exons, is ubiquitously expressed and acts as a methylation-dependent transcriptional repressor (1, 2). In addition, MeCP2 has recently been shown to be involved in the regulation of splicing (3). Furthermore, *MECP2* is not only implicated

in RTT but in a broad range of neurological disorders. Specific mutations in *MECP2* or large duplications including *MECP2* have been associated with mild mental retardation in females and severe retardation as well as progressive neurological symptoms in males (4–6).

The effects of *MECP2* mutations on the pathogenesis of RTT are not well understood, although large numbers of mutations have been reported. Until recently, *MECP2* was thought to produce a single transcript with a translation start site in exon 2, and it has been shown that about 80% of patients with clinically defined RTT harbor mutations in the coding region of exons 3 and 4 (7, 8). About 15% of patients carry large deletions of *MECP2* (9–11). These detection rates are influenced by the criteria applied to select the patients, and the percentage of deletions might be even higher in classic RTT (12). The high frequency of rearrangements reflects the presence of repetitive sequence elements particularly at the C-terminus of *MECP2* as well as the recombinogenic nature of the Xq28 genomic region more generally (13). So far, no mutations have been identified in exon 2 and since exon 1 was previously considered to be non-coding, it had not been included into routine *MECP2* analysis.

Surprisingly, however, a new *MECP2* transcript including exon 1 was identified in 2004 (14, 15). This novel transcript variant, termed MeCP2_e1, is generated by skipping of exon 2 and usage of an upstream start codon resulting in a transcript containing exons 1, 3, and 4. It encodes a protein that is 12 amino acids longer than the one encoded by the exon 2-containing transcript (MeCP2_e2) (Fig. 1). The two splice variants also differ in translation efficiency and are expressed at different relative amounts in different tissues, with the MeCP2_e1 isoform being more prevalent in brain (14, 15). Increasing evidence suggests that the novel transcript may play an important role in the pathogenesis of RTT. However, since only few patients with mutations involving exon 1 have been reported so far (Table 1), this hypothesis expects proof.

The aim of the present study was to identify novel mutations in exon 1 of *MECP2* and to assess the detailed clinical profiles of patients carrying such mutations.

Materials and methods

Selection of patients and assessment of clinical data

Twenty girls with a clinical diagnosis of RTT were selected for this study. Twelve girls met the criteria for classic RTT and eight for variant RTT, according to the revised clinical criteria for diagnosis of RTT described by Hagberg et al. (16). In these girls, previous sequencing of exons 2–4 had not revealed any mutations. The girls belonged to a larger cohort of affected individuals of north-eastern Switzerland and were examined at several time points during development by one of the authors. Missing data on development were collected from medical records. The first examination usually took place before the age of 18 months and subsequent clinical follow-up occurred once or twice a year. Depending on the age of the patient, the period of observation covered 4–19 years. For all four patients in whom we identified a mutation, severity was scored using the guidelines for reporting clinical features in cases with *MECP2* mutations (17) (Table 2). Scoring was related to the entire period of observation of an individual and not to a given time point of assessment.

Molecular analysis

Informed consent of the parents was obtained prior to molecular testing. Patients' DNAs were screened for the presence of mutations within exon 1 by DNA sequencing as well as for large deletions by multiplex ligation-dependent probe amplification (MLPA). X-inactivation patterns were determined using the polymerase chain reaction (PCR)-based androgen receptor assay. Total DNA prepared from peripheral blood leukocytes according to standard procedures was used as template for the molecular analyzes.

Exon 1 was amplified and sequenced using 1× Q-solution (Qiagen, Hilden, Germany) and the previously reported primers X1F and X1R with a final concentration of 4 and 0.8 μM of each primer, respectively (14, 18). PCR amplifications, PCR product purification, and cycle sequencing were performed under routine conditions (details are available upon request). The detected mutation was verified by repeated sequencing on newly amplified PCR product.

Multiplex ligation-dependent probe amplification was carried out with 100 ng DNA using the SALSA P015B and/or P015C kit, commercially available from MRC-Holland (Amsterdam, the Netherlands), on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) according to the manufacturers' instructions. Alterations were suspected if a relative peak area of a probe target sequence deviated more than 30% from the female control. Alterations were verified by repeated (greater than four times) MLPA analysis using both P015B (four
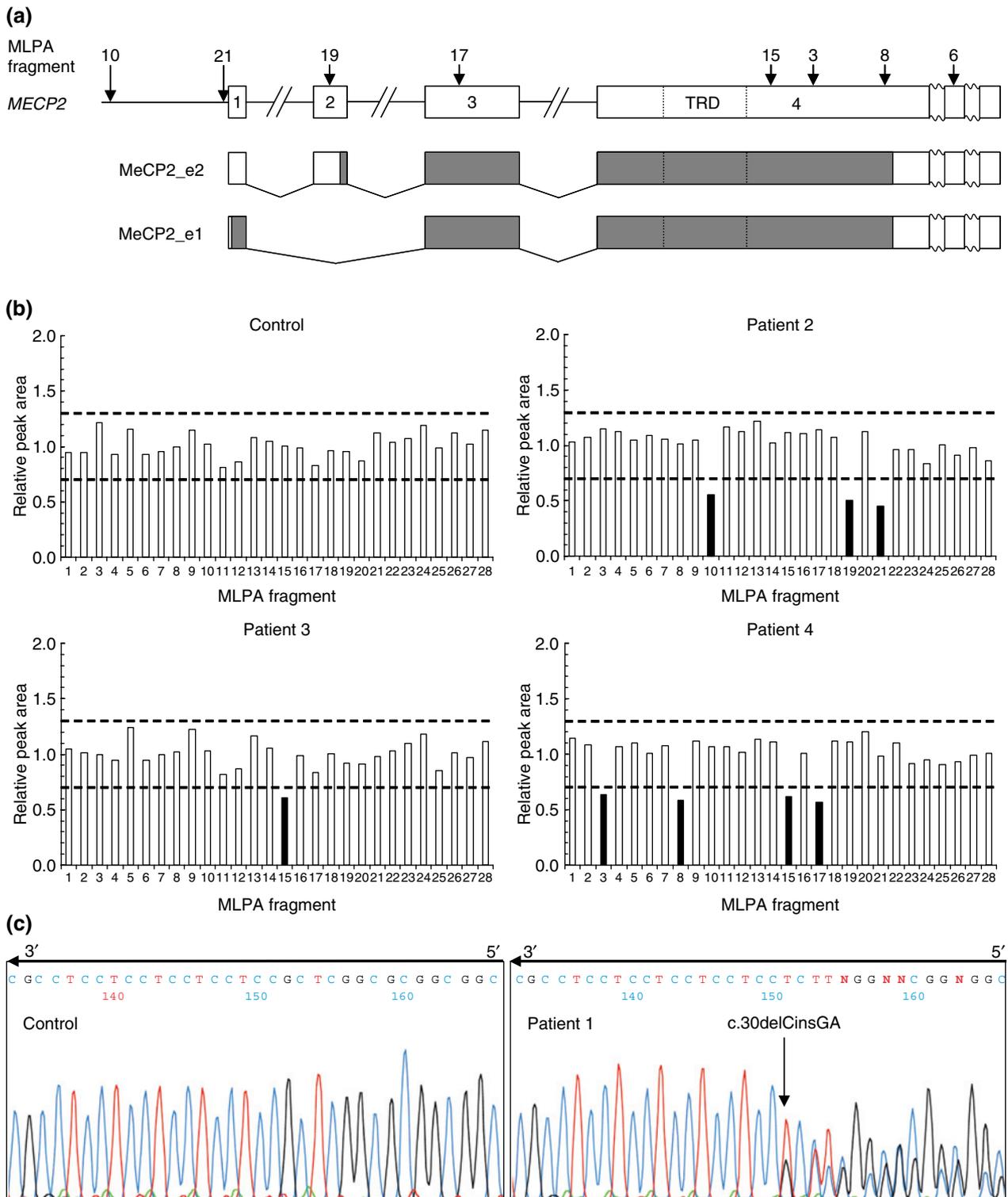
Fig. 1. MECP2 mutations identified in this study by multiplex ligation-dependent probe amplification (MLPA) analysis (a, b) and sequencing (c). The binding sites of *MECP2*-specific MLPA probes, the Transcription Repression Domain (TRD) as well as the structure of both MeCP2 isoforms (MeCP2_e1 and MeCP2_e2) are displayed schematically, representing the coding region with gray boxes. The 3′ untranslated region is not displayed in scale, as indicated by the symbols flanking the binding site of MLPA probe number 6. The normal range of normalized MLPA signals (white bars) is given by dotted lines (±0.30) and signals out of this range are marked by black bars. The direction of translation is indicated with an arrow (5′-3′) above the sequence of exon 1 (control and patient 1).

Table 1. Mutations affecting exon 1 of the *MECP2* gene in Rett syndrome (RTT)

Mutation		Patient		
Type[a]	Detection rate[b]	Age (year)	Phenotype[c]	Reference
c.3_4insGCCGCC[d]	1/97	–	classic RTT	Evans et al. (23)
c.23_27dup5nt (p.Ser10ArgfsX36)	1/10	25	classic RTT	Ravn et al. (24)
c.30delCinsGA (p.Ser10ArgfsX32)	1/20	19	classic RTT	This study (patient 1)
c.47_57del11nt (p.Gly16GlufsX22)	1/19	27	typical RTT	Mnatzakanian et al. (14)
c.47_57del11nt (p.Gly16GlufsX22)	1/10	37	classic RTT	Ravn et al. (24)
c.47_57del11nt (p.Gly16GlufsX22)	1/63	–	atypical RTT	Amir et al. (18)
c.47_57del11nt (p.Gly16GlufsX22)	1/20	13	atypical RTT	Saxena et al. (19)
c.62+1delGT	1/63	8	classic RTT	Amir et al. (18)
Deletion exon 1	1/19	20	typical RTT	Mnatzakanian et al. (14)
Deletion exon 1	1/90	14	atypical RTT	Archer et al. (12)
Deletion exons 1–2	3/90	4, 16, 45	classic RTT	Archer et al. (12)
Deletion exons 1–2	1/45	28	classic RTT	Ravn et al. (11)
Deletion exons 1–2	1/11	–	classic RTT	Erlandson et al. (9)
Deletion[e] exons 1–2	1/20	7	classic RTT	This study (patient 2)

[a]Novel mutations found in this study are indicated with boldface type.
[b]Detection rate (#1/#2) = number of cases with the respective mutation (#1) among patients previously tested negative for mutations (#2).
[c]RTT = Rett syndrome.
[d]c.3_4insGCCGCC found in a classical RTT patient and her unaffected mother has been predicted by Evans et al. (23) to be non-pathogenic.
[e]Based on decreased relative peak heights of corresponding MLPA fragments.

of 20 probes for *MECP2*) and P015C (eight of 28 probes for *MECP2*) kits.

The androgen receptor (*AR*) assay was performed according to standard methods. Briefly, *Hpa*II-digested and mock-digested genomic DNA samples were subjected to PCR amplification using primers specific for a segment of the *AR* gene. The primers encompass a region of the *AR* gene that contains a highly polymorphic microsatellite and a critical *Hpa*II site. The

Table 2. Clinical profiles of patients with Rett syndrome (RTT) carrying a *MECP2* mutation identified in this study

	Patient 1	Patient 2	Patient 3	Patient 4
MECP2 mutation	c.30delCinsGA	del exons 1–2[a]	del exon 4[a]	del exons 3–4[a]
Period of clinical follow up	until age of 19 years	until age of 7 years	until age of 4 years	until age of 14 years
Head circumference during 1st year	1	1	1	1
Development from birth to 12 months	1	1	1	1
Present head circumference	2	2	2	0
Weight (kg)	2	2	2	0
Height (cm)	2	2	2	1
Muscle tone	2	2	2	1
Spine posture	2	1	2	0
Joint contractures	2	1	1	1
Gross motor function	2	1	1	1
Hand stereotypies	2	2	2	2
Other involuntary movements	2	1	0	0
Voluntary hand use	2	2	2	1
Oro-motor difficulties	2	2	2	2
Intellectual disability	2	2	2	2
Speech	2	2	2	2
Epilepsy	2	2	0	0
Disturbed awake breathing rhythm	1	2	2	0
Peripheral circulation of extremities	2	1	1	0
Mood disturbances	2	2	0	2
Sleep disturbances	2	2	0	2
Total score[b]	37	33	27	19

[a]Deletion (del) is assumed based on decreased relative peak heights of corresponding MLPA fragments.
[b]Scoring of 20 features commonly associated with RTT was done according to previously published guidelines (17). Two points were given when the abnormality was severe, one if it was perceptible but not extreme and none if there was no abnormality. To take present head circumference as an example: Below third centile: 2 points, third to 10th centile: 1 point, above 10th centile: 0 points. Maximum score was 40 points. Age must be taken into consideration because of changes in function over time.

relative amount of each allele was calculated according to the peak areas obtained after separation of the PCR products using an ABI Prism 310 Genetic Analyzer.

Results
Patient 1, 19 years of age

Molecular analysis
Sequencing showed c.30delCinsGA, a combination of deletion and insertion in exon 1 (Fig. 1), which is predicted to lead to a frameshift and a premature stop codon (p.Ser10ArgfsX32). Neither of the unaffected parents harbored this mutation.

X-inactivation studies demonstrated borderline skewing (70%:30%) with preferential activation of the paternal allele.

Clinical course
Spontaneous delivery 2 weeks before term, birth weight, length, and head circumference were between the 10th and 50th centile. Mild developmental delay was noticed during the first year of life. The girl learned to sit unaided and used two words at the age of 12 months. Normal hand use (palmar and pincer grasp) until the age of 24 months. She learned to crawl with about 9 months and pulled herself to stand at the age of 15 months but she did not learn to walk. A hiatal hernia with reflux was diagnosed at 18 months, and she developed severe feeding difficulties. She discontinued to use of the words mama and papa and displayed stereotypic body movements (head banging). Deceleration in rate of head growth became evident. Weight and length dropped below the third centile at the age of 2 years, head circumference at the age of 2½ years. She developed stereotypic hand movements with loss of purposeful hand use between the second and third year of age. At the same time, regression of motor ability occurred and she was not able any longer to sit or stand unaided. Scoliosis was noticed at the age of 3 years and she developed severe sleeping disturbances. Epileptic seizures started at the age of 4½ years and were poorly controlled. During the following years, the girl remained bound to the wheelchair and was not able to sit or stand unaided. She developed severe joint contractures and generalized muscular wasting. Scoliosis was progressing, leading to displacement of the lower rib margin into the pelvis (parents refused surgery). She did not display any purposeful hand use and had no speech at all. Menarche occurred at the age of 17 years. At that time, her weight was 23 kg and the height/length could not be determined due to the severe scoliosis. The patient died at the age of 19 years from pneumonia.

Severity score
Completion of scoring revealed 37 out of 40 points, indicating severe abnormality in 17 out of 20 features (Table 2). The high score reflects the very severe phenotype and the long follow-up, covering 19 years.

Patient 2, 7 years of age

Molecular analysis
Multiplex ligation-dependent probe amplification resulted in decreased relative peak heights of fragments corresponding to exons 1 and 2, suggesting the deletion of these exons (Fig. 1). The alteration was not detected in the DNAs of the unaffected parents.

X-inactivation studies revealed clear skewing (93%:7%) with preferential activation of the maternal allele.

Clinical course
Delivery occurred at term by cesarian section due to breech presentation. Birth measurements were within normal ranges (weight and length on the 50th centile, head circumference between 10th and 50th centile). Mild dysmorphisms were noted at birth (fetal finger pads, partial cutaneous syndactyly between toes 2/3, extension deficit of the proximal interphalangeal joints). The girl was a lethargic infant, and distinct development delay was already noticed at the age of 10 months. She did not learn to sit unaided and started to shuffle with 12 months. She achieved only borderline hand use (incomplete palmar grasp, no pincer grasp) and did not produce any words. During the second year of life deceleration in rate of head growth became evident and the head circumference dropped below the third centile at 24 months. She developed stereotypic hand movements with loss of any purposeful hand use. Between the third and fourth year of life pronounced feeding difficulties became manifest and weight and length dropped below the third centile. Locomotion was severely disturbed (bound to the wheelchair). The first generalized epileptic seizures occurred at the age of 4 years. Mild scoliosis was diagnosed. At 7 years slight progress with respect to motor function was observed. She learned to walk a few steps with

support (very unsteady). She did not show any recovery of hand use nor did she develop speech.

Severity score
Scoring revealed 33 out of 40 points (Table 2), reflecting the severe phenotype of this girl.

Patient 3, 4 years of age

Molecular analysis
Multiplex ligation-dependent probe amplification revealed a decreased relative peak height for one of the three fragments corresponding to the coding region of exon 4, suggesting a partial deletion of this exon (Fig. 1). The DNAs of unaffected parents did not harbor this alteration.

X-inactivation studies revealed a random pattern (66%:34%).

Clinical course
Pregnancy was uneventful, delivery occurred by vacuum extraction 10 days after term. Birth measurements were within normal ranges (weight and length on 50th centile, head circumference between 50th and 90th centile). During the first year of life severe hypotonia was noticed (floppy infant). She learned to sit with 15 months, achieved normal hand use (pincer grasp), but did not use any words at all. Deceleration in rate of head growth became manifest at 16 months and head circumference dropped below the third centile at 2 years. At the same age stereotypic hand movements, major feeding problems, and breathing irregularities became apparent. Weight went below the third centile a few months later. Scoliosis was diagnosed at the age of 3 years, height dropped below the third centile at the same age. No epileptic seizures were reported until the age of 4 years. She never creeped and was able to stand with support at 3 years of age. At present, she shows marked gait ataxia but is able to walk without support. She does not display purposeful hand use and does not speak at all.

Severity score
Scoring revealed 27 out of 40 points, indicating intermediate severity (Table 2). However, the relatively low score might underestimate clinical severity because of the young age. It is likely that this girl will develop some other features associated with RTT in the future (e.g. epileptic seizures).

Patient 4, 17 years of age

Molecular analysis
Multiplex ligation-dependent probe amplification showed decreased relative peak heights of fragments corresponding to exon 3 and the coding region of exon 4, suggesting at least partial deletion of these exons (Fig. 1). Only the mother's DNA was available for MLPA analysis and revealed a normal profile.

This patient exhibited skewed X-inactivation (83%:17%) with preferential activation of the maternal allele.

Clinical course
Pregnancy was uneventful, spontaneous delivery occurred 12 days after term. Birth measurements were within normal ranges (weight and length on 10th centile, head circumference between 10th and 50th centile). The girl was described as being an unresponsive infant, and delay of gross motor function and language became already apparent during the first year of life. However, she displayed normal hand use (reaching objects, pincer grasp) at 12 months. During the second year of life marked autistic behavior with lack of eye contact became apparent; she did not speak and developed stereotypic hand movements. She lost pincer grasp but kept purposeful hand use. She learned to sit at the age of 18 months and to crawl at 20 months. Free ambulation was achieved by the age of 26 months. At the same time the parents observed chronic vomiting, irritability, and long-lasting crying episodes. Deceleration in rate of head growth became apparent between the second and third year of life, but head circumference did not drop below the third centile. At 4 years, acceleration in rate of head growth occurred and head circumference remained stable between the 10th and 50th centile from then onwards. She gained a lot of weight at the age of seven years, but the height remained stable between the third and 10th centile. At the age of 14 years, she was not able to speak any words, but displayed normal hand use and free locomotion. She never suffered from epileptic seizures and had not developed scoliosis up to this age. No follow-up could be obtained after the age of 14 years.

Severity score
This girl obtained 19 out of 40 points, reflecting a mild phenotype with near-normal hand use, locomotion and lack of microcephaly (Table 2). Since the follow-up period covered 14 years, the low score is most likely a reliable indication for the low severity.

Discussion

In the current study, we investigated the effect of distinct novel mutations of the human *MECP2* gene on RTT phenotypes. Firstly, we were interested whether mutations of exon 1 lead to different clinical profiles from those affecting other parts of the gene. Secondly, we wanted to study the effect of large deletions on RTT phenotypes. Out of 20 patients with classic or variant RTT, we identified one girl with an exon 1 insertion-deletion mutation and three girls carrying genomic rearrangements affecting exons 1–2, 3 and/or 4 (Fig. 1). Our results thus extend the spectrum of *MECP2* mutations associated with RTT.

Several groups have screened girls with RTT for mutations within the novel MeCP2_e1 transcript. However, only 7 patients harboring point mutations within exon or intron 1 have been identified so far, and information about their phenotypes is scarce (Table 1). Four out of the seven patients published until now carry the same 11-bp deletion (c.47_57del) and it is interesting to note that classification of the four girls does not match (two of them were classified as classic or typical and two as atypical RTT). The exon 1 mutation we identified in patient 1 is a combination of a deletion and insertion (c.30delCinsGA) and it has not been described before. It is predicted to induces a frameshift and a premature stop codon, very similar to the above mentioned 11-bp deletion and the recently reported small duplication (c.23_27dupCGCCG) in a patient with classic RTT (Table 1). From a clinical point of view, our patient showed a classical course of RTT during the initial stages (Table 2). Interestingly, however, she did not show any stabilization or recovery of motor function at later stages but exhibited pronounced delay in somatic growth and wasting, leading to a particularly severe end stage form of RTT with death at the age of 19 years.

Our second patient (patient 2) carries a genomic rearrangement encompassing exons 1 and 2, most likely affecting both MeCP2 isoforms at the N-terminus. To our knowledge, only five patients harboring similar mutations have previously been reported (Table 1). All where classified as classic RTT. Patient 2 is a seven year-old girl. In contrast to patient 1, developmental delay became obvious very early in this girl. She never learned to sit unaided, achieved only borderline hand use during the first year of life and did not speak any words.

Patients 3 and 4 carry a rearrangement of exon 4 and exons 3–4, respectively, potentially leading to C-terminal deletion or early truncation of both MeCP2 isoforms. Interestingly, the girl with the early truncation mutation (patient 4) showed a much milder phenotype than the girl with the C-terminal deletion (patient 3) and by far the mildest phenotype of all girls reported in this study. She developed neither microcephaly nor epileptic seizures. Somatic growth did not decelerate and no wasting occurred.

Comparison of the clinical courses of the four patients in this study shows that the girls carrying a mutation involving exon 1 (patients 1 and 2) are more severely affected than the girls with rearrangements not affecting exon 1 (patients 3 and 4). We are aware of the fact that the number of exon 1 mutations reported herein is far too small to draw final conclusions with respect to genotype–phenotype correlations. One could speculate, however, that the putative severe effect of exon 1 mutations is due to the fact that the isoform with translation start in exon 1 (MeCP2_e1) is more abundantly expressed in brain than the exon 2 containing transcript (MeCP2_e2). Specific disruption of MeCP2_e1 might therefore exert a particularly deleterious effect on neurological function and might indeed be sufficient to cause RTT. This hypothesis is supported by two recent publications on the effect of exon 1 mutations on transcription and translation of MeCP2 (19, 21). The authors show that mutations affecting exon 1 do not only disrupt the MeCP2_e1 isoform but also abolish translation (but not transcription) of MeCP2_e2. These novel findings on translational interference might explain the severe phenotypes of the girls carrying mutations of exon 1, because they lead to the suggestion that exon 1 mutations are associated with total lack of MeCp2 protein from the mutated allele.

We are aware of the fact that X-inactivation has to be taken into account if we would draw any conclusions about phenotype-genotype correlation (20). Although we performed X-inactivation studies, we think that the interpretation of these results is very delicate, because the pattern found in peripheral blood leukocytes (skewed in patients 2 and 4) might not necessarily reflect the pattern in brain tissue. Furthermore, it remained undermined whether the mutations identified occurred on the active or inactive allele.

Our data emphasize the need for further clinical studies to elucidate the role of *MECP2* in the pathogenesis of RTT. One major point will be the use of generally accepted scoring systems in order to reliably compare the phenotypic features of different individuals. This point is particularly challenging in RTT since the clinical features

change over time. The scoring applied in this study does therefore not allow direct comparison between girls of different ages, because it leads to underestimation of severity in younger girls. In our opinion, however, this was the most appropriate procedure to assess the disease profile of a given individual. Standardized assessment of clinical profiles needs even further attention since mutations in *MECP2* are not only linked to RTT, but to a broad spectrum of human developmental disorders, including mild mental retardation, Angelman-like syndrome, autism and severe mental retardation with spasticity in males (reviewed in Ref. 22). Appropriate classification of *MECP2* mutations and assignment to a given disorder will be crucial to provide adequate genetic counseling to the families.

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