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ARTICLE

De novo MECP2 duplication in two females with random X-inactivation and moderate mental retardation

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Xq28 duplications including *MECP2* are a well-known cause of severe mental retardation in males with seizures, muscular hypotonia, progressive spasticity, poor speech and recurrent infections that often lead to early death. Female carriers usually show a normal intellectual performance due to skewed X-inactivation (XCI). We report on two female patients with a *de novo MECP2* duplication associated with moderate mental retardation. In both patients, the *de novo* duplication occurred on the paternal allele, and both patients show a random XCI, which can be assumed as the triggering factor for the phenotype. Furthermore, we describe the phenotype that might be restricted to unspecific mild-to -moderate mental retardation with neurological features in early adulthood.
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INTRODUCTION

The X-linked Rett syndrome (OMIM #312750) has been known for a long time as a mental retardation syndrome, with its first clinical description in 1966/1977, and the identification of its underlying cause with loss-of-function mutations of the *MECP2* gene in 1999.[1–3] The Rett syndrome is almost always sporadic because of *de novo* mutations and is seen almost exclusively in girls. Typical findings of classic Rett syndrome are a regression of motor skills, communicative abilities and especially a loss of purposeful hand movements, accompanied by secondary microcephaly after a period of (near to) normal development. The few affected males with deleterious *MECP2* mutations usually present with clinical features consisting of fatal seizures and severe mental retardation including microcephaly that often leads to death during the initial years. The genetically related Lubs syndrome (OMIM #300260) was first described in 1999, and etiologically resolved in 2005 as the *MECP2* duplication syndrome.[4–6] Xq28 duplications of different sizes spanning the *MECP2* gene have been identified to date as a common cause of mental retardation in males. In familial cases with X-linked pedigrees, the asymptomatic female carriers show a significant skewing of X-inactivation (XCI).[7–9] Besides mental retardation, the main features of the affected boys are muscular hypotonia, progressive spasticity, seizures, poor speech and recurrent infections. The reported duplications vary from 0.1 to 2.6 Mb with the minimal critical region containing the *IRAK* and *MECP2* genes.[7] In familial cases, carrier females might suffer from endocrinological abnormalities, autoimmune diseases, as well as psychiatric disorders.[10] A phenotype with mental retardation has been reported only two times.[11,12]

We report on two unrelated female patients with a *de novo MECP2* duplication and a random XCI, revealing a moderate but unspecific mental retardation in childhood and the development of neurological features in the second decade of life.

MATERIALS AND METHODS

Patients

Both patients were examined for diagnostic evaluation because of mental retardation. Karyotype analysis was performed for each patient on metaphase chromosomes from cultured blood lymphocytes using Giemsa banding at 550 bands resolution with normal results. The parents of patient 1 also showed normal karyotypes. Informed consent for the investigation was obtained from both families.

SNP microarray

Genomic DNA was isolated from peripheral blood leukocytes using routine procedures. In both samples, we used the Affymetrix Genome-Wide Human SNP Array 6.0 (Affymetrix, Santa Clara, CA, USA) with an average distance of 1.3 kb between neighboring probes. Samples were processed according to the manufacturer's instructions. Genotypes were called with Affymetrix Genotyping Console Software v3 (GTC) using the Birdseed algorithm with a default calling threshold of 0.5 and a prior size of 10 000 bases in a simultaneous analysis of the patient–parents trio. Interpretation was based on Human Genome Build 36 (NCBI).

Multiplex ligation-dependent probe amplification (MLPA)

In patient 2, *MECP2* duplication was confirmed by multiplex ligation-dependent probe amplification with the test kit P015D2 Human Probe Mix by MRC Holland, Amsterdam, The Netherlands (http://www.mlpa.com),

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according to the manufacturer's instructions. The multiplex ligation-dependent probe amplification profiles were obtained using normal and pathological DNA samples as controls.

Quantitative PCR (qPCR)

In patient 2, the relative copy number of selected genomic regions was determined by quantitative PCR as described previously (data not shown).

X-inactivation (XCI)

The highly polymorphic (CAG)n motif within the human androgen receptor gene (*HUMARA*) was used to distinguish and compare the methylation activity of the X chromosomes in both patients as described previously.[13]

Fluorescence *in situ* hybridization (FISH)

To investigate the localization of the duplicated segment, FISH analyses were performed on metaphase spreads and interphase nuclei of both patients and the parents of patient 2. Using standard two-color FISH protocols, a BAC clone from the duplicated region Xq28 (RP11-218L14, kindly provided by Joris Vermeesch, Center for Human Genetics, Leuven, Belgium) was hybridized together with a whole or partial chromosome painting probe for chromosome X (wcpX or pcpXq).[14] Microscopic evaluation of 20 metaphase spreads and 100 nuclei from each patient and the parents of patient 2 was performed using a DM RXA epifluorescence microscope (Leica Microsystems, Bensheim, Germany) equipped with a Sensys CCD camera (Photometrics, Tucson, AZ, USA).

Microsatellite analysis

The parental origin of the duplication was determined by microsatellite analysis, and the signal intensities for specific probes were determined on the 6.0 array. Microsatellite analysis was performed with four di-nucleotide sequences within the region of interest. Motifs were selected using the UCSC (University of California) genome browser and repeat-spanning primers were designed with a 5′-fluorescence tag for the forward primer. PCR amplicons for trios were analyzed using capillary electrophoresis (Beckman Coulter CEQ8000; Beckman Coulter, Brea, CA, USA) and amplicon lengths were determined. Besides genotyping, we measured peak heights for all duplication carriers compared with normal controls in order to identify duplicated and non-duplicated alleles as described previously.[15]

The SNP-microarray data were evaluated considering the genotype and the corresponding probe signals within the duplicated region, thus providing a correlation of a specific genotype and the respective signal height. The origin of the duplication was identified by an allele-specific elevation of signal intensity in the patient in comparison with the parental genotypes.

RESULTS

All position coordinates given below are based on Human Genome Build 36.

Case reports

Patient 1. The girl was born to healthy, non-consanguineous parents of Turkish origin at 39 weeks gestation via forceps delivery with a birth weight of 2900 g (−1.0 SD), a length of 48 cm (−1.3 SD) and a head circumference of 34 cm (−0.3 SD). She had constipation from birth, for which she had to be treated with a laxative. During her initial years of life, she presented with a high frequency of respiratory infections. She learned to walk and spoke her first words at the age of 19 months. Her further psychomotor development was delayed. The parents noted autistic behavioral features in their daughter – for example, that she mostly played alone.

We examined patient 1 for diagnostic evaluation at the age of 6 $\frac{2}{12}$ years and again at 7 $\frac{6}{12}$ years. At second examination, her body measurements were still normal: her height was 128 cm (0.3 SD), her weight was 28.5 kg (0.9 SD) and her OFC was 52.5 cm (0.7 SD). She visited a school for the mentally retarded, and was not able to write or count. Her non-verbal IQ was 58. Her constipation improved, but sometimes a drug therapy was necessary. Adenoids were removed at the age of 5 years, but overall there was no history of recurrent or severe infectious diseases. She still displayed autistic behavior, spending most of her time engaged in role-playing games with her dolls. Her psychomotor development was slow, but the parents did not report regression, and no epilepsy or stereotypies were reported. She had no facial dysmorphism, but facial similarity to her father, who also presented with synophrys. Bilateral simian creases as well as a sacral hemangioma were noted. No other abnormalities were present.

Patient 2. Patient 2 was born at 40 weeks of gestation by spontaneous vaginal delivery as the third of four children of non-consanguineous Turkish parents following an uncomplicated pregnancy. Birth measurements were normal with a weight of 3650 g (0.4 SD), a length of 52 cm (mean) and an OFC of 35.5 cm (0.5 SD). Her three brothers were healthy. She achieved normal motor milestones. Speech development was delayed and started at 3 years. At the age of 5 years, the parents reported a stagnation of development, but no regression. She mostly played alone and revealed some autistic features. After a first year at regular school, she changed to a special school. She never learnt to read or write. At the age of 10 years, standardized non-verbal testing (SON) showed a developmental age of 6 years and a cognitive score of 55. On physical examination at the age of 10 years, her weight was 36.8 kg (BMI: 17.6, 50–70th centile), her height was 145 cm (0.9 SD) and her OFC was 54 cm (1.2 SD). She showed a primary enuresis until the age of 12 years, and, furthermore, a vesicourethral reflux II° was diagnosed. The EEG at that age was irregular, with alpha-rhythm and abnormal beta waves. The brain MRI was normal.

At the age of 18 years, a spastic hypertonia of the legs appeared, which led to gait disturbances. EEG at the age of 20 years was physiological; MRI of the myelon revealed no pathological aspects. At 20 years, the patient's weight was 67 kg (BMI: 26.2, 90–97th centile), her height was 160 cm (−1 SD) and her OFC was 54 cm (mean). Patient 2 did not present with any dysmorphic features. Her course of puberty was described as normal and no endocrinological problems were reported.

Detection of *MECP2* duplications

The *de novo* duplication of Xq28 material, including the *MECP2* gene, was identified in both patients using array analyses. In patient 1, a 266-kb duplication (chrX: 152 892 781–153 158 642, 266 kb, 71 array markers) containing seven reference sequence genes was detected (RefSeq, according to the genome browser of the UCSC). The proximal breakpoint was located within the *TMEM187* gene, centromeric to the *IRAK* gene, and the telomeric breakpoint was located within the *TEX28* gene (Figure 1). In addition, patient 1 showed a second *de novo* duplication of 2q37.3 material (chr2: 241 714 171–241 825 938, 112 kb, 71 array markers), including the genes *ANO7*, *HDLBP*, *PASK* and*PPP1R7*. The genotype for patient 1 according to the International System for Human Cytogenetic Nomenclature 2009 (ISCN) is arr 2q37.3(241 714 171–241 825 938)×3 dn, Xq28(152 892 781–153 158 642)×3 dn.

In patient 2, we found a duplication of chromosome Xq28 material (chrX: 152 925 421–153 403 154, 478 kb, 215 array markers) containing 22 RefSeq genes (UCSC), with the proximal breakpoint centromeric to the *IRAK* gene and the distal breakpoint telomeric to the *FAM3A* gene (Figure 1). The nomenclature according to ISCN for patient 2 is arr Xq28(152 925 421–153 403 154)×3 dn.

Confirmation and delineation of *MECP2* duplications

In patients 1 and 2, the duplication in Xq28 was confirmed by interphase FISH based on the presentation of three hybridization signals

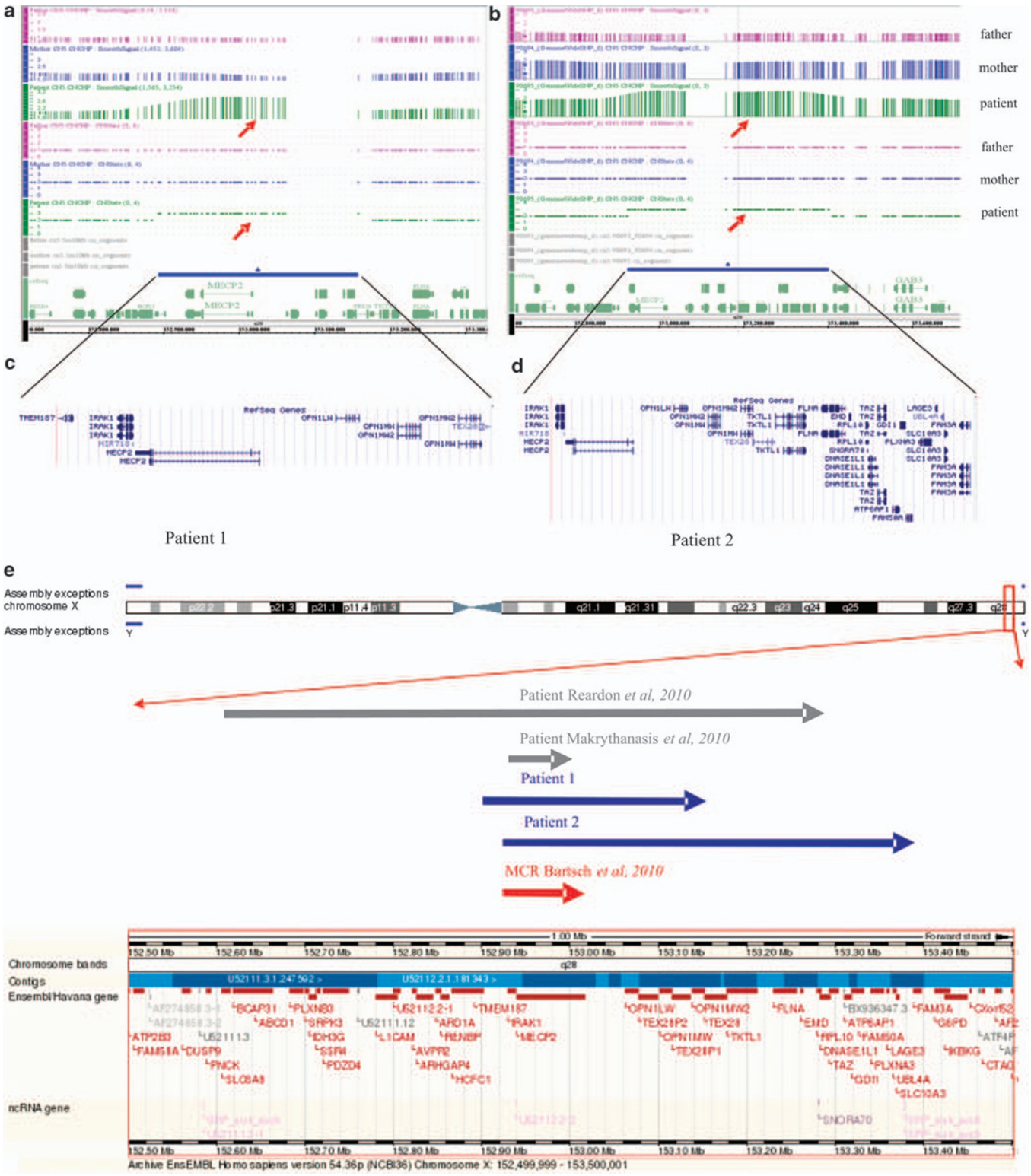

Figure 1 Illustration of the genomic aberration of the *MECP2* region with the 6.0 Affymetrix SNP Array and schematic representation of the duplicated region. (**a** and **b**) Image of the parents–patient trio analysis, showing the *de novo* occurrence of the duplication (red arrows) in both cases; (**c** and **d**) mapping of the duplicated regions according to UCSC; (**e**) schematic representation of part of the genomic region Xq28 with the location of the duplication of our two novel patients (blue arrows), the female patients of Reardon *et al*[11] and Makrythanasis *et al*[12] (gray arrows) as well as the minimal critical region (MCR) defined by Bartsch *et al*[7] (red arrow). Gene content of the region is shown from the Ensembl genome browser version 54.36p (NCBI36).

for the BAC clone RP11-218L14 (Figure 2) A localization of the duplicated region on an autosome was excluded for both patients in metaphase spreads. In the parents of patient 2, FISH analyses for the Xq28 BAC clone revealed only normal signals on the X chromosomes. Furthermore, duplications of the genes *IRAK*, *MECP2* and *GDI1* were confirmed in patient 2 according to the gene-specific probes from the

multiple ligation-dependent probe amplification kit, whereas a normal signal was obtained for the probe against the proximal flanking *IRAK* gene. In patient 2, the duplication of *MECP2* was confirmed by quantitative PCR (data not shown).

XCI studies showed a random XCI in both patients, with a status of 61:39 and 71:29, respectively (Figure 3). Microsatellite analysis revealed two alleles with different repeat lengths for the polymorphic marker in position chrX: 153 050 087–153 050 120 (17×TG) in the father's and in the mother's DNA of patient 2. Semi-quantitative analysis indicates a paternal origin of the *MECP2* duplication in patient 2 (data not shown). This marker and all other tested markers were not informative for patient 1. The analysis of the SNP-microarray data regarding the correlation of genotype and signal height within the duplicated region indicates a paternal origin in both patients.

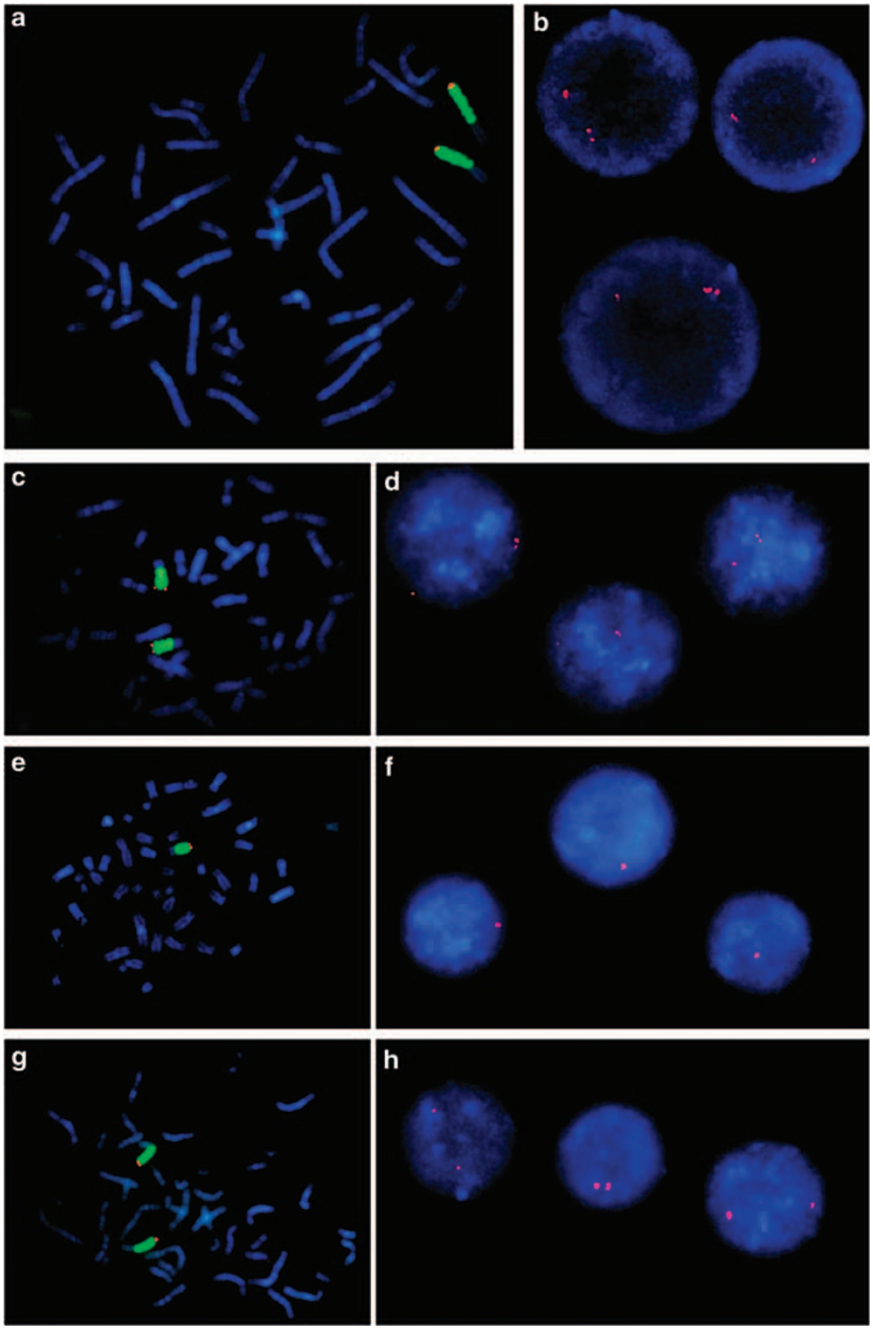

Figure 2 Metaphase and interphase FISH results. (**a**) Metaphase spread of patient 1 hybridized with the BAC clone RP11-218L14 localized in the duplicated chromosome region Xq28 (red) together with a partial chromosome painting probe for the long arm of the X chromosome (pcpXq, green). Signals are only present on both X chromosomes. (**b**) Interphase nuclei of the same patient, presenting a normal hybridization signal and a duplicated signal for the Xq28 BAC clone (red). (**c**) Metaphase spread of patient 2 after hybridization of the Xq28-BAC clone (RP11-218L14, red) together with a chromosome-painting probe for the X chromosome (wcpX, green). Signals are only present on both X chromosomes. (**d**) Interphase nuclei of patient 2 showing three hybridization signals, a normal signal and a duplicated signal for the Xq28 BAC clone (red). (**e**) Metaphase spread of the father of patient 2 presenting only one signal on the X chromosome and (**f**) a normal signal in the interphase nuclei with the Xq28 BAC clone. (**g**) Metaphase spread of the mother of patient 2 showing a specific hybridization signal on both X chromosomes and (**h**) two normal signals in the interphase nuclei.

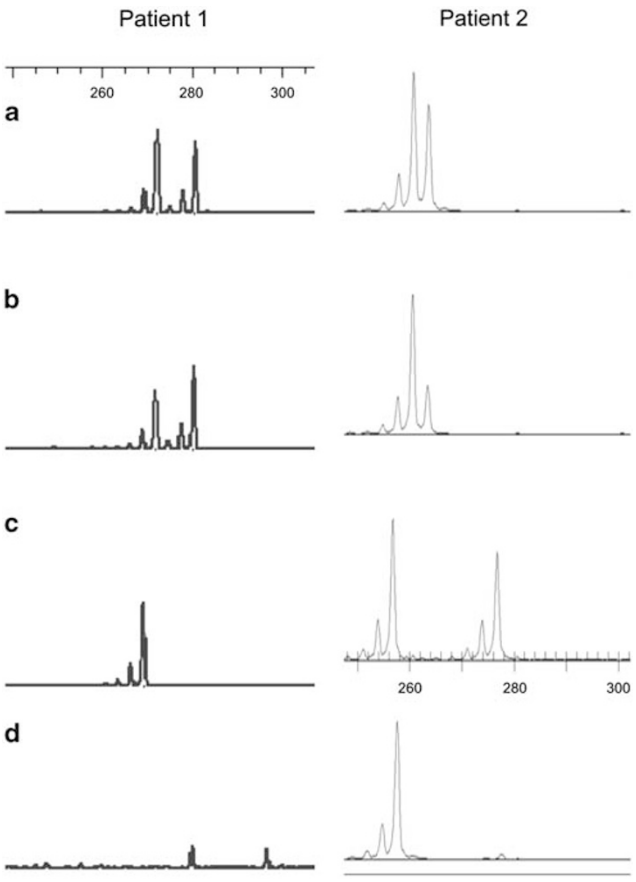

Figure 3 XCI results. Results showing random X inactivation in both patients and proof of complete digestion with methylation-sensitive restriction endonuclease *Hpa*II after PCR amplification of *HUMARA* (male control and female positive control, respectively). For patient 1 (performed on ABI3100, Genotyper v3.7), trace (**a**) shows the undigested sample, trace (**b**) the digested fragments. The lower two traces show the male control (**c**) undigested and (**d**) after digestion. The XCI in patient 1 is 39:61. For patient 2 the upper two traces show the patient's sample (**a**) undigested and (**b**) digested (CEQ8000, Beckman Coulter), revealing a random XCI of 29:71. The lower traces show a female positive control with complete skewing of X-inactivation in (**c**) undigested and (**d**) digested samples.

DISCUSSION

There were a considerable number of reports on mentally retarded boys with *MECP2* duplications during the years following the first publication.[6] However, to the best of our knowledge, only two mentally retarded females with an assured duplication have been reported.[11,12] This underrepresentation of girls may have different reasons. An investigation bias favoring affected boys to be investigated for *MECP2* duplications may have occurred, while girls were simply not investigated for this disorder. The fact that all females carrying the duplication were described as intellectually normal could have added to that effect. There is a recent report that considers certain clinical findings in those 'carrier mothers' as an affinity to endocrinological abnormalities (eg, hypothyroidism, menstrual irregularity and diabetes), autoimmune diseases, as well as psychiatric disorders (especially depression, anxiety and compulsion).[10] Nevertheless, the intellectual performance of all investigated females in that study was in the normal range (full scale IQ 81–123). During the previous years, large cohorts of mentally retarded patients of both sexes were investigated with high-resolution arrays for submicroscopic genomic imbalances through different array technologies. These should have shown *MECP2* duplications in females, and yet no further affected girls have been reported.[16] This contradicts the investigation bias as an explanation for the rarity of those cases. We conclude that *MECP2* duplications are a comparatively rare cause of mental retardation in females. This is confirmed by the identification of only two cases out of our cohort of 1000 unselected patients with mental retardation (incidence of 0.002).

In this study, we demonstrate two more female patients with an *MECP2* duplication, and further delineate the aspect that, as Reardon *et al*[11] first suggested, the random XCI may be causative for the phenotype. Most of the published carrier women who were informative for the XCI test presented a highly skewed XCI, with the duplication-bearing X chromosome being preferentially inactivated.[10] One can reasonably assume that the random XCI is the crucial point leading to the mental retardation phenotype in these patients, whereas a highly skewed XCI with a preferential inactivation of the duplication-bearing X-chromosome is a protective factor. The impact of the XCI in our patients is further emphasized by the fact that a translocation of the duplicated segment to an autosome was excluded by FISH investigation. Another remarkable aspect of our patients, besides their retardation and the random XCI, is that the *MECP2* duplication occurred *de novo* in both cases. We propose that *de novo* occurrence of the *MECP2* duplication, as in our cases, might have an effect on the XCI by preventing a protective 'mutation-induced' skewed XCI by a yet unrecognized mechanism. This would mean that there could be a different trigger for the methylation process from an inherited *MECP2* duplication than from the one that occurred *de novo*. Furthermore, analysis of the microsatellites and the signal intensities of the microarrays for our patients showed that the *de novo MECP2* duplication had been derived from the paternal X chromosome, which is a novelty in the literature.

For all but one female case reported so far, a duplication on the maternal X-chromosome can be assumed because of an X-linked pedigree. In this case, the duplication occurred *de novo* on an autosome (chromosome 10), thus completely escaping XCI.[12]

A post-zygotic mosaic occurrence of the duplication is a feasible explanation for a random XCI, and mosaicism cannot be excluded by any means. However, both patients showed no clinical signs of mosaicism, such as pigmental abnormalities of the skin or asymmetry, and the SNP-arrays gave no evidence for a mosaic state of the *MECP2* duplication. This contradicts a high-level post-zygotic mosaicism as a cause for the random XCI in our cases.

It has to be mentioned that in patient 1, a second *de novo* duplication was detected spanning 112 kb of chromosome 2q37 material with the RefSeq genes *PASK*, *PPP1R7*, *ANO7* and *HDLBP* (UCSC:NCBI36/hg18). Only weak *PASK* (OMIM *607505) expression is found in human thymus and testis. *PPP1R7* (OMIM *602877) is expressed in all human tissues including the brain. *ANO7* (OMIM *605096) is expressed only in the prostate. The haploinsufficiency of two of the genes within this region has been associated to neuropsychiatric disorders (*HDLBP* and *PASK*[17]), but to our knowledge no phenotype has been demonstrated for a duplication of these genes. Thus, the significance of the 2q37 duplication remains unclear. An effect of the 2q37 duplication on the phenotype of the patient seems to be unlikely, but cannot be completely excluded. Regarding that, the phenotypic effect of a *MECP2* duplication is already well documented, and patient 1 presents no clinical signs of a neuropsychiatric disorder

Table 1 Clinical findings in female patients with *MECP2* duplication and random X-inactivation

	Patient 1	*Patient 2*	*Reardon et al*[12]	*Makrythanasis et al*[13]
Age at last examination (years)	7 $\frac{6}{12}$	20	12	18
MECP2 duplication	*De novo*	*De novo*	Maternal	*De novo*
Mental retardation	Moderate	Moderate	Mild	Apparent, not specified
Autistic features	+	+	n.r.	–
Speech	Good	Good	Good	Simple
Seizures	–	–	–	–
Neurological symptoms	–	Leg spasticity	–	Poor coordination, eye movements
Facial dysmorphism	–	–	–	–
Constipation	+	–	n.r.	–
Frequent infections in early childhood	+	–	n.r.	n.r.
Bilateral simian creases	+	–	n.r.	–
Sacral hemangioma	+	–	n.r.	–
Vesicourethral reflux	n.r.	+	n.r.	–
Endocrinological problems	n.r.	–	n.r.	+

Abbreviation: n.r., not reported.

exceeding the phenotype of patient 2; we feel it safe to assume that the phenotype is caused by the *MECP2* duplication in both patients.

The typical symptoms of affected boys, such as seizures, poor speech development and recurrent severe infections, are not present in the females. The clinical signs in female patients with *MECP2* duplication and random XCI consist of unspecific mild-to-moderate mental retardation, with autistic features, recurrent infections in early childhood, constipation and late-onset neurological features as variable symptoms. Facial dysmorphism was absent in all females (Table 1).[18] This unspecific phenotype does not allow the diagnosis of females with a *MECP2* duplication and random XCI on clinical grounds alone. Nevertheless, the stagnation of development at the age of 5 years in patient 2, as well as the onset of spasticity in this patient at the age of 18 years, seems remarkable, especially regarding the progressive spasticity in males. This underlines the necessity to consider late-onset neurological features as a possible aspect in affected females.

In conclusion, we report on two female patients with a *de novo* *MECP2* duplication and a mental retardation. Both patients show a random XCI, which can be postulated as the triggering factor for the phenotype. Furthermore, these are the first patients with a duplication derived from the paternal X chromosome and this report gives a description of the phenotype of affected females.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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