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Influence of Mutation Type and X Chromosome Inactivation on Rett Syndrome Phenotypes

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We screened 71 sporadic and 7 familial Rett syndrome (RTT) patients for *MECP2* mutations by direct sequencing and determined the pattern of X chromosome inactivation (XCI) in 39 RTT patients. We identified 23 different disease-causing *MECP2* mutations in 54 of 71 (76%) sporadic patients and in 2 of 7 (29%) familial cases. We compared electrophysiological findings, cerebrospinal fluid neurochemistry, and 13 clinical characteristics between patients carrying missense mutations and those carrying truncating mutations. Thirty-one of 34 patients (91%) with classic RTT had random XCI. Nonrandom XCI was associated with milder phenotypes, including a mitigated classic RTT caused by a rare early truncating mutation. Patients with truncating mutations have a higher incidence of awake respiratory dysfunction and lower levels of cerebrospinal fluid homovanillic acid. Scoliosis is more common in patients with missense mutations. These data indicate that different *MECP2* mutations have similar phenotypic consequences, and random XCI plays an important role in producing the full phenotypic spectrum of classic RTT. The association of early truncating mutations with nonrandom XCI, along with the fact that chimeric mice lacking methyl-CpG-binding protein 2 (MeCP2) function die during embryogenesis, supports the notion that RTT is caused by partial loss of MeCP2 function.

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Rett syndrome (RTT), initially described by Andreas Rett in 1966,[1] is an X-linked dominant disorder. One of the leading causes of severe developmental delay in girls, it affects approximately 1 in 10,000 to 15,000 females.[2] Patients with classic RTT appear to develop normally during the first 6 to 18 months of life before entering a period of rapid regression, during which they lose acquired speech and purposeful hand use, their cranial growth slows, and they experience seizures, autistic features, ataxia, gait apraxia, and breathing abnormalities.[3,4] Repetitive hand movements—wringing, clasping, clapping, or other stereotyped motions—begin during this regression and are a hallmark of the disease. By puberty, most patients stabilize and some may recover some skills. Most survive into the third decade. There are patients with much milder phenotypes who may, for example, retain the ability to speak or walk; such patients have been described as having a variant of Rett syndrome.[5,6] Neuropathology and imaging studies show that the brains of RTT patients are smaller than normal, primarily because of decreased individual neuronal size and increased packing density (number of neurons per 0.1 mm^3).[7] Certain brain regions also show a decrease in dendritic number and arborization,[8–10] but lack of evidence of neuronal loss provided early indication that Rett is not a neurodegenerative disorder.

The recent discovery that RTT is caused by mutations in the *MECP2* gene, located on Xq28, proved the developmental origin of the disease.[11] *MECP2* encodes the methyl-CpG-binding protein 2 (MeCP2), a ubiquitous DNA-binding protein that is thought to act as a global transcriptional repressor.[12–14] The protein's 84–amino acid (aa) methyl-binding domain (MBD) binds 5-methyl cytosine (5-mC) residues in CpG dinucleotides.[15,16] The 104-aa transcriptional repression domain (TRD) interacts with the corepressor Sin3A to recruit histone deacetylases, which in turn results in

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deacetylation of core histones and transcriptional silencing.[17,18] An aberration in MeCP2 function could thus lead to dysregulation of a number of other genes.

Amir and colleagues[11] and Wan and co-workers[19] found that approximately 40% of a small group of sporadic and familial RTT patients have missense or truncating mutations. Consistent with the sporadic occurrence of RTT, most of these mutations occurred de novo at CpG mutation hotspots, and a preferential paternal origin for de novo *MECP2* mutations has been suggested.[19] All reported missense mutations change evolutionarily conserved amino acids in either the MBD or the TRD, while most nonsense and frameshift mutations truncate MeCP2 distal to the MBD. We propose, then, that RTT is due to either partial or complete loss of function of MeCP2.

Because the *MECP2* gene undergoes X chromosome inactivation (XCI),[12] it seems likely that different XCI patterns would contribute to differences in phenotype. Although several studies have shown that classic RTT patients have a random XCI pattern, there are reports of nonrandom XCI in some patients.[20–24] A nonrandom XCI pattern has been observed in 3 carrier females and in the unaffected member of a pair of monozygotic twins discordant for the Rett phenotype.[20,25–27] Two of the carrier females were found to have *MECP2* mutations, but their phenotypic manifestations were quite different: 1 had no symptoms, but the other suffered from a mild learning disability.[19,26,27]

In this study, we investigated the possibility that different types of mutations or patterns of XCI explain the phenotypic variability of Rett syndrome. We studied a large group of classic RTT patients by identifying their disease-causing mutation and XCI pattern and correlating these with a variety of phenotypic parameters. The results of these analyses may provide insight into the effect of the mutations on MeCP2 function.

Methods

Subjects and DNA Samples

Patients were evaluated at the Blue Bird Circle Rett Center at Baylor College of Medicine, Houston, TX. Blood samples were obtained from patients and their parents under a protocol approved by the Institutional Review Board of Baylor College of Medicine and its affiliated hospitals. We studied 69 sporadic patients and 8 RTT families (2 cases in each family), all of whom fulfilled the criteria for the diagnosis of Rett syndrome[4] except for one RTT variant.[5] The familial cases included 5 pairs of full sisters (unpublished), 2 pairs of half sisters, and a pair of second half cousins.[28,29] In the family with affected second half cousins, we found two independent mutations, demonstrating that these were sporadic rather than familial cases. We therefore corrected the final number of patients in each group to 71 sporadic and 7 families (14 cases).

Clinical Evaluation

We used the clinical profiles of the patients as recorded during their most recent evaluation. We noted the ethnicity and age of each patient, as well as the cause of death for those who were deceased. We evaluated common clinical features of RTT, electroencephalographic (EEG) findings, electrocardiographic (ECG) recordings (Table 1), and levels of cerebrospinal fluid (CSF) β-endorphins and biogenic amines. The clinical characteristics were scored for severity using a scale of 1–5, 0–2, or 0–3, depending on the variable. Because RTT patients have been reported to have prolonged QTc intervals, we also scored the severity of the EEG abnormalities and the length of the QTc interval on ECG.[30,31] A Composite Clinical Severity Score (CCSS) was calculated by adding scores for the 13 clinical characteristics; patients missing values for any characteristic were excluded. Finally, we evaluated the levels of CSF biogenic amines—homovanillic acid (HVA), 3-methoxy-4-hydroxyphenylglycol (MHPG), and 5-hydroxyindoleacetic acid (5HIAA)—in 29 patients and of β-endorphin in 26 patients.

Mutation Analysis

We prepared total genomic DNA from peripheral blood leukocytes (PBLs) or from lymphoblastoid cell lines (LCLs) using standard protocols.[32] Somatic cell hybrids retaining either the maternal or the paternal X chromosome were generated as described previously.[33,34] Polymerase chain reaction (PCR) amplification of *MECP2* coding exons was performed using primer pairs and conditions described elsewhere.[11,19] PCR products were purified using a Qiagen PCR purification kit (Qiagen, Los Angeles, CA). Amplimers were sequenced directly using the ABI PRISM big dye terminator cycle sequencing ready reaction kit (Perkin-Elmer, Applied Biosystems, Foster City, CA). Automated sequencing was performed on an ABI 377 DNA sequencer (Applied Biosystems). Sequences were compared with reference to human genomic and cDNA *MECP2* sequences (GenBank accession No. X99686 and AF030876) and were analyzed using GCG software, Wisconsin package version 10.0-Unix. Nucleotides are numbered from the first nucleotide of the translation initiation ATG codon.

To determine the parental origin of three de novo mutations, a similar mutation analysis was carried out on total genomic DNAs derived from somatic cell hybrids retaining either the paternal or the maternal X chromosome from three sporadic RTT patients. Restriction enzyme digestion of PCR products was performed to screen for the common R168X mutation, since the nucleotide change C to T at position 502 creates a new cleavage site for the *Hph*I restriction enzyme.[19] We also analyzed DNA samples from the parents and from normal individuals to confirm the de novo origin of mutations in the sporadic cases.

Reverse transcriptase PCR (RT-PCR) was performed to analyze the mutant mRNA from a patient with a splicing mutation. We isolated total RNA from lymphoblastoid cell lines using Trizol (Gibco-BRL, Life Technologies, Gaithersburg, MD), and reverse-transcribed 1 to 2 μg of total RNA with the Superscript II reverse transcriptase kit (Gibco-BRL) using 500 ng of random oligohexamer primers according to the manufacturer's recommendations.

Table 1. Severity Score Definition for Evaluated Variables

Variable	Score	Definition
Age at onset of regression	1	>30 mo
	2	18–30 mo
	3	12–18 mo
	4	6–12 mo
	5	<6 mo
Ambulation	0	Walks alone
	1	Walks with help
	2	Used to walk
	3	Never walked
Seizures	0	None
	1	Well controlled
	2	Uncontrolled
	3	Infantile spasms
Respiratory dysfunction	0	None
	1	Minimal cyanosis
	2	Intermittent cyanosis
	3	Constant cyanosis
Head growth	0	None to minimal deceleration
	1	Deceleration > 10th percentile
	2	2nd–10th percentile after 24 mo
	3	2nd percentile by 24 mo
	4	<2nd percentile by 24 mo
Somatic growth	0	No growth failure
	1	Mild failure
	2	Moderate failure requiring oral supplements
	3	Severe failure requiring (naso)gastric feeding
Motor	0	None to mild ↑ or ↓ tone
	1	Moderate tone change and mild ↓ ambulation
	2	Severe impairment of ambulation
Hand use	0	Feeds self
	1	Holds objects
	2	May reach for objects
	3	None
Communication	0	Makes choices
	1	Inconsistent eye gaze
	2	No communication
Autonomic dysfunction	0	None
	1	Mild color/temperature changes
	2	Moderate
	3	Severe
Electroencephalogram	0	Normal pattern
	1	Multifocal and/or slow spike
	2	Slow spike; slow background
	3	Hypsarrhythmia
QTc interval on electrocardiogram	0	<0.045 sec
	1	0.045–0.050 sec
	2	>0.050 sec
Scoliosis	0	None
	1	Minimal: <20°
	2	Moderate: 20°–70°
	3	Severe: >70°
Self-abuse	0	None
	1	Intermittent
	2	Constant
Screaming	0	None
	1	< Weekly
	2	Weekly or more

X Chromosome Inactivation Assay

The phosphoglycerate kinase gene (PGK) and the hypoxanthine phosphoribosyl transferase gene (HPRT) methylation assays were performed as described elsewhere.[20] For the androgen receptor gene (AR) methylation assay, we modified a previously described protocol.[35] Briefly, genomic DNA (1 μg) from PBLs was digested overnight with the methylation-sensitive restriction enzyme *Hpa*II (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's recommendations. Digested and undigested genomic DNA were used as a template for PCR amplification of a DNA fragment containing the *Hpa*II site as well as the highly polymorphic trinucleotide repeat in the AR gene.[35] Since the *Hpa*II sites on the AR gene are methylated on the inactive X chromosome, this is the only allele that is amplified after digestion.[35] Radioactively labeled γ-^{32}P-ATP was used in a PCR with the following conditions: 28 cycles of 45 seconds at 95°C, 30 seconds at 55°C, and 45 seconds at 72°C. PCR products were separated on 6% denaturing polyacrylamide gels and autoradiographed on Hyperfilm (Amersham International Plc, Buckinghamshire, UK). The gels were also exposed to Fuji phosphorimaging screens for 8 to 16 hours and the intensity of the bands was analyzed using a Model Storm 860 Phosphorimager with imageQuaNT software version 4.1 (Molecular Dynamics, Sunnyvale, CA). Because the smaller allele amplifies more efficiently, a correction factor was generated using the undigested samples so that both alleles were represented equally in the calculations.[36] The density of the smaller allele divided by the sum of the densities of both alleles was used to determine the pattern of XCI.[37] The density of the smaller allele is thus rendered as a percentage; if this number is 20% or less or 80% or higher, the XCI is considered skewed.[38]

Genotype–Phenotype Correlation Analysis

Patients were divided into two groups (A and B), according to whether they had a missense or a truncating mutation. The association between each group and each individual characteristic as well as the CCSS was assessed by using either a χ^2 test (when the analyzed parameter was measured as a dichotomous (present/absent) variable) or a t test (when the parameter was given a numerical value—eg, age of onset of regression, CCSS, and CSF levels of biogenic amines and β-endorphins). We controlled for subject age by using binary logistic regression for dichotomous variables and analysis of covariance for numerical variables. Most individual clinical characteristics were also measured on a more detailed scale, such as absent, mild, moderate, or severe (see Table 1). These were analyzed using ordinal logistic regression in addition to χ^2 analysis. Because data on each characteristic were not available for every patient, the number of patients for all dichotomized variables ranged from 15 to 18 in the missense group and 23 to 27 in the truncating group. This sample size is sufficient to detect important differences between the two groups with respect to the presence of a given characteristic. The power to detect a difference of 90% versus 10% occurrence of a specific characteristic between two groups with this sample size is 99% with a type I error rate of 1%. A smaller difference such as 80% versus 20% can be detected with a power of 90% and type I error of 5%. $p < 0.05$ was considered significant.

Results

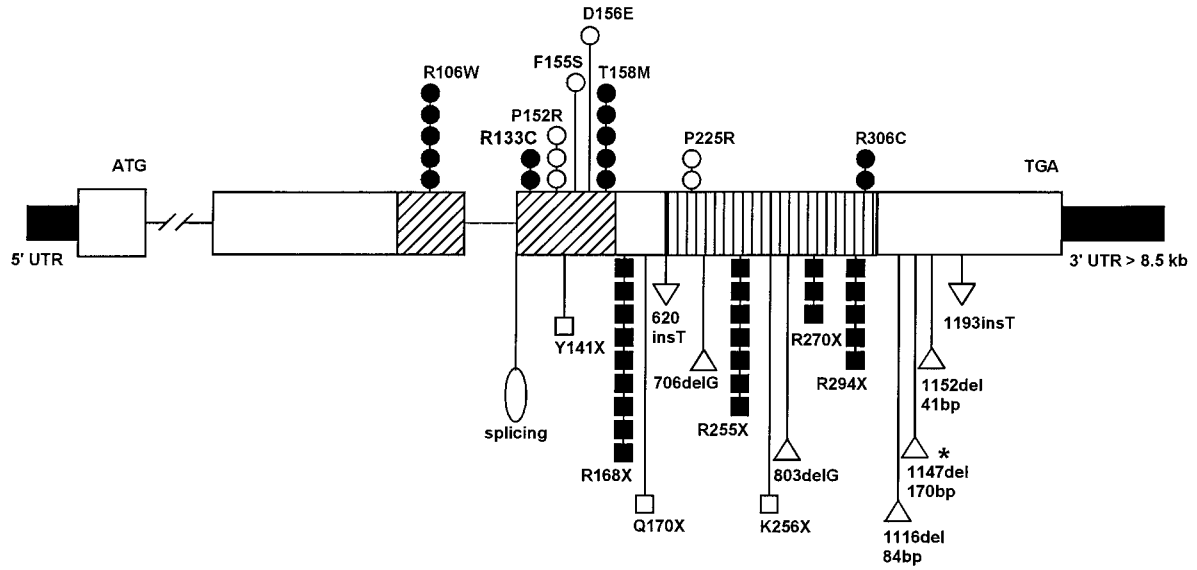
Mutational Analysis

We identified a total of 56 disease-causing *MECP2* mutations in 54 of 71 sporadic patients (76%) and in 2 of 7 (29%) familial cases (Fig 1). There were 23 different mutations: 15 (65%) truncating and 8 (35%) missense. Nine of the 23 mutations have been previously described,[11,19] and 14 are novel.

Truncating mutations occurred in 35 patients—27 nonsense, 7 frameshift, and 1 splicing mutation. All except two truncating mutations (94%) were distal to the MBD. Of the two that affected the MBD, one was a splicing mutation that changed the acceptor splice site of exon 3 and therefore is predicted to cause the earliest truncation reported yet. RT-PCR using lymphoblast RNA from this patient failed to amplify the mutant allele and yielded only wild-type sequence. The lack of amplification of the mutant allele could have been due to a nonrandom pattern of XCI in the patient's LCLs, or it may indicate that the mutant transcript was unstable. We were not able to determine which was the case because the patient was uninformative for the AR methylation assay as well as for several polymorphisms within *MECP2*. The other early truncating mutation, Y141X, caused a premature termination resulting in a protein that is missing 22 aa of the MBD. Although the truncation does not directly affect residues from the DNA-binding surface, the mutant MBD lacks several amino acids and, presumably, cannot achieve functional conformation.[39] Even though the pattern of XCI in this patient was random by conventional criteria,[38] we found a tendency for skewing (75:25) that suggests this mutation is more detrimental to the function of MeCP2. In all other truncating mutations, the MBD was left intact and termination occurred within the TRD or in the C-terminus of the protein. The truncated protein containing these mutations is predicted to bind 5-mC, but it may not interact with Sin3A or other proteins of the corepressor complex. The frameshift mutations included an insertion (T) or deletion (G) of a single nucleotide as well as larger deletions: 1116del 84 bp, 1147del 170ins 3 bp, and 1152del 41 bp.

Missense mutations, all occurring in highly conserved amino acids in the protein, were found in 21 patients. In contrast to the nonsense mutations, most of the missense mutations (81%) occurred in the MBD. The R106W and F155S mutations affect residues with long side chains that are mostly buried in the protein and thus contribute to the globular core of the MBD; the R133C and D156E mutations affect amino acids of the DNA-binding surface (Fig 2).[39] The R306C mutation in the TRD may alter the native structure of the domain or affect its interaction with other proteins. Using available DNA samples from parents and normal individuals, we confirmed the de novo origin of all mutations but one. In 1 patient, the missense mutation (P225R) was inherited from her asymptomatic mother, who has a nonrandom pattern of XCI (90:10) according to the AR methylation assay. Be-

Fig 1. MECP2 *mutations in Rett syndrome. A schematic diagram of the gene shows exons in* boxes, *noncoding regions in* black, *the methyl-binding domain in* diagonal lines, *and the transcriptional repression domain in* vertical lines. *Missense mutations are shown in* circles above *the gene; truncating mutations are shown* below *the gene. This latter group includes nonsense mutations* (squares), *frameshift mutations* (triangles), *and an* oval *for the splicing mutation. Recurrent mutations are lined together. CpG hot spot mutations are indicated by* filled *circles or* squares. **Insertion of 3 bp in the frameshift mutation 1147del 170 bp.*



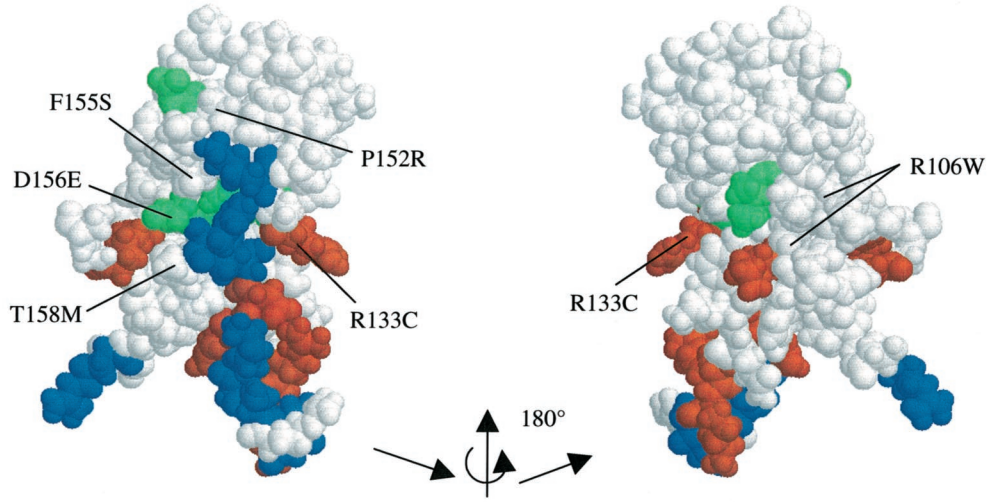


Fig 2. Missense mutations in the methyl-binding domain (MBD) of MeCP2. Rasmol software was used to visualize the three-dimensional structure of amino acids (aa) 90–164 of MeCP2, which include residues of the MBD (aa 78–162).[39] Significant conformational change induced by DNA binding is shown in green; significant chemical shift (a change in the magnetic environment) caused by DNA binding is shown in blue; and residues implicated in both are shown in red. Mutations identified in patients with Rett syndrome are indicated by lines and cluster mostly at the DNA-binding site. Both R133C and D156E affect residues implicated in DNA binding.

cause the same mutation occurred de novo in another sporadic patient and was not found in more than 100 normal chromosomes, we believe that this is a disease-causing mutation and not a rare polymorphism. Among the familial cases we identified a truncating mutation (K256X) in a pair of affected full sisters that was not detected in their mother or father, suggesting that this represents a case of parental germline mosaicism. Our previous identification of a missense mutation (R106W) in a pair of affected half sisters but not in their common mother presents another case of germline mosaicism.[11] To our surprise, we found two different nonsense mutations (R168X and R294X) in the family with a pair of second half cousins,[28] indicating that these are not true familial cases.

Ten different mutations occurred de novo in more than 1 patient or family. The R255X and the R168X mutations appeared with particular frequency (7 and 9 independent occurrences, respectively). Eight of the 10 recurrent mutations and 38 of 48 (79%) single nucleotide substitutions were C→T transitions at CpG mutation hotspots, suggesting that these are paternally derived.[40] We therefore analyzed somatic cell hybrids retaining either the maternal or the paternal X chromosome from three sporadic patients with de novo mutations. The R168X and R270X mutations were found in somatic cell hybrids retaining the paternal X chromosomes; another patient showed the R168X mutation in somatic cell hybrids retaining the maternal X chromosome. Our previous finding of the R106W mutation in two half sisters who share the same maternal haplotype near *MECP2*[11] indicates that the R106W mutation occurred in the maternal germline. Thus, C→T transitions may have either paternal or maternal origin.

X-Chromosome Inactivation

To determine whether patterns of XCI contribute to phenotypic variability in classic RTT, we evaluated XCI in 39 patients with *MECP2* mutations for whom we had peripheral blood leukocyte DNA. We demonstrated previously that 14 patients, informative for the PGK or the HPRT loci, have random patterns of XCI.[20] An additional 25 patients were analyzed using the AR methylation assay: 20 were informative for the locus and 17 of these had random patterns of XCI (Fig 3). In total, we found random XCI patterns in 31 of 34 patients (91%) and a borderline pattern of XCI (78:22) in 1 patient. For practical analysis of genotype–phenotype correlation, this patient was considered to have a nonrandom pattern to avoid bias in the analysis. One patient showed a nonrandom XCI pattern (87:13), and a patient with an inversion of the X chromosome between Xp22.2 and Xq26.1 showed a highly skewed pattern of XCI (99:1) (Amir and Zoghbi, unpublished data) (see Fig 3). Interestingly, analysis of somatic cell hybrids in the latter patient demonstrated that all hybrids retaining the active X chromosome contained the X chromosome with the inversion, which had the normal *MECP2* sequence. The mutated *MECP2* must be on the intact X chromosome that is preferentially inactivated and thus never retained in somatic cell hybrids that select for the active chromosome.

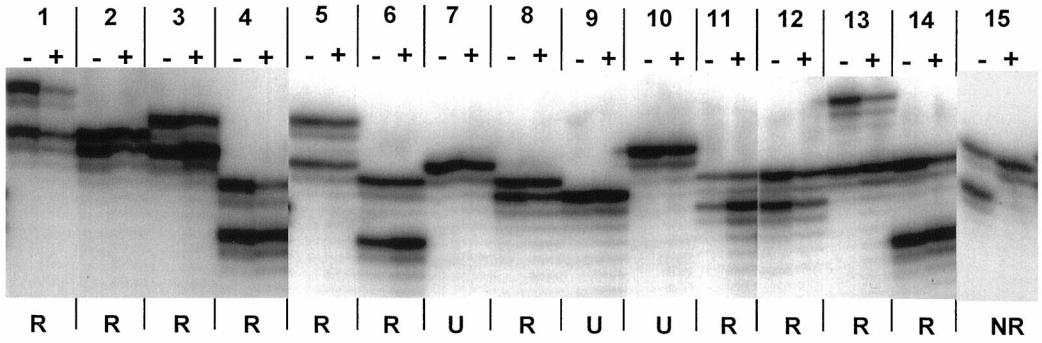


Fig 3. Analysis of X chromosome inactivation (XCI) patterns using the androgen receptor methylation assay: autoradiograph of labeled polymerase chain reaction (PCR) products from 15 patients. Peripheral blood leukocyte DNA from each patient was either used directly as a template (− lanes) or digested with Hpa*II prior to PCR amplification (+ lanes). When the patients have alleles with different size repeats (informative), the two alleles are present in the undigested samples (− lanes); after* Hpa*II digestion, the alleles from the active X will not amplify, and only alleles from the inactive X chromosome amplify and can be seen in + lanes. Patients 7, 9, and 10 are uninformative (U); patient 15 has a nonrandom (NR) pattern of XCI (99%:1%); and all other patients have a random (R) pattern of XCI.*

This XCI pattern explains the milder phenotype of this patient, who has good eye contact and normal gait.

Clinical Evaluation

Clinical data were available for 52 of 55 RTT patients in whom we found *MECP2* mutations. Since the pattern of XCI may influence the phenotype, we excluded 3 patients with nonrandom or borderline patterns of XCI from the analysis. The patient with the splicing mutation was also excluded, because we were not able to prove that this mutation truncates the protein. The mean age at the most recent evaluation was 13.0 ± 7.43 years (± SD). Of the 48 patients, there were 47 who met all criteria for classic RTT. One patient was considered to be an RTT variant because of some preserved language and hand use and a narrow-based gait atypical of RTT. Of 45 patients, 25 (56%) were able to walk independently, 5 (11%) walked with assistance, 9 (20%) had lost the ability to walk, and 6 (13%) had never walked. Data regarding seizures, respiratory dysfunction, and somatic growth were available for 44 patients. Fourteen (32%) had seizures at the time of evaluation that were not controlled by therapy, 18 (41%) had histories of seizures, and 12 patients (27%) never had seizures. Forty-one patients (93%) had some degree of breathing dysfunction characterized by hyperventilation, apnea, or both. Nineteen patients (43%) showed mild to moderate growth failure, 15 (34%) had severe growth failure, and 10 (23%) showed no significant growth failure. Thirty-six of 42 patients (86%) had clinical findings suggesting autonomic nervous system dysfunction (eg, skin color or temperature changes). Information on the presence or absence of scoliosis was available for 42 patients: 23 (55%) had mild to moderate scoliosis, 7 (17%) had severe scoliosis requiring surgical intervention, and 12 (28%) had no scoliosis. ECGs had been done on 25 patients, 5 (20%) of whom had prolonged QTc intervals (>0.45 seconds). CSF had been collected from 29 patients and analyzed for biogenic amines and β-endorphins; these results have been reported elsewhere.[41]

Genotype–Phenotype Correlation

Forty-eight patients were included in the analysis: group A consisted of 18 patients with a missense mutation and group B comprised 30 patients with a truncating mutation (Table 2). We found significant differences in the occurrence of awake respiratory dysfunction and in the levels of CSF HVA between the two groups. Awake respiratory dysfunction occurs more frequently in patients with truncating mutations than in patients with missense mutations (100% vs 82.4%; $p = 0.024$), and this group of patients has lower levels of CSF HVA ($p = 0.02$). Because respiratory dysfunction and low levels of CSF HVA can be interdependent, we analyzed the relationship between the two characteristics, but because of the small number of patients ($n = 2$) with known levels of CSF HVA and no respiratory dysfunction, we could not exclude an association between the two ($p = 0.074$). We also found an association between the presence of scoliosis and the group with missense mutations (93.3% vs 59.3%; $p = 0.019$). Other characteristics such as mortality, age at onset of regression, ambulation, seizures, and somatic growth failure were not significantly different between the two groups. We found no relationship between the CCSS and the type of mutation, nor did levels of MHPG, 5HIAA, and β-endorphins in the CSF differ significantly between patients with missense mutations and those with truncating mutations. None of these findings were significantly affected by controlling for patient age.

Table 2. Clinical and Laboratory Features versus Genotype

Feature	Patients with Missense Mutation	Patients with Truncating Mutation	*p*
Onset (mo)[a]	19.6 ± 8.1	17.6 ± 5.5	0.346
Mortality[b]	25% (4/16)	8.7% (2/23)	0.165
Able to walk[b]	61.1% (11/18)	70.4% (19/27)	0.519
Seizures[b]	76.5% (13/17)	70.4% (19/27)	0.658
Respiratory dysfunction[b]	82.4% (14/17)	100% (27/27)	0.024[d]
Somatic growth failure[b]	76.5% (13/17)	77.8% (21/27)	0.920
Scoliosis[b]	93.3% (14/15)	59.3% (16/27)	0.019[d]
CCSS[a,c]	18.4 ± 5.2	18.4 ± 5.7	0.975
CSF HVA[a] (ng/ml)	87.9 ± 15.2	65.3 ± 5.7	0.020[d]
CSF MHPG[a] (ng/ml)	8.2 ± 2.6	7.7 ± 1.6	0.521
CSF 5HIAA[a] (ng/ml)	30.6 ± 4.9	30.5 ± 16.6	0.985
CSF β-endorphins[a] (pg/ml)	125.9 ± 21.4	108.2 ± 44.0	0.293

[a]Data presented as mean ± SD.
[b]Percentage of patients with a feature with the actual number shown in parentheses.
[c]Composite clinical severity score.
[d]This *p* value was significant.

CSF = cerebrospinal fluid; HVA = homovanillic acid; MHPG = 3-methoxy-4-hydroxyphenylglycol; 5HIAA = 5-hydroxyindoleacetic acid.

Discussion

To our knowledge, this study presents the first detailed analysis of *MECP2* mutations and clinical correlations in a large number of Rett syndrome patients (71 sporadic and 7 familial cases). We established the incidence and spectrum of mutations and their phenotypic expression, taking into account the role of XCI. Extending the results of our[11] and Wan and colleagues'[19] initial studies, in which mutations were found in only 25 to 40% of a relatively small number of patients, we have shown that at least 76% of RTT patients carry *MECP2* mutations. This is a high proportion of patients, given that our analysis was limited to the coding region and did not search for large DNA rearrangements.

The distribution of missense, nonsense, frameshift, and splicing mutations within different domains of MeCP2 is unequal. All missense mutations change amino acids that are conserved among species, with most occurring in the MBD (17 of 21 patients; $p < 0.001$). As shown by our modeling, the mutations in the MBD map to the globular core or the DNA binding surface[39] (see Fig 2); we speculate that they disrupt the integrity of the domain or interfere with MeCP2's ability to bind methylated DNA. The earliest truncation mutation (Y141X) is distal to the putative DNA-binding interface of the gene's MBD. Most other truncating mutations occur in the region distal to the MBD, and all except two of these keep the MBD intact (33/35 patients vs 2/35 patients; $p < 0.001$). Although we have not yet found direct evidence that these mutations result in the expression of truncated proteins (the available antisera did not permit such analysis), this seems the most likely scenario, and such truncated proteins could still bind the methylated cytosines. Mutations involving the TRD, on the other hand, could interfere with the assembly of the transcriptional silencing complex, abolishing interactions with the Sin3A corepressor or histone deacetylase recruitment. It is not clear how mutations causing truncations distal to the TRD influence the protein's function. We speculate that they could still affect the quaternary structure of the DNA-MeCP2-Sin3A complex and thus affect the silencing complex. We take the preponderance of missense mutations in the MBD and the near absence of early truncations of the protein (17/21 patients vs 2/35 patients; $p < 0.001$) to indicate that RTT results from a partial rather than complete loss of MeCP2 function.

A high proportion of mutations involve C→T transitions at CpG dinucleotides (79% of single-nucleotide substitutions). All of these (eight different mutations) recurred in unrelated patients, reflecting the hypermutability of these sites.[42] According to our previous report on germline mosaicism in a mother of an RTT family and the results from 3 patients in this study, two C→T transitions at CpG originated in the paternal and two in the maternal germline. The number of patients studied is too small to draw conclusions, but it is interesting that half of the C→T transitions at CpG are of maternal origin.

There were several small deletions (ranging from 41–170 bp) in the region between bp1116 and 1152. This may be due to a number of palindromic and quasi-palindromic sequences within this region, which are believed to form secondary structures that render the sequences vulnerable to deletions.[43]

At this time, only the coding region has been screened for mutations. The noncoding regions and the promoter region are also possible sites for disease-

causing mutations, and large DNA rearrangements have not yet been ruled out. The 3′UTR, more than 8.5 Kb long with several evolutionarily conserved regions, may be important for posttranscriptional regulation of *MECP2*: alternate polyadenylation in the 3′UTR results in a variety of transcripts with variable abundance in the human brain during development.[14] Given that even our limited search has revealed 76% of the disease-causing mutations, we believe there is unlikely to be another major RTT locus. Adding our data to the results of Wan and colleagues,[19] *MECP2* mutations have been identified in 3 of 4 of the RTT families that were used for the exclusion mapping studies. Because the gene in these families is known to be linked to Xq28, all of them are expected to have *MECP2* mutations. The discovery of mutations in only 75% of the X-linked families perhaps indicates that some *MECP2* mutations have not yet been identified and that their real occurrence in RTT remains underestimated.

We found a random pattern of XCI in PBL-DNA in most patients in whom this could be evaluated. A previous study found that 3 classic RTT patients (2 of whom we show have *MECP2* mutations) have random patterns of XCI in brain tissue as well.[20] This finding of random XCI in brain tissue and in most samples analyzed in this study indicates that most classic RTT patients have a random pattern of XCI and that it is the expression of mutant *MECP2* in a high proportion of their cells that leads to the RTT phenotype. Conversely, a nonrandom pattern of XCI most likely protects against the consequences of *MECP2* mutations, as indicated by a totally asymptomatic female carrier who has the P225R mutation and the presence of less severe clinical symptoms in 2 of 3 patients with a nonrandom or borderline pattern of XCI. In 1 of these 2 patients we confirmed that the mutant X chromosome has been preferentially inactivated. Wan and co-workers[19] also described a patient with incomplete diagnostic features of RTT and localized skin lesions resembling incontinentia pigmenti. This patient has a mutation (411delG) that causes an early truncation of MeCP2, which should result in a severe phenotype. Her XCI status ranges from borderline (70:30) to nonrandom (90:10).[19] Recent analysis of somatic cell hybrids from this patient demonstrated that the X chromosome carrying the mutant allele is predominantly inactivated (Amir and Zoghbi, unpublished data), which could contribute to her unusual phenotype.

All except 1 of the patients for whom phenotypic data were analyzed met the necessary diagnostic criteria for classic RTT. Focusing on classic RTT patients with varying supportive criteria allowed us to evaluate whether the variability of phenotype correlated with different types of mutations. We found a positive correlation between truncating mutations and two parameters: breathing abnormalities and low levels of CSF HVA. Breathing dysrhythmia, with periods of hyperventilation or apnea (breath-holding) interrupting normal breathing, is a striking feature of RTT and occurs during wakefulness.[44] Patients with missense mutations were more likely to have scoliosis than patients with truncating mutations. The scoliosis in these patients is probably neurogenic and can be influenced by other factors. All other analyzed characteristics, including major clinical features of RTT and the degree of severity, were similar regardless of the type of mutation.

We surmise that all mutations cause at least partial loss of MeCP2 function. The human phenotypes resulting from *MECP2* mutations vary from the barely noticeable to the astonishingly severe: there have been largely unaffected females with favorable XCI who are either asymptomatic or display only the mildest learning deficit, and there have been a few males born into Rett families with such severe neonatal encephalopathy that they survived little more than 1 year. One of these males had an 803delG mutation, which causes a truncation at aa 288 in the TRD.[19] Chimeric male mice engineered to bear an early truncation of the MeCP2 protein upstream of the MBD fail to gastrulate and die in utero.[45] Why do the chimeric embryos that are mosaic for MeCP2 deficiency die in utero, while female RTT patients who are mosaic because of a random pattern of XCI survive? Humans may have some protective mechanism that rescues them from lethality; alternatively, the human patients we see survive because they retain some *MECP2* function. Our study found no early truncating mutations that would definitely cause a complete loss of function. The viability, however short-lived, of the human male infant with the late truncating mutation is consistent with this notion. The 2 patients with early truncating mutations described here and by Wan and associates[19] (Y141X and 411delG) survive because of their nonrandom pattern of XCI, with the normal allele being activated in most cells.

The exact mechanism by which loss of MeCP2 function leads to the pathogenesis of Rett syndrome is still unknown. Given that MeCP2 is a global transcriptional repressor that prevents unscheduled transcription of other genes,[13] its loss of function may result in excessive transcriptional "noise" and overexpression of target genes. We propose that the dysregulation or misexpression of these genes, especially during the development of the nervous system, contributes to the RTT phenotype. It is not clear, however, why mutations in this abundantly expressed gene affect the CNS more than other tissues. MeCP2 is only one member of a family of mammalian proteins that bind specifically to methylated DNA. Two other members of this family have been shown to repress transcription: MBD2 is part of the MeCP1 histone deacetylase complex[46] and MBD3 is a component of the Mi-2 deacetylase complex.[47] It is possible that in some tis-

sues, transcriptional regulation is taken over by these related proteins to compensate for MeCP2 deficiency. Alternatively, the CNS may depend more on this form of epigenetic regulation than other tissues. Future studies in animal models should elucidate the pathogenesis of Rett syndrome as well as the role of *MECP2* in neurodevelopment.

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