MeCP2 mutations in children with and without the phenotype of Rett syndrome

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Article abstract—*Background:* Rett syndrome (RTT) is a neurodevelopmental disorder caused by mutations in the X-linked methyl CpG binding protein 2 (*MeCP2*) gene. *Methods:* One hundred sixteen patients with classical and atypical RTT were studied for mutations of the *MeCP2* gene by using DHPLC and direct sequencing. *Results:* Causative mutations in the *MeCP2* gene were identified in 63% of patients, representing a total of 30 different mutations. Mutations were identified in 72% of patients with classical RTT and one third of atypical cases studied (8 of 25). The authors found 17 novel mutations, including a complex gene rearrangement found in one individual involving two deletions and a duplication. The duplication was identical to a region within the 3′ untranslated region (UTR), and represents the first report of involvement of the 3′ UTR in RTT. The authors also report the identification of *MeCP2* mutations in two males; a Klinefelter's male with classic RTT (T158M) and a hemizygous male infant with a Xq27-28 inversion and a novel 32 bp frameshift deletion [1154(del32)]. Studies examining the relationship between mutation type, X-inactivation status, and severity of clinical presentation found significant differences in clinical presentation between different types of mutations. Mutations in the amino-terminus were significantly correlated with a more severe clinical presentation compared with mutations closer to the carboxyl-terminus of MeCP2. Skewed X-inactivation patterns were found in two asymptomatic carriers of *MeCP2* mutations and six girls diagnosed with either atypical or classical RTT. *Conclusion:* This patient series confirms the high frequency of *MeCP2* gene mutations causative of RTT in females and provides data concerning the molecular basis for clinical variability (mutation type and position and X-inactivation patterns).

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Rett syndrome (MIM 312750) is a neurodevelopmental disorder affecting postnatal brain growth, with a prevalence estimated to be 1:10,000 to 22,000 females.[1] Rett syndrome (RTT) is thought to be the one of the most common genetic causes of mental retardation in girls, second only to Down syndrome.[2] The disorder almost exclusively affects females, with fewer than a dozen putative cases reported in males.[3] Typically, patients show an apparently normal neonatal period, followed by developmental regression and deceleration of head growth.[4-6] The failure of postnatal brain growth is accompanied by loss of communication skills, including learned words and nuanced babble, loss of purposeful hand skills, and apraxia. Stereotypic hand washing or hand wringing behaviors and marked breathing dysfunction (hyperventilation and periodic apnea) are common in patients with RTT. After regression between infancy and the fifth year of life, the clinical course becomes more stable.[4-6]

Through genetic linkage studies, we showed X-linked dominant inheritance of RTT and mapped the disease trait to the Xq28 region of the X chromosome.[7] The Rett gene was recently identified as the methyl CpG binding protein 2 (*MeCP2*) gene.[8] The MeCP2 protein, initially characterized by its ability to bind single methylated CG nucleotides,[9] plays a significant role in the transcriptional silencing of genes.[10-13] MeCP2 has been shown to promote the association of histone deacetylases and transcriptional repressors with methylated DNA.[11,12]

Initially, six different mutations in the *MeCP2* gene were described in both sporadic and familial cases of RTT.[8] Subsequent studies have identified *MeCP2* mutations in approximately 65% to 80% of patients with classic RTT, although familial cases and clinically atypical cases show a lower incidence

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of *MeCP2* mutations.[14-24] *MeCP2* mutations are predicted to result in a loss of function by either disrupting the methylated DNA–binding properties of the protein or interfering with its association with transcriptional co-repressors.[25,26] Most studies have reported a relatively high prevalence of de novo mutations in the *MeCP2* gene in RTT.[14,16-24] These findings are consistent with a high sporadic mutation rate and high incidence of isolated cases.[8,14] Inheritance of *MeCP2* mutations has been described in several cases in which the mother was either a germline mosaic or an asymptomatic carrier of an *MeCP2* mutation.[8,14,16-18,27,28] Nonrandom patterns of X-inactivation have been shown for several asymptomatic carriers of *MeCP2* mutations.[7,14,17,18,28] In these cases, the pattern of X-inactivation most likely protected the mutation carriers from expression of the disease by preferential inactivation of the mutant *MeCP2* allele.

We report a genotype and phenotype correlative study of 116 patients carrying the diagnosis of either classical or atypical RTT. We describe 17 novel mutations, including a C-terminal deletion in a male patient with an unusual presentation and a complex gene rearrangement involving the 3'UTR. We correlated specific clinical features with types of mutations and X-inactivation patterns and provide evidence that both of these factors influence phenotype.

Methods. *Patients.* Patients were ascertained and examined at the Rett Syndrome Clinic at Kennedy Krieger Institute. In some cases, patient samples were referred to Children's National Medical Center by primary care physicians. Patients were scored on the five following clinical features based on either clinical examination at the Rett syndrome clinic (38 patients) or review of medical history sent by primary care physicians (six patients). Patients ranged in age from 2 to 34 years.

Head growth:

0 = no deceleration, head circumference near or above 50th percentile

1 = mild deceleration, head circumference between the 25th and 50th percentile

2 = moderate deceleration, head circumference between the 5th and 25th percentile

3 = microcephaly, head circumference below the 5th percentile.

Frequency and manageability of seizures:

0 = no seizures

1 = easily managed with medications

2 = managed with medications but breakthroughs occur

3 = recalcitrant seizures requiring multiple medications for control.

Respiratory irregularities:

0 = not present

1 = consist of minimal breath-holding spells

2 = breath-holding and hyperventilation for less than half the period

3 = hyperventilation and breath-holding for more than half the wake period, with or without cyanotic episodes.

Scoliosis:

0 = not present

1 = less than 20 degrees

2 = less than 30 degrees

3 = greater than 30 degrees or if surgical correction had taken place.

Ability to walk:

0 = normal gait

1 = mildly apraxic

2 = severely apraxic or requiring to be held when patient walked independently

3 = requiring support to stand and/or wheelchair bound.

Statistical analysis. Analysis of variance (ANOVA) was used to examine the differences in the mean clinical scores between five mutation groups. These analyses were performed on the individually scored clinical features, in addition to the total clinical score (the summation of the five clinical features discussed in the previous section). Patients were divided into five mutation groups according to the type of *MeCP2* mutation: Group 1, 19 patients with missense mutations within the methyl-binding domain (MBD; R106W, R133H, P152R, T158M); Group 2, six patients with nonsense mutations within the region between the MBD and the transcriptional repression domain (TRD; R168X and S204X); Group 3, nine patients with nonsense and frameshift mutations within the TRD [Q244X, R255X, R270X, R294X, 855(del4), 747(insC)]; Group 4, five patients with TRD missense mutations ([K305R and R306C); Group 5, six patients with frameshift deletions in the C-terminus [1160(del26), 1163(del43), 1011(del191), (1156(del41), 1454(del4)].

To evaluate the effects of both X-inactivation status and age, two additional analyses were performed. Differences in clinical scores in only patients with random X-inactivation among the five mutation groups were examined by ANOVA. For these analyses, six patients with skewed X-inactivation (arbitrarily defined as greater than 85% of one allele active) were excluded from the five mutation groups previously described. All ANOVA procedures also were performed adjusting for age, and this covariant was significant for only one outcome measure, scoliosis. Higher clinical scores in the scoliosis category were associated with older patients.

Analysis of covariance (ANCOVA) was used to detect differences in the slope and y-intercepts of the X-inactivation ratio and total clinical score between two mutation groups, Group 1 (mutation groups 1 and 2 from the previous analysis) and Group 2 (mutation groups 3, 4, and 5 from the previous analysis).

Genomic DNA isolation, PCR, and genotyping. Genomic DNA was isolated[29] from peripheral blood samples or lymphoblast cell lines established as previously described.[30] PCR conditions and primers have been previously described for six PCR fragments (exon 3ab, exon 4a, 4b, 4cd, 4d, 4e).[8] PCR conditions and primers for additional PCR fragments (exon 2,

exon 3bc, exon 4l, and the 3′ UTR) are as follows: approximately 100 ng genomic DNA was amplified in a 25-μL reaction volume containing 1× Gene Amp PCR Gold buffer (Applied Biosystems, Foster City, CA), 1.5 mmol/L $MgCl^2$, 160 μmol/L dNTP, 1 unit of Amplitaq Gold DNA polymerase (Applied Biosystems), and 10 pm of each primer (2F 5′ctatgtgtttatcttcaaaatgtc; 2R 5′cagatggccaaaccaggacatata; 3cR 5′ggagttgctcttacttacttgatc; 4lF 5′gattgcgtacttcgaaaaggtaggc; 4lR 5′gcgtttgatcaccatgacctgggtg; UTR 1aF 5′ cgacaagcacagtcaggttgaag; and UTR 3R 5′ gagccctgaggaggcctt). PCR reactions were cycled 30 times with annealing temperatures ranging between 54 and 60 °C. Genotyping was performed as previously described,[31] using primers for CA repeat markers DXS8033, DXS984, DXS998, DXS8086, DXS1073, and DXS8087 (Research Genetics).

Mutation analysis, DNA sequencing, and X-inactivation studies. PCR products were screened for base changes by using a Transgenomic Wave DNA Fragment Analysis System (San Jose, CA). Denaturing high-pressure liquid chromatography (HPLC) was performed as previously described,[32] except the linear gradient of solvent B (0.1 mol/L triethylammonim acetate [TEAA]–25% acetonitrile) ranged between 48% and 68% over 6 to 8 minutes for analysis of PCR fragments. Melting temperature profiles were determined by sequence analysis of PCR products by using WaveMaker 4.0 software (Transgenomics, Inc., San Jose, CA). Because of the presence of more than one melting domain in several PCR fragments (exon 3ab, exon 4a, 4b, 4cd), additional PCR fragments were amplified (exon 3cb, exon 4l, exon 4d) and analyzed by denaturing HPLC (DHPLC). These PCR fragments were smaller, overlapping fragments designed to amplify one melting domain (3cb overlapping with 3ab; 4l overlapping with both 4a and 4b, and 4d overlapping 4cd). Column temperatures for each PCR fragment were as follows: exon 2, 59 °C; 3ab, 62 °C; 3cb, 65 °C; 4a, 61 °C; 4l, 63 °C; 4b, 65 °C; 4cd, 64 °C, 4d, 65 °C, 4e, 60 °C. Mutation-negative controls and mutation-positive controls were run with every DHPLC analysis, with the exception of exon 2 (no mutations have been identified in this exon). PCR products amplified from genomic DNA of male patients were combined with equal amounts of PCR products amplified from control DNA for heteroduplex detection.

Direct, automated sequencing was performed by using a Thermo Sequenase cycle sequencing kit (Amersham, Arlington Heights, IL), and reactions were run on the LiCor automatic sequencer (Gene Reader 4200). Data were analyzed by using BaseImagerIR and AlignIR software (LiCor, Inc., Lincoln, NE). Mutations were confirmed by reverse sequencing or restriction digestion of a second PCR product.

X-inactivation was performed as we have previously described,[33] except PCR products were analyzed on a LiCor automatic sequencer, and peak heights and areas were calculated by using GeneImagIR software (LiCor).

Results. *Mutation analysis of the* MeCP2 *gene.* We analyzed 116 patient samples (79 classical, 25 atypical [fulfilling some but not all inclusion criteria], and 12 with incomplete medical records) for mutations by using a combination of DHPLC and direct sequencing. DHPLC is an efficient, yet sensitive method for mutation analysis, based on the detection of heteroduplexes by ion-pair reverse phase HPLC.[34] DHPLC has been successfully used for detection of mutations within the *MeCP2* gene and was found to be as sensitive as direct sequencing.[23] Overlapping PCR fragments encompassing the protein coding region and the exon/intron boundaries of the *MeCP2* gene were analyzed by DHPLC. Heteroduplex positive samples were targeted for direct sequencing. Eighteen mutation-positive samples, representing 10 different *MeCP2* mutations previously identified by direct sequencing, were included in DHPLC analysis as controls. Heteroduplex peaks were identified for every mutation-positive sample by DHPLC analysis (18 of 18), confirming the sensitivity of this method for mutation detection.

Table 1 *Novel MeCP2 mutations and polymorphisms*

Mutation	Amino acid	Recurrence	Region
G298T	D97Y	1	MBD
G398A	R133H	1	MBD
A914G	K305R	1	TRD
C730T	Q244X	1	TRD
704(insG)	Frameshift	1	TRD
747(insC)	Frameshift	1	TRD
808(delC)	Frameshift	1	TRD
855(del4)	Frameshift	1	TRD
1011(del191)	Frameshift	1	C-terminus
1038(del82)*	Frameshift	1	C-terminus
1169(del171)*	In frame	1	C-terminus
1169(dup137)*	Frameshift	1	C-terminus
1154(del32)	Frameshift	1	C-terminus
1156(del41)	Frameshift	1	C-terminus
1160(del26)	Frameshift	3	C-terminus
1163(del43)	Frameshift	1	C-terminus
1454(del4)	Frameshift	1	C-terminus
Polymorphism			
C426T	No change	1	MBD
C590T	T197M	1	Between MBD/TRD
C834T	No change	1	TRD
G1968C	N/A	1	3′ UTR

* Mutations identified in one patient.

Thirty different mutations were identified in the *MeCP2* gene in 73 of 116 (63%) of patients (tables 1 and 2). We found 17 novel mutations: three missense mutations (D97Y, R133H, K305R), one nonsense mutation (Q244X), two 1-bp insertions, one 137-bp duplication, nine frameshift deletions, and one in-frame deletion (table 1). With the exception of two sets of identical twins, all mutation-positive patients were unrelated and isolated cases. Mutation-positive subjects included 73% (58 of 79) who displayed the diagnostic hallmarks of classic RTT, 32% (8 of 25) of atypical patients, and 7 of 12 patients for whom inadequate clinical information was available to clearly assign to either classical or atypical groups. With the exception of one male infant with a 32-bp deletion, most of the atypical cases with *MeCP2* mutations had some common features (table 3), including some preserved language (5 of 7), some intact hand use (4 of 7), higher cognition (4 of

Table 2 *Recurring MeCP2 mutations*

Mutation	Amino acid	Recurrence	Region	Reference
C316T	R106W	7	MBD	8
C397T	R133C	2	MBD	8
C455G	P152R	3	MBD	16
C473T	T158M	12	MBD	8
C916T	R306C	6	TRD	14
C965T	P322L	1	C-terminus	19
C502T	R168X	6	Between MBD/TRD	14
C763T	R255X	7	TRD	8
C808T	R270X	8	TRD	16
C880T	R294X	2	TRD	17
AG→GG (A9963G*)	N/A	1	Exon 4 splice acceptor site	This report, other splicing mt 17,19

* MeCP2 genomic DNA, numbering according to Gen Bank accession number AF031078.

7), normal head circumference (4 of 7), and most were ambulatory (7 of 7). Most of these cases (75%, six of eight cases studied, including the male variant) had mutations within the transcriptional repression domain (TRD) or deletions within the C-terminus. Nonrandom X-inactivation was found in 33% (two of six informative cases) of atypical female cases (table 3).

Most *MeCP2* mutations were caused by C→T transitions at CpG dinucleotides (71%), which was similar to other reports.[14,16-24] For 17 cases, DNA samples from the mother or both parents were available for genotyping. All cases with the exception of one (the infant boy with the 32-bp deletion whose mother was a carrier with skewed X-inactivation) represented de novo *MeCP2* mutations. Six recurring mutations (R106W, R306C, T158M, R255X, R168X, and R270X), all caused by C→T transitions, accounted for 63% (46 of 73) of all mutation-positive cases.

Mutations were not detected in 43 of 116 (37%) after the entire coding region of the *MeCP2* was screened by DHPLC, or both DHPLC and sequencing in some patients. Most of these were isolated cases (39 of 43). Four were familial cases of RTT (4 of 43), two sister–sister pairs and two brother–sister pairs. Our findings that four of a total of six familial cases of RTT included in this study did not show *MeCP2* mutations suggested that genetic causes independent of *MeCP2* mutations may cause the RTT phenotype. Atypical clinical features also correlated with lack of *MeCP2* mutations; 68% (17 of 25) of patients with atypical RTT tested negative for mutations in the *MeCP2* coding region. These included most of the males (7 of 9) with some Rett-like features.

MeCP2 mutations were identified in two males. A heterozygous T158M mutation was identified in a 13-year-old Klinefelter's XXY male with classical RTT. His clinical history included developmental delay, loss of social and language skills between 1 and 2 years of age, hand wringing, respiratory irregularities, and seizures. Some pubertal delay, typical of Klinefelter's syndrome, was evident at 13 years of age. Genotyping of X-linked markers was performed to determine the parental origin of X chromosomes.

Table 3 *Clinical findings of atypical Rett syndrome patients with MeCP2 mutations*

Patient	MeCP2 mutation	Sex	X-inactivation	Clinical findings
39	1154(del32)	M	N/A	Neonatal apnea, gastroesophageal reflux, hypotonia. Significant developmental delay and microcephaly, died at 21 months of age of respiratory complications
74	R306C	F	Random	Higher cognition with expressive language, ambulatory
96	R133H	F	Random	Autistic behavior, no clear period of regression, some limited hand use, nonverbal, ambulatory, normocephalic
116	R306C	F	Skewed	Later onset of hyperventilation and seizures, good hand use, excellent gait, no hand stereotypes
122	1160(del26)	F	Not informative	Some expressive language, ambulatory normocephalic
150	T158M	F	Skewed	Developmental delay, some preserved language, higher cognition, some preserved hand use, ambulatory
154	R294X	F	Random	Some preserved language, ambulatory, good hand use, normocephalic
180	R306C	F	Random	Moderately preserved language, good gait, ambulatory, higher cognition (can perform simple math), and mild hand wringing

The boy showed two distinct X chromosome haplotypes, one of which was shared by his mother. These studies suggested that the nondisjunction occurred in the father. DNA from the father was not available.

A novel 32 bp deletion 1154(del32) was found in a hemizygous infant boy with a paracentric Xq27-28 inversion who presented at birth with severe neonatal apnea, gastroesophageal reflux, and hypotonia. His developmental history included significant developmental delay and microcephaly (head circumference below the 5th percentile at 45 days, 51/2 months, and 9 months). The boy died at 21 months of age of respiratory-related complications. An older brother, who died at 18 months of age of complications from syncytial virus, had shown a similar clinical presentation. The older brother and the mother harbored the paracentric Xq27–28 inversion, and the mother tested positive as a carrier for the same 1154(del32) mutation. Because the mother is an asymptomatic carrier, quantitative X-inactivation assays were performed and showed skewed X-inactivation (ratios measured in duplicate: 86:14 and 88:12). This suggested that the mother's inverted X chromosome with the 1154(del32) was preferentially inactivated, protecting her from the symptoms of the disease. The inversion and 32bp deletion in this family may have been caused by the same mutation event. However, direct sequencing did not identify other rearrangements in the coding region of the *MeCP2* gene in either the affected son or his mother, indicating that these rearrangements may not be contiguous. The 1154(del32) causes a frameshift, resulting in a nonsense codon eight amino acids downstream of the deletion. This should truncate the protein in a region 3′ of the highly conserved methyl-binding domain (MBD) and transcription repression domain (TRD). The resulting MeCP2 protein would still contain both characterized functional domains but would lack 100 amino acids at the carboxyl-terminus (C-terminus) of the protein.

An extraordinarily complex gene rearrangement, involving two deletions and a duplication of a 3′ UTR region, was identified in the *MeCP2* gene in a 3½-year-old girl. Her clinical history was consistent with classic RTT and included developmental regression, hand stereotypes, and hypotonia. An MRI performed at 18 months of age showed dysplastic changes to the left cerebellar hemisphere. Direct sequencing of the *MeCP2* gene in this patient showed the presence of two deletions (1038[82del] and (1169[171del]), and a 137-bp duplication (1169[137dup]). The nucleotide sequence of the duplication was identical, with the exception of one nucleotide, a G→C transversion, to a region in the 3′ UTR (cDNA 1915–2052), 750 bp downstream of the 5′ breakpoint of the duplication. Sequencing of the 3′ UTR region indicated no other sequence differences, except for a heterozygous G1968C polymorphism, the same sequence variation found in the duplication. Neither parent is a carrier of the deletions nor the duplication identified in their daughter. However, the father is a carrier of the G1968C polymorphism identified in his daughter, suggesting the rearrangements occurred in the paternal allele and probably originated in the father's germ line.

A 26-bp deletion (1160[del26]) was identified in two girls with classical RTT and a pair of 12-year-old female monozygotic twins who were clinically disconcordant for RTT. The twins harbored the same heterozygous 1160(del26) deletion, although only one of the two twins was diagnosed with RTT.[35] The 1160(del26) deletion was not detectable in either parent. Quantitative X-inactivation showed skewed X-inactivation in peripheral blood cells from the unaffected twin (ratio 99:1), and random X-inactivation in the blood cells from the twin diagnosed with RTT (ratio 40:60). The *MeCP2* allele that was inherited from the father was preferentially inactivated in the asymptomatic twin, indicating a paternal germ line origin of the *MeCP2* mutation.

A novel 4 bp deletion [1454(del4)], 3 bp upstream of the MeCP2 stop codon, was found in a 4-year-old with classic RTT. The 4-bp deletion was a frameshift deletion predicted to result in a read-through of the *MeCP2* stop codon and the addition of 23 amino acids on the end of the MeCP2 protein. This is only the second patient reported with a mutation resulting in the addition of extraneous amino acid sequence to the C-terminus of the MeCP2 protein.[18]

Two novel missense mutations were identified in the methyl-binding domain (table 1). The first, a G398A transition, was found in a girl with atypical RTT. The mutation caused the substitution of a histidine for a conserved arginine (R133H). A C397T missense mutation, resulting in the replacement of the same conserved arginine for a cysteine (R133C), has been previously reported[8] and found in two patients in this study. The second novel missense mutation found in the MBD was a G289T transversion in a girl with classic RTT. The mutation causes the replacement of a conserved aspartic acid for tyrosine (D97Y). One other mutation (D97E) has been reported which causes the same aspartic acid to be replaced by a tyrosine.[15] A K305R (A914G) missense mutation was identified in the transcriptional repression domain of the *MeCP* gene in a 4-year-old girl with classic RTT. Neither parent was a carrier of the K305R mutation; thus, the mutation most likely was a de novo occurrence. The K305R was reported as an unclassified sequence variant in a recent paper,[23] because parental samples were not available for testing.

A mutation of the *AG* splice acceptor site of exon 3 (AG→GG) was identified in a patient with classic RTT. The mutation was a de novo occurrence, and was not present in any family members, including a third cousin (paternal lineage) clinically diagnosed with RTT. There are two other reports of splice site mutations in RTT,[17,19] but it is unclear from these reports whether the splice site mutations resulted in the same A→G transition found in our patient.

Genotype/phenotype. To examine the relationship between specific *MeCP2* mutations and clinical presentation, 44 mutation-positive patients were scored for five clinical features: deceleration of head growth, frequency and manageability of seizures, respiratory irregularities, scoliosis, and motor ability (see Methods). Patients were categorized into five mutation classes according to the type of mutation and location of the mutation within the gene. Scores for each of five clinical features and the total score were averaged for each mutation group and plotted against mutation class. Additional analyses were performed using 1) age-corrected clinical scores and 2) clinical scores from patients with random X-inactivation.

When the five clinical features were analyzed separately, significant differences among mutation groups were observed for one clinical feature, deceleration of head growth (figure 1, bottom panel). These differences remained significant when clinical scores were corrected for

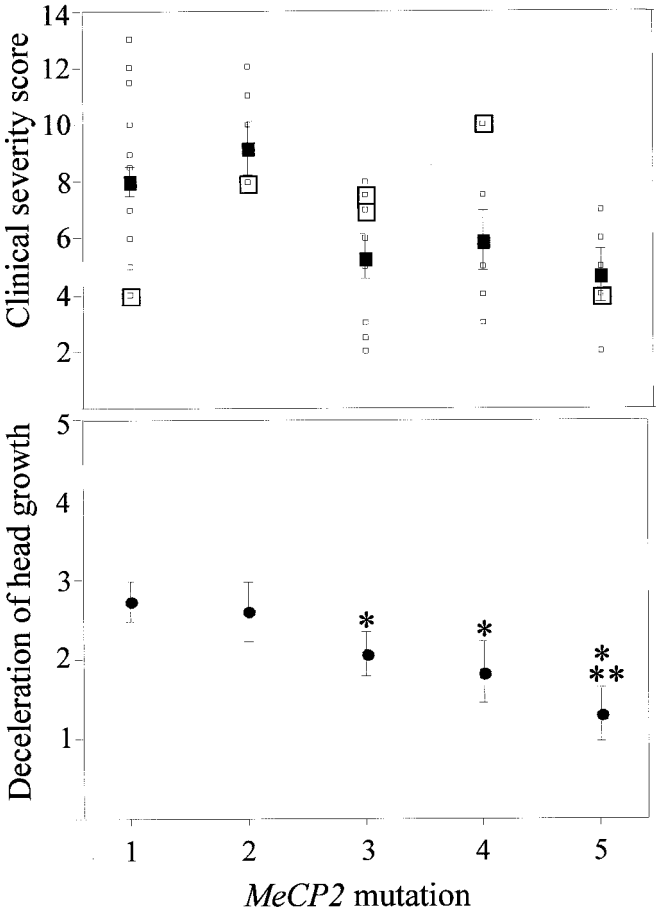

*Figure 1. Correlation between clinical severity and type of MeCP2 mutation. A representative scatter plot (upper graph) shows significant differences in clinical severity (y-axis) between five different mutation groups (groups 1–5, x-axis); the individual scores are shown by the open squares. For these analyses, five clinical features were scored for clinical severity (0 being normal, 3 being most severe), and these five scores were then totaled for each patient. The patients were then divided into five mutation groups: MBD missense mutations (Group 1), nonsense mutations between MBD and TRD (Group 2), TRD nonsense mutations (Group 3), TRD missense mutations (Group 4), and C-terminal deletions (Group 5). Analysis of variance (ANOVA) was used to examine differences in total clinical scores between the mutation groups. Means (black squares) and standard error bars are shown for each mutation group. Differences were found between mutation groups 1 and 3 (*p = 0.0042), groups 1 and 5 (p = 0.0021), groups 2 and 3 (p = 0.0003), groups 2 and 4 (p = 0.0047), and groups 2 and 5 (p = 0.0002). Six patients included in these analyses were found to have skewed X-inactivation (data points with two squares). A representative comparison of the five different mutation groups (groups 1–5, x-axis) and the clinical severity of head growth (y-axis) is shown (lower graph). Clinical severity was scored (0 being normal, 3 being most severe), and scores from individual patients were averaged for each mutation group. Means (black circles) with 95% confidence intervals are shown. Differences were found between mutation groups 1 and 3–5 (*p < 0.05) and mutation groups 2 and 5 (** p < 0.05).*

age and X-inactivation status. Deceleration of head growth was more severe (p = 0.0361 [groups 1 vs 3], 0.0274 [groups 1 vs. 4], 0.0002 [groups 1 vs 5]) in patients with MBD missense mutations (Group 1) compared to missense and nonsense mutations within the TRD and frameshift deletions within the C-terminus (mutation groups 3 through 5). Patients with early truncations (mutation Group 2) also showed a more severe deceleration of head growth compared with patients with deletions in C-terminal region of the protein (mutation Group 5; p = 0.0047). Head circumferences for 16 of 19 patients with missense mutations in the MBD were below the fifth percentile, with deceleration of head growth. In contrast, head circumferences for four of six patients with C-terminal deletions were within the normal range (50th percentile or above) or between the 25th and 50th percentiles and with very minimal or no deceleration of head circumference. As expected, we found the severity and presence or absence of scoliosis to be entirely age dependent, with only patients 6 years of age and older showing significant scoliosis (20 degrees or greater).

Statistical differences were observed when scores from all five clinical features were totaled and compared for each mutation group (figure 1, top panel). Patients with missense mutations within the MBD and mutations truncating the entire TRD (mutation groups 1 and 2) had a more severe clinical presentation compared with patients with missense and nonsense mutations within the TRD and frameshift deletions within the C-terminus (mutation groups 3 through 5). These results were similar when the data were corrected for age and limited to patients with random X-inactivation. These results are suggestive of a correlation between the severity of clinical phenotype and the position of the mutation within the gene (MBD missense mutations or mutations truncating the entire TRD compared with mutations further downstream).

Despite significance of the mutation site and clinical severity scores, the scatter plot (figure 1, top panel) showed considerable variation in total scores for patients within each mutation group. For example, two patients with a MBD missense mutation (Group 1) scored 4 out of a total of 15 compared with the mean of 8 ± 2.48 (1 SD) for Group 1. We hypothesized that some of this variability could be explained by differences in X-inactivation patterns (as shown below).

X-inactivation. All female patients are functionally mosaic for the *MeCP2* defect, a proportion of cells have normal amounts of MeCP2 (normal X active), and the remaining cells are deficient for MeCP2 (abnormal X active). Because of differences in lyonization, there is variability in the proportions of mutant cells, which influences clinical presentation. To examine the relationship between X-inactivation patterns and disease manifestations, X-inactivation ratios in peripheral blood cells from 39 patients were quantitated by using a fluorescent X-inactivation assay we have previously described.[33]

The assay was informative for 35 of the 39 patients studied. Most patients (29 of 35) had random X-inactivation patterns, with approximately equal numbers of normal and abnormal cells. Six patients showed preferential use of one X chromosome (>85% of cells with same X active). Two of these six patients with preferential X-inactivation had a diagnosis of atypical RTT, a girl with

Table 4 *Rett syndrome (RTT) patients with skewed X-inactivation*

Patient	MeCP2 mutation	Mt Group	X-inactivation	Diagnosis
35	R270X	3	92:8; 90:10	Classical RTT
111	R270X	3	88:12; 86:14	Classical RTT
116	R306C	4	86:14	Atypical RTT
126	1160(del26)	5	92:8	Classical RTT
132	S204X	2	86:14; 88:12	Classical RTT
150	T158M	1	92:8; 94:6	Atypical RTT

a T158M mutation and a girl with a R306C mutation (tables 3 and 4). Both girls showed some purposeful hand movements and higher cognition, and both were ambulant with little or no sign of an apraxic gait. The girl with the T158M mutation also had some preserved speech and was the most mildly affected in the mutation class (Group 1), in comparison with the classic severe RTT presentation of five other girls with the same T158M mutation and random X-inactivation (figure 1, top panel). The atypical patient with the R306C was the most severely affected compared with others in her mutation class (Group 4) in our genotype/phenotype comparisons (figure 1, top panel). She showed moderate to severe seizures, hyperventilation, and scoliosis; however, the progression of these symptoms was slower than that normally seen in girls with classical RTT. This patient also showed purposeful hand movements and higher cognitive ability, clinical features not included in our genotype/phenotype study but relatively high functioning for RTT.

The other four patients who showed preferential use of one X chromosome had a diagnosis of classic RTT (table 4). The clinical symptoms of two of these four patients were milder in comparison with others in their mutation class (figure 1, top panel, groups 2 and 5). In contrast, the other two patients were more severely affected in their mutation class (figure 1, top panel, mutation Group 3). These results suggested that nonrandom X-inactivation could result in either a milder or more severe phenotype depending on which X chromosome (the normal or abnormal) was preferentially active.

To further investigate the relationship between X-inactivation patterns and clinical disease, X-inactivation ratios and total clinical score were correlated for 28 patients (figure 2). Patients were divided into two larger mutation groups, based on our previous results; Group 1 (mutation classes 1 and 2 from previous analyses) and Group 2 (mutation classes 3, 4, and 5). We found that clinical severity was correlated with X-inactivation status through a linear relationship for only one of the two mutation groups (Group 1, mutations closer to the N-terminus of the MeCP2 protein). No relationship was observed between X-inactivation status and clinical severity for mutation Group 2 (mutations within the TRD and C-terminus). These data suggested that the X-inactivation status has some effect on clinical severity, especially in cases of skewed X-inactivation. However, one of the limitations of these analyses was the fact that we did not know phase in most patients; for example, the normal X chromosome could not be unambiguously identified.

The two mutation groups also exhibited different slopes and the Y-intercepts ($p = 0.006$ for slopes and $p < 0.0001$ for Y-intercepts), with mutation Group 1 having a steeper slope and a larger Y-intercept (figure 2). These data verified our previous findings indicating that patients with MBD missense mutations and early truncations (mutation Group 1) showed a more severe clinical presentation compared with patients with missense and nonsense mutations later in the gene (mutation Group 2).

Discussion. We have described mutation analysis of the complete coding region of the *MeCP2* gene in 116 patients with both classic and atypical RTT. This study is one of the largest to date and the most inclusive of atypical cases. We found that most classic RTT cases (73%) and one third of atypical cases (8 of 25) were caused by *MeCP2* mutations. We report the identification of causative mutations in two males (one with Klinefelter's syndrome and classical RTT and a hemizygous male neonate with a severe presentation). We described 17 novel mutations including the first *MECP2* mutation involving the

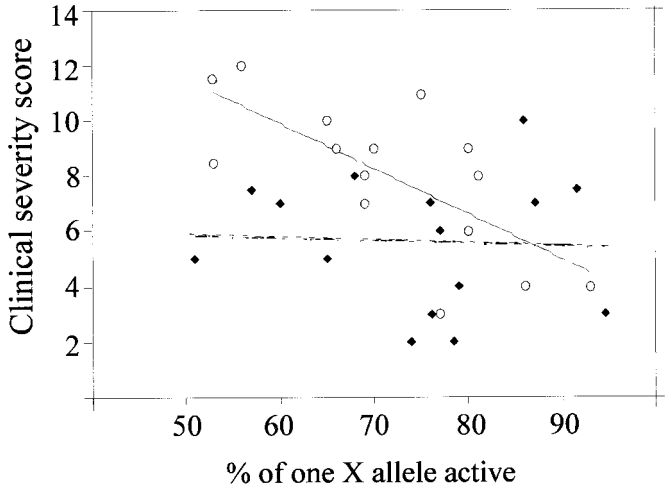

Figure 2. X-inactivation patterns and clinical phenotype. A comparison of clinical severity (total clinical score; y-axis) and X inactivation patterns (percentage of one X active, x-axis) for 28 patients are shown. Patients were divided into two larger mutation groups, Group 1 (Missense mutations in the MBD and nonsense mutations between the MBD and TRD, gray circles) and Group 2 (TRD missense and nonsense mutations and C-terminal deletions, black triangles) for comparison. Fitted regression line for Mutation Group 1 (straight line) and Mutation Group 2 (dashed line) are shown.

3′ noncoding region (3′ UTR). Finally, we show positive correlations between mutation type and position, clinical features, and X-inactivation patterns.

We identified 30 different mutations in the *MeCP2* gene, including four novel missense and nonsense mutations (D97Y, R133H, K305R, Q244X), 11 novel frameshift insertions and deletions, a novel in-frame deletion, and a novel duplication. Including this study, more than 75 different mutations in the *MeCP2* gene have been described in patients with both classic and atypical RTT.[8,14-24,36] Other reports and this study have shown that, despite this large number of mutations identified in the MeCP2 gene, six to eight "common" mutations account for 64% to 77% of mutation-positive cases.[8,14,16-24,36]

Of the novel mutations described in this study, a particularly complex rearrangement, involving two de novo deletions and a 137-bp duplication, was found in a girl with classic RTT. The duplication was almost identical in sequence to a region in the 3' UTR, 750 bp downstream of the rearrangement. This rearrangement and five other novel deletions were found in the C-terminal region of the gene, a region that may be a "hotspot" for deletions because of the presence of repetitive sequences, especially deoxy cytosine repeats. There are more than 15 reports of small deletions in this region of the *MeCP2* DNA.[15-19,23,24]

One third of atypical cases studied (8 of 25) were found to have *MeCP2* mutations, including a male infant with a C-terminal 32-bp frameshift deletion. Most of the atypical cases studied (seven of eight) have shown a milder clinical course, with individual cases showing preserved language, intact hand use, higher cognition, and head circumference in the normal range, and most were ambulatory. Nonrandom X-inactivation and *MeCP2* mutations within the TRD or near the C-terminus were found with a higher incidence in these milder atypical cases, indicating that mutations in this region of the gene and X-inactivation may protect against some of the more severe clinical manifestations. These data were consistent with our findings from our genotype/phenotype studies, which showed that mutations in the TRD and C-terminus were associated with milder clinical presentation.

Of the eight mutation-positive atypical RTT patients we reported, the single clinically severe male patient considerably extends the clinical manifestations resulting from *MeCP2* mutations. This male infant showed a 1154(del32) deletion and presented with severe microcephaly, developmental delay, and respiratory distress. An older male sibling had died at 18 months of age with similar symptoms. We showed the mother to be an asymptomatic carrier of the mutation, with skewed X-inactivation protecting her from RTT. In this case, an Xq27-28 inversion was observed in the mother and both affected infant boys. The chromosome with the inversion was also shown in both mother and son to have the 32 bp deletion in the *MeCP2* gene. Presumably, the inversion event and deletion event occurred simultaneously; however, we successfully amplified over the deletion, suggesting that these abnormalities may not be contiguous.

Five additional families with affected males with *MeCP2* mutations have been reported.[14,27,28,37,38] The spectrum of clinical presentations of *MeCP2* mutations in males includes severe neonatal encephalopathy in an infant boy with a frameshift deletion (806delG)[14]; severe neonatal encephalopathy and apnea in two infant brothers, one who showed a T158M mutation (DNA was not available for testing one brother)[29]; severe mental retardation in four brothers with an A140V missense mutation[37]; severe mental retardation with progressive spasticity in an uncle–nephew pair with a E406X nonsense mutation[27]; and a nonfatal neurodevelopmental disorder with similarities to RTT in a boy showing somatic mosaics for 2 bp deletion.[38] In two families, a sister and an aunt–niece pair were carriers of *MeCP2* mutations and were diagnosed with classic RTT.[14,28] Interestingly, two *MeCP2* mutations identified in affected males (A140V; E406X) were associated with a milder phenotype in a mother–sister pair[37] or no phenotype in two carrier females with random X-inactivation.[27] These mutations may only have a subtle effect for MeCP2 function,[37] or tissue-specific differences in X-inactivation may account for the mild phenotype in female carriers.[27]

Nine males with Rett-like symptoms were included in this study, and we did not identify a *MeCP2* mutation in most cases (seven of nine males). With the exception of the male with Klinefelter's syndrome, the males we studies only partially fulfilled the diagnostic criteria used for RTT. Our data suggest that most RTT-like cases in males are probably caused by some other gene defect. Genetic heterogeneity also may be responsible for the mutation-negative cases (girls with both atypical and classical RTT), and the four cases of sibling pairs (two brother–sister pairs and two sister–sister pairs) that showed a positive family history of RTT. Preliminary data from two other reports suggests a lower incidence of MeCP2 mutations in familial cases of RTT.[15,17] However, it is possible that some of these mutation-negative cases have a mutation in the 3'UTR and promoter regions of the MeCP2 gene, regions which were not screened by any study to date.

Our results confirm recent studies of our laboratory and others showing that nearly all cases represent new mutation events, and rare asymptomatic female carriers escape symptomatic expression of the disease through preferential inactivation of the X chromosome containing the MeCP2 mutation.[7,14,16-18] Asymptomatic RTT female carriers may have two X-linked genetic defects; one gene causing skewed X-inactivation and the second, the mutation in the *MeCP2* gene. In these women, the X-linked mutant locus leading to skewed X-inactivation must be on the same chromosome (in cis) with the mutant *MeCP2* gene, leading to preferential inactivation of

the X chromosome with the *MeCP2* mutation. We have recently shown in other studies that many females showing highly skewed X-inactivation are carriers of X-linked recessive lethal disorders, which result in death or a growth disadvantage of cells having the abnormal X active[39,40] (Lanasa et al., in press). Similarly, we have found patterns of skewed X-inactivation in clinically manifesting carriers of X-linked recessive traits such as Duchenne dystrophy. In these cases, the X-linked mutant locus leading to skewed X-inactivation would be "in trans" (the opposite X chromosome) with the X-linked phenotypic.[32]

We found that most mutation-positive RTT patients had random patterns of X-inactivation, which is consistent with results of another study.[17] However, six patients displayed skewed X-inactivation patterns in their peripheral blood cells, showing ratios of greater than 85% of one X active. Two girls with atypical RTT and skewed X-inactivation showed milder, atypical clinical symptoms, including some purposeful hand use and preserved speech (1 girl). Four girls with skewed X-inactivation and a diagnosis of classical Rett were either more mildly affected or more severely affected when compared with others with the same type of mutation and random X-inactivation. These results suggested that skewed X-inactivation could result in a milder or more severe clinical phenotype, depending on which chromosome, the normal X chromosome or the mutated X chromosome, was preferentially active in most of the cells. Three girls (two with milder phenotype, one classic Rett) showed levels of preferential X-inactivation (ratios $>$ 90% of one X chromosome active) usually observed for asymptomatic carriers of RTT.[7,14] These apparently contradictory results of similar X-inactivation ratios in both asymptomatic carriers and patients with a milder Rett phenotype raised several questions. First, we have assumed that in milder RTT cases with skewed X-inactivation, the normal chromosome was preferentially active, although we have no direct method to determine this (phase not known). Second, all X-inactivation studies were performed on peripheral blood cells from patients. It is possible that different tissues in the body may have different X-inactivation patterns, for example, the brain may have a more random pattern of X-inactivation compared with blood. Previous studies in our laboratory have shown excellent concordance in X-inactivation patterns of 45 women between two types of tissues, peripheral blood cells (mesoderm) and oral mucosal cells (endoderm),[39] suggesting that tissue-specific differences in X-inactivation patterns may be rare. A limited study of X-inactivation patterns in brain tissue have shown random X-inactivation patterns for all patients.[41] X-inactivation patterns in brain tissue may not be inherently different from those in other tissues.

Two previous studies have examined the relationship between specific genotypes (type or region of *MeCP2* gene mutation) and severity or range of clinical symptoms, with conflicting results. One study found few significant differences between the clinical presentation of patients with *MeCP2* missense mutations and truncating *MeCP2* mutations, with the exception of respiratory dysfunction and levels of cerebrospinal fluid neurochemistry.[17] A second study found significant differences in clinical severity, and patients with early truncating mutations showed a more severe clinical presentation.[16] To the contrary, the patient series presented here showed significant correlations between specific genotypes and phenotypes. Mutations closer to the N-terminus (methyl-binding domain and truncations of the entire transcriptional repression domain) were associated with more severe clinical presentations than those in the C-terminal (nonsense and missense mutations within transcriptional repression domain and C-terminal deletions). This study also compared the severity of individual clinical features with mutation type, and one clinical feature, deceleration of head growth, showed the same type of correlation of clinical severity with mutation type. Most patients with C-terminal deletions showed minimal or no deceleration of head circumference, in contrast most patients with missense mutations in the methyl-binding domain (N-terminus) who showed deceleration of head circumference, below the fifth percentile for most. The explanation for differences in our study results is likely attributable to several factors: 1) Our inclusion of a large number of atypical cases; 2) The consistency of clinical investigations on most patients (Kennedy Krieger RTT Research Study); 3) the quantitation of X-inactivation patterns in most patients included in the phenotype/genotype study; and 4) our consideration of both type of mutation and location of mutation (five mutation groups) in our study.

Future challenges for RTT research include the identification of the genetic cause in the 30% to 40% of patients who have not shown *MeCP2* mutations in our study and that of others. Additionally, we need to extend analysis to more "atypical" cases, including clinically mild females showing only a subset of the diagnostic hallmarks of RTT, and clinically severe males showing CNS symptoms not previously considered consistent with RTT.

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