Increased *MECP2* gene copy number as the result of genomic duplication in neurodevelopmentally delayed males

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Purpose: Mutations in the *MECP2* gene are associated with Rett syndrome, an X-linked mental retardation disorder in females. Mutations also cause variable neurodevelopmental phenotypes in rare affected males. Recent clinical testing for *MECP2* gene rearrangements revealed that entire *MECP2* gene duplication occurs in some males manifesting a progressive neurodevelopmental syndrome. **Methods:** Clinical testing through quantitative DNA methods and chromosomal microarray analysis in our laboratories identified seven male patients with increased *MECP2* gene copy number. **Results:** Duplication of the entire *MECP2* gene was found in six patients, and *MECP2* triplication was found in one patient with the most severe phenotype. The Xq28 duplications observed in these males are unique and vary in size from approximately 200 kb to 2.2 Mb. Three of the mothers who were tested were asymptomatic duplication carriers with skewed X-inactivation. *In silico* analysis of the Xq28 flanking region showed numerous low-copy repeats with potential roles in recombination. **Conclusions:** These collective data suggest that increased *MECP2* gene copy number is mainly responsible for the neurodevelopmental phenotypes in these males. These findings underscore the allelic and phenotypic heterogeneity associated with the *MECP2* gene and highlight the value of molecular analysis for patient diagnosis, family members at risk, and genetic counseling. *Genet Med* 2006:8(12):784–792.

The *MECP2* gene located on chromosome Xq28 encodes the methyl-CpG-binding protein 2 (MeCP2), which normally functions as a transcriptional repressor.[1] The MeCP2 protein preferentially binds methylated CpG dinucleotides and functions as a co-repressor of target gene transcription.[2] *MECP2* encodes two isoforms: MeCP2_e2 (exons 2–4) and MeCP2_e1 isoform (exons 1, 3, and 4), which is more abundant in brain tissue.[3–5] Mutations in the *MECP2* gene cause Rett syndrome (Online Mendelian Inheritance in Man [database] 312750), a progressive neurodevelopmental disorder that affects approximately 1 in 10,000 females.[6] Classic Rett syndrome is charac-

terized by apparently normal development in girls 6 to 18 months, followed by regression. Clinical features include loss of purposeful hand use replaced by stereotypic hand wringing, loss of speech, acquired microcephaly, mental retardation, autistic features, seizures, ataxia, and breathing dysrhythmias.[7,8] In addition to classic Rett syndrome, *MECP2* mutations have been identified in patients with a broader range of phenotypes including severe or mild Rett variants, Angelman syndrome-like phenotype, mental retardation with seizures, autistic phenotypes, or mild learning disabilities.[9,10] The clinical phenotype in females can be modulated significantly by nonrandom X-inactivation patterns.[11]

Molecular testing currently involves sequencing of the entire *MECP2* coding region, which detects the majority of mutations in Rett syndrome. Additional tests for *MECP2* gene rearrangements, such as deletions and duplications, have been developed to augment overall test sensitivity. Our previous analysis on a cohort of 216 females with classic Rett syndrome identified *MECP2* mutations in 96% of cases. Sequencing of *MECP2* exons 1 to 4 detected mutations in 86%, and additional testing by dosage-sensitive Southern analysis identified *MECP2* partial gene deletions in another 10% of females with classic Rett syndrome.[12]

Rett syndrome was initially considered to be an X-linked dominant lethal condition in males. However, *MECP2* gene

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mutations were identified in males affected with a broader range of neurodevelopmental phenotypes.[13,14] Hemizygous *MECP2* point mutations that inactivate the protein are associated with a severe neonatal encephalopathy phenotype in affected males.[15] When such mutations occur in males with somatic mosaicism or a 47, XXY karyotype, the presentation is more consistent with a classic Rett phenotype because of a compensating normal X-chromosome.[16–18] Hypomorphic mutations, which barely cause a phenotype in females, cause mental retardation, tremors, and a variety of neuropsychiatric features in affected males.[13,19,20] More recent clinical testing for *MECP2* gene rearrangements revealed that entire *MECP2* gene duplications occur in some males manifesting a progressive neurodevelopmental syndrome.[21–23]

Diagnostic studies at the Baylor Medical Genetics Laboratories have identified seven affected males with increased copy-number of the *MECP2* gene. These include two males who were referred specifically for mutation analysis of the *MECP2* gene and five males who were referred for microarray comparative genomic hybridization analysis for deletions or duplications of multiple genomic regions involved in clinical genomic disorders.[24,25] Confirmatory testing using complementary methods were performed in all seven cases.

MATERIALS AND METHODS

Patient material

Peripheral blood samples from patients were submitted for clinical testing to the Baylor Medical Genetics Laboratories for either *MECP2* deletion/duplication analysis (patients 1 and 2) or chromosomal microarray analysis (CMA) (patients 3–7). Patients' clinical features are summarized in Table 1. Genomic DNA was extracted from blood leukocytes using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN), and DNA concentration was measured using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Rockland, DE).

MECP2 Southern analysis

Genomic DNA (2 μg) was digested with *Pst*I or *Ban*I restriction enzymes (New England Biolabs, Ipswich, MA), electrophoresed on a 0.8% agarose gel, and blotted onto a Hybond XL nylon membrane (Amersham Pharmacia, Buckinghamshire, UK). Blots were hybridized to ^{32}P-labeled probes corresponding to *MECP2* exon 2, 3, and 4 polymerase chain reaction (PCR) products. Dosage differences indicative of *MECP2* whole gene duplication by Southern analysis were detected by visual inspection of autoradiogram band intensities compared with ethidium bromide-stained agarose gel images.

Quantitative real-time polymerase chain reaction

Quantitative real-time PCR (qRT-PCR) analysis for the *MECP2* gene was performed using 20 ng of genomic DNA, and PCR primers and Taqman MGB probes were designed using Primer Express software (Applied Biosystems, Foster City, CA). Reactions were done in triplicate, wherein each target region was coamplified with an internal control (*RNaseP*) us-

ing the ABI 7500 RT-PCR system (Applied Biosystems). Relative gene copy number was determined by the comparative threshold cycle method (ddCt).[26] qRT-PCR analysis of multiple loci flanking *MECP2* on Xq28 was performed using the SYBR-Green chemistry, which measured the fluorescent signal at the end of the elongation phase. Data were normalized against an endogenous reference gene (GAPDH), and a melting curve analysis was performed to verify PCR product specificity.

Multiplex ligation-dependent probe amplification

Dosage analysis of the *MECP2* gene and flanking loci was performed by multiplex ligation-dependent probe amplification (MLPA) analysis using commercially available reagents and instructions from MRC-Holland (Amsterdam, The Netherlands). Ligation products were amplified using a Gene Amp PCR System 9700 (Applied Biosystems). Products were resolved on an ABI-3100 genetic analyzer, and data were analyzed using Genescan software (Applied Biosystems). Patient data were normalized to controls for copy-number differences using the Gene Marker Software (Softgenetics, State College, PA).

Chromosomal microarray analysis

Microarray-based comparative genomic hybridization for selected genomic regions involved in clinical genomic disorders was performed at our institution as previously described.[27] Patients 1 and 2 and their respective mothers were analyzed with our version 4 microarray containing 366 fluorescence in situ hybridization (FISH)-verified bacterial artificial chromosome (BAC) clones that cover genomic regions involved in more than 40 deletion/duplication disorders.[27] Subsequent patients 3 to 7 were analyzed with an expanded microarray version 5 containing 860 FISH-verified BAC clones, including four clones for *MECP2* and flanking loci (http://www.bcm.edu/cma/). Reciprocal dye reversal experiments were performed for each patient sample, and hybridized arrays were scanned into 16-bit tiff image files using an Axon two-color microarray scanner 4000B and quantified by using GenePix Pro 6.0 software (GenePix 4000B from Axon Instruments, Union City, CA). Data analysis was performed by a web-based software platform with the capability of displaying the raw, normalized, and integrated data of all clones to detect genomic copy-number changes for each patient relative to a sex-matched control, as previously described.[27]

Fluorescence in situ hybridization

FISH experiments on interphase or metaphase nuclei chromosome preparations were performed to confirm increased copy-number changes in the *MECP2* or other loci identified by CMA. Miniprep BAC DNA (100 ng) was labeled with Spectrum Orange-dUTP or Spectrum Green-dUTP (Vysis, Downers Grove, IL), according to the manufacturer's instructions, and used as probes for FISH analysis using standard protocols.[28]

X-inactivation studies

Skewing of X-inactivation patterns in duplication carrier mothers was assessed with the androgen receptor methylation assay.[29] One microgram of genomic DNA was digested at 37°C

Table 1
Comparison of clinical findings observed in male patients with increased *MECP2* dosage

Clinical features	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Literature cases (N = 53)[a]
Age at testing	8.5 y	11.5 y	1.1 y	3 mo	4 y	8.3 y	16 y	
Developmental delay	+	+	+	+	+	+	+	53/53 (100%)
Infantile hypotonia	+	+	+	+	+	+	+	47/47 (100%)
Absent speech	+	+	+	+	+	+	−	27/32 (84%)
Lack of ambulation	−	−	+	−	−	−	−	14/31 (45%)
History of recurrent infections	+	−	−	+	+	−	+	40/48 (83%)
Breathing dysrhythmias	−	−	−	−	−	−	−	0/53 (0%)
Stereotypic hand movements	−	−	−	−	+	−	−	1/53 (2%)
Autistic-like features	−	+	−	−	−	+	+	1/53 (2%)
Seizures	−	−	−	+	−	−	−	27/48 (56%)
Microcephaly	−	−	+	− (Mac)[b]	−	−	−	19/48 (40%)
Brachycephaly	+	+	+	−	+	+	−	5/28 (18%)
Triangular face	−	−	−	+	−	−	−	0/28 (0%)
Narrow forehead	−	−	−	−	−	−	−	1/28 (4%)
Flat midface	+	+	−	+	−	+	+	6/28 (21%)
Large ears	−	−	−	+	+	−	−	8/28 (29%)
Broad nasal root	−	+	−	−	−	−	−	5/28 (18%)
Anteverted nares	−	+	−	−	−	−	−	1/28 (4%)
Prominent nasal bridge	−	−	−	−	−	−	+	4/28 (14%)
Upslanting palpebral fissures	−	+	−	−	−	−	−	0/28 (0%)
Epicanthal folds	−	+	−	−	−	−	−	2/28 (97%)
Ptosis	+	−	−	+	−	−	−	0/28 (0%)
Hypertelorism	−	−	−	−	−	+	−	5/28 (18%)
Deep-set eyes	−	−	−	−	−	−	+	0/28 (0%)
Proptosis	−	−	−	−	−	−	−	4/28 (14%)
Widely spaced teeth	−	−	−	−	+	−	−	0/28 (0%)
High arched palate	−	−	−	−	−	−	+	1/28 (4%)
Bifid uvula	−	−	−	−	−	−	+	0/28 (0%)
Digital abnormalities[c]	+	+	+	+	−	−	+	10/28 (36%)
Genital abnormalities[d]	−	+ (Cr)	−	−	+ (Hs)	−	−	19/28 (68%)
Other organ systems[e]	−	−	−	+ (Tr, Hn)	DU	−	+ (AD)	4/28[f] (14%)

[a]Sanlaville et al.,[23] Van Esch et al.,[22] and Friez et al.[35]
[b]Mac, macrocephaly.
[c]Fifth finger clinodactyly, 2–3 toe syndactyly, broad thumbs/great toes, long/slender fingers.
[d]Cr (cryptorchidism), Hs (hypospadias).
[e]Tr (tracheomalacia), Hn (hydronephrosis), DU (duplex urethra), AD (aortic root dilatation).
[f]Hearing loss, peripheral vasomotor disturbance, coarctation of the aorta, tracheomalacia, and laryngomalacia.

overnight with 10 units of the methylation-sensitive restriction enzyme *Hpa*II (New England Biolabs, Ipswich, MA) followed by heat inactivation at 65°C for 20 minutes. Undigested and digested DNA were used as templates for ^{32}P-labeled PCR amplification of the Androgen Receptor CAG$_n$ repeat.[29] Resulting PCR products were analyzed on a 6% (39:1 acrylamide/bisacrylamide) denaturing gel, which was dried and exposed to x-ray film overnight at −80°C.

RESULTS

In the course of DNA diagnostic testing for *MECP2* mutations, our laboratories received referrals for *MECP2* deletion/duplication testing for neurodevelopmentally delayed males. Of 122 males referred specifically for *MECP2* gene rearrangement testing within a 17-month period, two patients tested positive for duplication of the entire *MECP2* gene. Represen-

tative data are shown for patient 1. Our standard test for deletion/duplication consisted of a dosage-sensitive Southern analysis for *MECP2* exons 1 to 4. Figure 1A shows the results of *MECP2* Southern analysis for patient 1 using a genomic restriction digestion with the enzyme *Pst*I. The resulting hybridization pattern consists of a 7.60-kb *Pst*I fragment containing *MECP2* exons 1 and 2, and a second fragment of 2.5 kb containing *MECP2* exons 3 and 4. Relative band intensities are internally consistent within this patient lane. However, the collective band intensities are disproportionately higher compared with a normal male and resemble a normal female with two X-chromosomes. Comparison of signal intensities on the *Pst*I autoradiogram (Fig. 1A) versus the corresponding ethidium bromide gel (Fig. 1B) is consistent with duplication of *MECP2* exons 1 to 4 in male patient 1. These findings were corroborated by Southern analysis using a second restriction enzyme, *Ban* I. Figure 1C shows the autoradiogram of three *Ban*I fragments of 2.14 kb, 1.74 kb, and 1.2 kb that correspond to *MECP2* exons 3, 2, and 4, respectively. Thus, Southern analyses using two restriction enzymes indicate duplication of the entire *MECP2* gene. Our laboratories performed confirmatory testing by independent dosage-sensitive methods. *MECP2* duplication in these patients was verified by MLPA analysis using commercially available reagents. Figure 1D shows the raw MLPA data for patient 1, and Figure 1E shows patient 1 data

normalized to a male control, which indicates a twofold increased copy number of probes corresponding to *MECP2* exons 1 to 4, plus flanking loci on Xq28 (*SLC6A8, IDH3G, L1CAM* and *IRAK*1). In addition to dosage-sensitive Southern and MLPA analyses, we developed qRT-PCR testing for *MECP2* exons 1 to 4 using a Taqman assay. Representative data are shown in Figure 1F, wherein *MECP2* exon 4 is coamplified with the autosomal *RNaseP* gene as an endogenous control. Patient 1 was determined to have a twofold *MECP2* gene copy number relative to a normal male using the comparative cycle threshold method on qRT-PCR. Thus, duplication of the entire *MECP2* gene was established by several DNA methods in two male patients referred specifically for *MECP2* gene rearrangement testing.

The Baylor Medical Genetics Laboratories have also been performing a highly multiplexed CMA for genetic disorders associated with deletions and duplications of specific chromosomal regions. We developed a microarray containing large-insert genomic DNA clones of clinically relevant regions as targets for comparative genomic hybridization (array-CGH).[27] The advantages of CMA include increased sensitivity for subchromosomal deletions or duplications that are likely to go undetected by conventional karyotyping or metaphase FISH, and simultaneous detection of a wide range of duplication and deletion syndromes in one highly multiplexed assay.[27,30–32] Our CMA microarray was
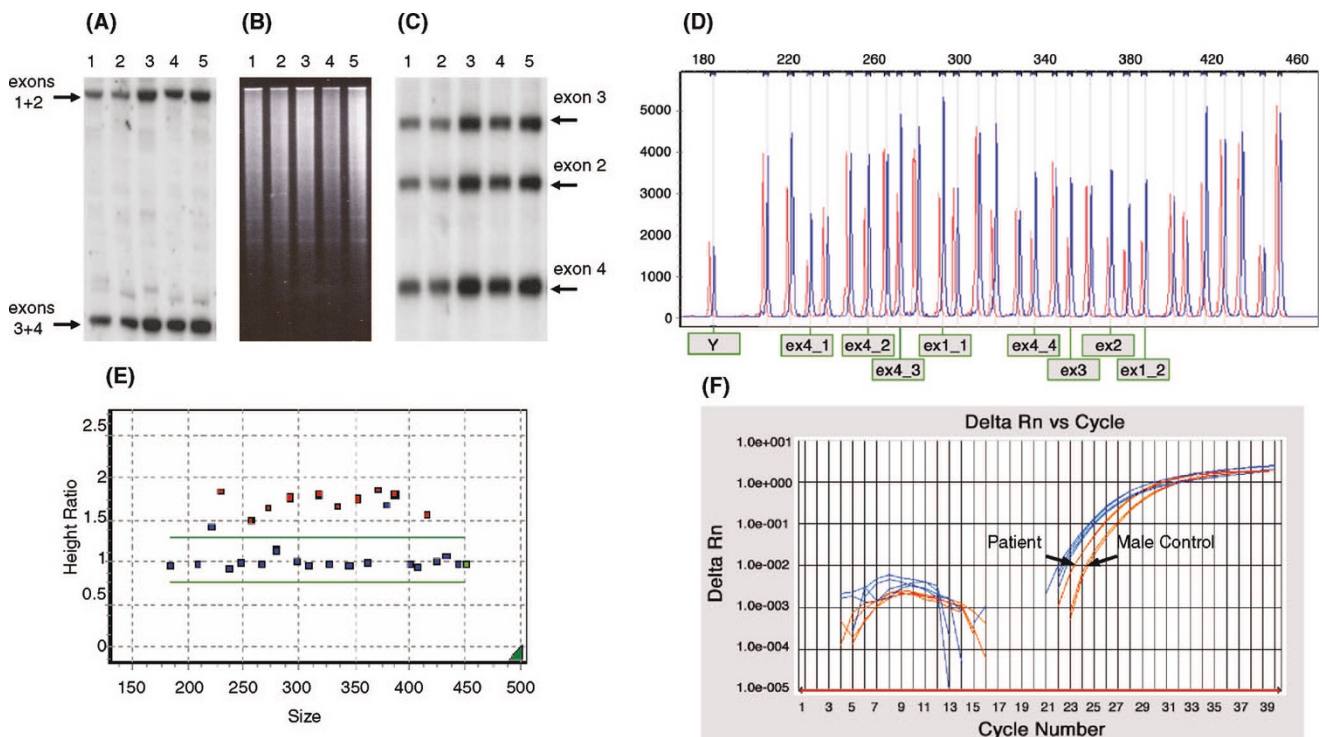

Fig. 1. Molecular detection of *MECP2* gene duplication. A: Southern analysis with *Pst*I-digested genomic DNA from male patient 1 (lane 3), normal male controls (lanes 1 and 2), and normal female controls (lanes 4 and 5). Hybridization with *MECP2* exon 2 probe generates a 7.6-kb fragment containing *MECP2* exons 1 and 2 and a 2.5-kb fragment containing *MECP2* exons 3 and 4. B: Corresponding ethidium bromide-stained gel of the *Pst*I-digested DNA. C: Southern analysis with *Ban*I-digested genomic DNA from male patient 1 (lane 3), normal male controls (lanes 1 and 2), and normal female controls (lanes 4 and 5). Hybridization with probes for *MECP2* exons 2 to 4 detects three fragments of 2.14 kb (exon 3), 1.74 kb (exon 2), and 1.2 kb (exon 4). D: MLPA data of male patient 1 with *MECP2* duplication. E: The same MLPA data normalized to a male control using Gene Marker Software (Softgenetics, State College, PA). *MECP2* duplicated exons appear as outliers (*blue*) from the data set, as points outside of the normal copy threshold lines. F: qRT-PCR analysis of *MECP2* exon 4 (*orange*) and RNAaseP control (*blue*) in a duplex assay. Results are shown for a male control and patient 1 with *MECP2* duplication (ddCt ratio ~2.00). ΔRn is plotted on the y-axis, and the cycle number is on the x-axis.

designed to include BAC clones for *MECP2*, based on the observation that increased *MECP2* levels cause progressive neurologic features in a mouse model.[33,34] CMA detection of *MECP2* duplications was demonstrated on our initial patients 1 and 2, along with their asymptomatic mothers, using our version 4 microarray (366 FISH-verified BAC clones).[27] Figure 2A shows representative CMA data from patient 1, which was cohybridized with a normal male genome; the results show a twofold increased copy number of the two Xq28 genomic clones on the CMA version 4 microarray (i.e., BAC RP11-119A22 including *MECP2*, and RP11-24410 including the flanking *L1CAM* gene). Corresponding DNA and CMA studies on the asymptomatic mother of patient 1 were positive for heterozygous carrier status (Fig. 2B). The magnitude of her deviation is relatively smaller, consistent with three copies of *MECP2* in the carrier mother versus two copies in the normal female. Similar results were obtained on the healthy carrier mother of male patient 2 with duplication (data not shown).

Clinical CMA testing using an updated version 5 microarray identified five additional male patients with increased *MECP2* gene copy number of 1380 cases tested by CMA within a 6-month period. Collectively, increased copy number for BAC clones RP11-119A22 (containing *MECP2*) and RP11-24410 (*L1CAM*) were seen in all seven patients. A third BAC clone on CMA version 5 (RP11-54120 centromeric to *MECP2*) was duplicated in patients 1, 3, 4, and 5. A fourth BAC clone RP11-157E12 (more proximally located) was duplicated in patients 3 and 4 (data not shown). Patient 4 had unusual CMA results that suggested triplication of the *MECP2* clone. This unexpected finding was confirmed by additional studies, with the most graphic evidence provided by FISH analysis.[28] Figure 2C and D show interphase FISH data for patient 4 using clone RP11-119A22, which clearly demonstrate three signals for *MECP2* (red) versus one signal for the control probe. In contrast, Figure 2E shows duplication of clone RP11-24410 flanking the *MECP2* gene in the same patient. Patient 4 has a complex rearrangement that involves triplication of the *MECP2* gene amid duplication of a flanking region of approximately 2 Mb on Xq28. The detection of increased *MECP2* gene copy number by CMA analysis in these five additional male patients was also confirmed by quantitative DNA testing methods in our laboratories. Figure 3 shows a composite duplication map based on collective data from Southern, MLPA, CMA, and qRT-PCR studies. Endpoint mapping was facilitated by qRT-PCR analysis performed on 25 loci flanking the *MECP2* gene. Individual duplications ranged from approximately 2.2 Mb as the largest to a minimal region of approximately 200 kb con-
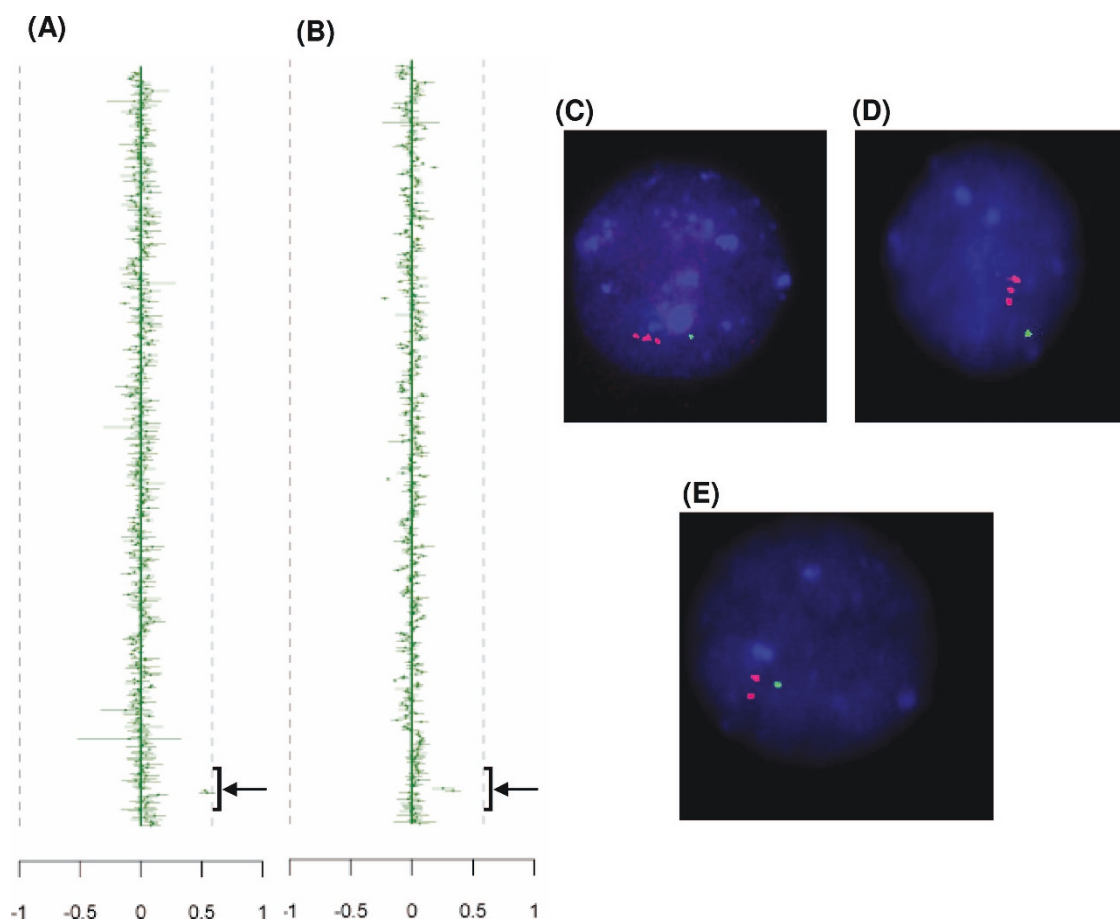

Fig. 2. Molecular cytogenetic detection of increased copy number of the *MECP2* gene. A: Chromosomal microarray detection of *MECP2* duplication in our male patient 1 using CMA version 4.0 microarray. B: Corresponding CMA data showing heterozygous *MECP2* duplication in the mother of patient 1. C and D: FISH detection of *MECP2* triplication in male patient 4 using RP11-119A22 clone containing the *MECP2* gene. E: FISH detection of a flanking duplication in male patient 4 using the RP11-24410 clone containing the *L1CAM* gene.
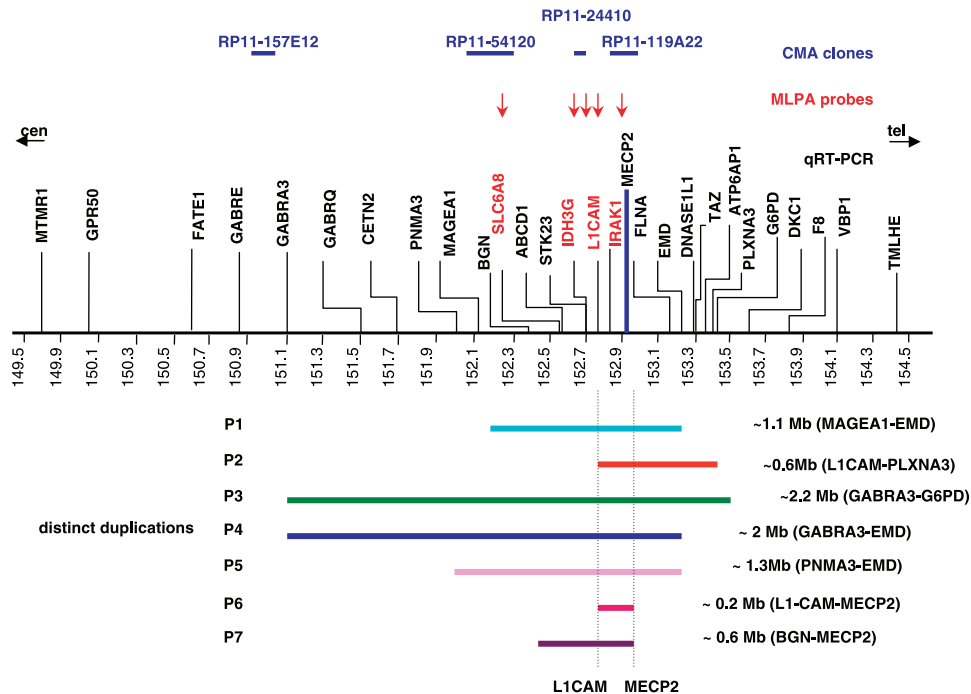
Fig. 3. Nonrecurrent duplications involving the *MECP2* gene in the Xq28 region among seven affected males determined by quantitative molecular methods. Array CGH BAC clones mapping in the Xq28 region (*blue bars*). Locations of MLPA probes (*red arrows*); Xq28 genes tested by qRT-PCR (*loci in black text*). The extent of the duplicated region based on combined molecular and cytogenetic data for each patient (1–7) (*colored horizontal lines*). The common duplicated region (*vertical dotted lines*) (~200 kb) including the *MECP2* and *L1CAM* genes. CMA, chromosomal microarray analysis; MLPA, multiplex ligation-dependent probe amplification.

taining the *L1CAM* and *MECP2* genes. The genomic rearrangements are unique and vary in size among our seven male patients.

The major clinical features in these patients are summarized in Table 1. Predominant features include developmental delay, hypotonia, mild to moderate dysmorphic features, and limited to absent speech. Some patients presented with autistic features, seizures, and progressive loss of ambulation. Four of seven patients had a history of respiratory infections. Involvement of other organ systems was seen in two of seven patients, whereas patient 4 had hydronephrosis and patient 7 had mild aortic dilatation. The most severe, early-onset presentation was seen in patient 4. This is a white male who was 3 months old at the time of testing, with dysmorphic facies, moderate developmental delay, hydronephrosis, tracheomalacia, and recurrent pneumonia. Notably, recurrent respiratory infections have been reported in a number of males with *MECP2* gene duplication.[35]

Our laboratories received samples from several of the patients' mothers, who were reported to be asymptomatic and tested positive as heterozygous duplication carriers. Unexpectedly, the mother of our complex patient 4 has a heterozygous duplication of the *MECP2* gene and the surrounding region of approximately 2 Mb (data not shown); this is in contrast with her affected son who has triplication of *MECP2* and duplication of the flanking approximately 2-Mb region. These unusual results underscore the complexity of the genomic rearrangement in this family, which has yet to be fully characterized. Given the normal clinical status of these carrier mothers, we performed X-inactivation stud-

ies at the androgen receptor locus. Genomic DNA samples were treated with and without predigestion with the methylation-sensitive restriction enzyme *Hpa*II, followed by PCR amplification of the androgen receptor CAG$_n$ repeat.[29] The X-inactivation results for three cases wherein sufficient material was available are shown in Figure 4. In all three cases, one of two alleles predominates after *Hpa*II digestion. These data are consistent with skewed X-inactivation that presumably inactivates the mutant allele in these unaffected carrier mothers.

DISCUSSION

Mutations in the *MECP2* gene encompass a wide range of mutation types that include inactivating and hypomorphic point mutations, as well as loss-of-function deletions[36] and gain-of-function duplications.[21,22] *MECP2* mutations are associated with a broad range of neurodevelopmental phenotypes that extend beyond classic Rett syndrome in females. We describe seven male patients with increased copy number of the *MECP2* gene and surrounding loci on Xq28 identified by quantitative molecular testing and CMA in our diagnostic laboratory. The phenotypic severity in these male patients did not correlate strictly with their duplication sizes, which ranged from approximately 200 kb to 2.2 Mb. We identified Xq28 duplications that involve *MECP2* and flanking genes such as *L1CAM* and *SLC6A8*, which are known to play a role in neuronal cell migration or nonspecific mental retardation.[37,38] Although flanking gene contribution is a theoretic possibility, these collective data suggest that increased *MECP2* gene dosage
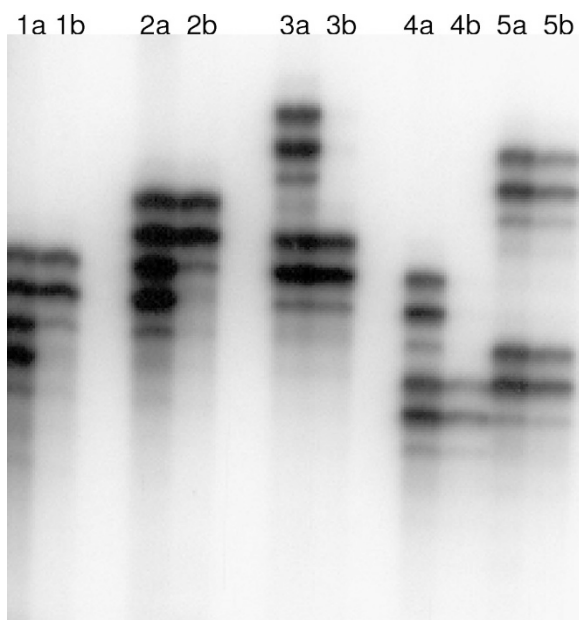
Fig. 4. X-inactivation studies by methylation analysis of the androgen receptor locus. Data for individual maternal DNA samples (lanes 1–3). PCR amplifications performed on template DNA without prior digestion with the methylation-sensitive *Hpa*II enzyme (lanes 1a–3a) and corresponding results with prior *Hpa*II digestion of template DNA (lanes 1b–3b). All samples are informative, and the observed predominance of one allele after *Hpa*II digestion indicates skewing of X-inactivation in the three tested carrier mothers. Data for female controls DNA with skewed X inactivation and random X inactivation before HpaII digestion (lanes 4a and 5a) and post-HpaII digestion (lanes 4b and 5b).

is specifically responsible for the phenotypes in male patients. Submicroscopic Xq28 duplications containing the *MECP2* gene with corresponding increase in RNA expression were reported in multiple male patients.[21,22] Those include one male who was duplicated for the entire *MECP2* gene but not the flanking *L1CAM* gene.[21] In our study, the most severely affected patient is triplicated for the *MECP2* gene. Taken together, the critical duplication region seems to be limited to the *MECP2* gene. These patient findings are consistent with data from transgenic mouse models overexpressing the wild-type human *MECP2* protein.[33,34] These mice appear clinically normal at birth but develop Rett-like progressive neurologic problems including motor dysfunction, hypoactivity, tremors, and ataxia, and die prematurely.[33] Higher MECP2 protein levels

were found to correlate with more severe phenotypes in these mice.[33]

Duplications within the human genome are increasingly recognized as a cause of neurodegenerative phenotypes. Such rearrangements are responsible not only for Mendelian traits such as Charcot-Marie-Tooth neuropathy type 1A[39] and Pelizaeus-Merzbacher leukodystrophy,[40,41] but also for conditions generally thought to be acquired in nature such as Parkinson[42] and Alzheimer[43] diseases. Recurrent rearrangements in Charcot-Marie-Tooth neuropathy type 1A are mediated by nonallelic homologous recombination,[44] wherein genome architectural features seem to lead to genomic instability.[44,45] In a similar manner, nonrecurrent rearrangements may also have predisposing features in the local genome.[46,47] Segmental duplications or low-copy repeats (LCRs) have been implicated in the cause of chromosomal rearrangements that are associated with several genomic disorders.[24,48] Some of these repeats have the potential to form cruciform structures that are highly susceptible to double-strand breaks, which could initiate recombination events leading to duplications within Xq28. To investigate this possibility we performed extensive *in silico* analysis of an approximately 4-Mb region surrounding the *MECP2* gene on Xq28 (149,400,000–154,824,264, Build 35). Figure 5 shows the presence of numerous LCRs in both the direct and inverted orientations, which range from approximately 31 kb to 59 kb in size. Similar analysis was performed by Lee et al.[49] on the region surrounding the *PLP1* locus, wherein duplications are associated with the X-linked Pelizaeus-Merzbacher disease. Their studies found a statistically significant association between this complex genomic architecture and the various duplication breakpoints. This suggests that the LCRs may stimulate the genomic rearrangements responsible for the majority of Pelizaeus-Merzbacher disease cases and supports an alternative role of genomic architecture in nonrecurrent rearrangements. Along these lines, we hypothesize that LCRs in the Xq28 region may be involved in nonrecurrent rearrangements observed in our male patients with *MECP2* duplication. Nonallelic homologous recombination[44,48,50] is the usual mechanism for recurrent rearrangements with breakpoints clustering in LCRs. However, nonrecurrent rearrangement breakpoints are also
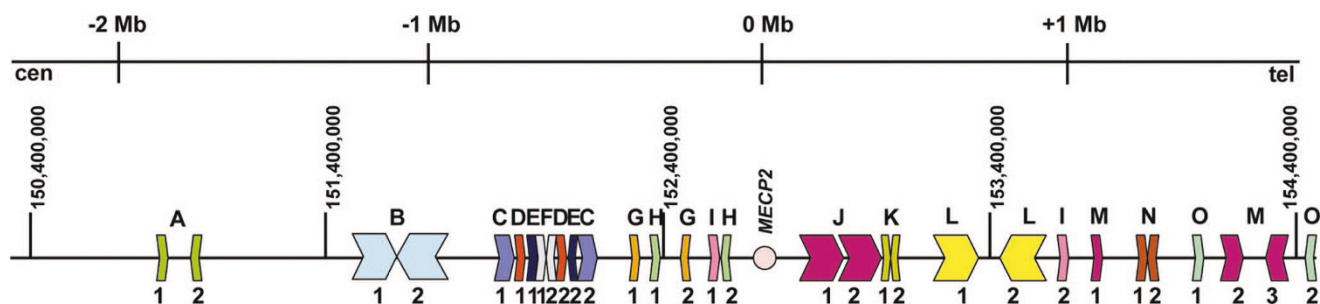

Fig. 5. *In silico* analysis using BLAST 2[55] on repeat-masked sequence reveals LCRs in an ~4-Mb region surrounding the *MECP2* gene. Novel LCRs are displayed centromeric (cen) to telomeric (tel) and labeled A to O. Those with apparent shared identity are shown graphically in like colors; the directions of the block arrows indicate relative orientations. The largest LCRs identified include LCR-*MECP2* B1 and B2 (49 and 59 kb, ~95.9% shared identity), J1 and J2 (40 and 41 kb, ~99.4% shared identity and highly complex), L1 and L2 (31 and 37 kb, ~99.6% shared identity), and M2 and M3 (37 and 38 kb, 98.9% shared identity). *MECP2* gene (*pink circle*). Genomic positions (Build 35) are given relative to the *MECP2* gene in Megabases.

associated with LCRs.[41,51] Sequencing of the actual *MECP2* duplication breakpoint junctions could confirm the mechanism of nonhomologous end joining,[41,51] which has been observed for other nonrecurrent rearrangements. Such experiments are challenged by the highly complex and repetitive nature of this genomic region, and lie beyond the immediate scope of our present study.

Males carrying hypomorphic mutations in the *MECP2* gene present with a broad and less predictable phenotypic range of mental retardation and diverse neurologic signs and symptoms.[52] In addition, *MECP2* hypomorphic mutations may be a less common cause of unexplained mental retardation, developmental delay, and associated neurologic features in males than *MECP2* duplications,[52,53] and to add further complexity, some of the reported *MECP2* hypomorphic mutations have been found to be normal genetic variants.[54]

Table 1 summarizes the clinical findings in our seven male patients and compares their features with literature reports of other males with *MECP2* gene duplication. Infantile axial hypotonia was a finding observed in all of our patients. This neurologic finding was also observed by Van Esch et al.[22] and Friez et al.[35]; however, our patients did not exhibit the facial hypotonia described by these two groups. The infantile axial hypotonia in this syndrome leads to progressive spasticity later on in childhood, and this finding was corroborated in our group. A majority of the patients with *MECP2* duplication in our study exhibited susceptibility to respiratory infections. Frequent childhood infections, in particular respiratory infections such as pneumonias, have been reported among males with *MECP2* duplication.[22,23,35] Immune deficiency has been postulated as a possible cause[35]; however, an extensive immunologic evaluation has not been carried out by other groups or our study to clearly establish immune deficiency as the underlying factor of frequent infections. Although only one of our patients (Table 1) had absent swallowing with aspiration pneumonias, it is not inconceivable to speculate that discoordinated swallowing leading to microaspiration could be responsible for the observed frequent respiratory infections in our group.

The dysmorphic features observed in our patients were heterogeneous. However, brachycephaly, midfacial hypoplasia, and large ears were among the most frequent dysmorphic features in our group. Although the clinical data for some of the patients were not available, Van Esch et al.[22] also mentioned prominent ears and flat nasal bridge as some of the most commonly observed mild dysmorphic features among the patients they described. A longitudinal clinical study enrolling a larger number of patients will be required to carefully establish the most characteristic dysmorphic features observed in this syndrome. Multiple congenital anomalies are not commonly found in this condition. The patient with the *MECP2* triplication (patient 4, Table 1) exhibited hydronephrosis and tracheomalacia, and another patient with *MECP2* duplication presented with mild aortic dilatation (patient 7, Table 1). Two of our patients had genital abnormalities (cryptorchidism and hypospadias). None of the cases described by Van Esch et al.[22] presented with hypoplastic genitalia; however, genital abnor-

malities were commonly reported in the cases described by Sanlaville et al.[23] and in the case reported by Meins et al.,[21] suggesting that they could be part of the clinical spectrum of this condition. In addition, digital abnormalities such as long, slender fingers were observed in some of our patients. Digital abnormalities have also been described in the published literature of Xq28 disomy.[22,23]

Three of our patients (Table 1) exhibited autistic-like features, including a limited range of facial expression, gaze avoidance, and repetitive behaviors. Autism or autistic-like features have been associated with Rett syndrome[10]. The male patient with *MECP2* duplication described by Meins et al.[21] exhibited autistic features. Autism or autistic-like features were not described in previous studies; however, it is not known whether the patients previously described underwent a neuropsychologic evaluation.[22,23] The current findings suggest that autistic-like features could also be found in patients with *MECP2* duplication expanding the neuropsychologic phenotype of *MECP2*-related disorders. Our patients did not exhibit the breathing dysrhythmias commonly observed in females with classic Rett syndrome. Only one patient in our study (patient 5, Table 1) developed stereotypic hand movements; hand stereotypies were only observed in the study described by Meins et al.,[21] but not in the studies carried out by other groups.[22,23] The carrier females with *MECP2* duplication did not manifest any evidence of cognitive dysfunction or abnormal neurologic findings, which could be a reflection of the skewed X-inactivation pattern.

These data collectively highlight the value of comprehensive *MECP2* clinical testing in both female and male patients with a diverse range of neurodevelopmental phenotypes, in addition to classic Rett syndrome. This includes sequence analysis for the majority of Rett syndrome mutations, plus quantitative analysis to assess *MECP2* deletions and duplications. Although we have a limited dataset to assess recurrence risks associated with *MECP2* duplications, our findings of three of three asymptomatic mothers tested to be duplication carriers with skewed X-inactivation raises important issues for carrier testing and genetic counseling in families with identified abnormalities in the *MECP2* gene. In conclusion, a large cohort study of patients with *MECP2* duplication will be required to establish the frequency of this microduplication in males with mental retardation and associated neurologic features, and to determine the variability in the clinical phenotype observed for this syndrome.

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