

Clinical and Molecular Cytogenetic Characterization of Two Patients With Non-Mutational Aberrations of the *FMR2* Gene

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We report on two patients; a female having mild mental retardation (MR) with a balanced translocation, 46,XX,t(X;15)(q28;p11.2), and a male diagnosed as having mucopolysaccharidosis type II (MPS II or Hunter syndrome) with atypical early-onset MR and a normal male karyotype. Molecular cytogenetic analyses, including fluorescence in situ hybridization and array-based comparative genomic hybridization using an in-house X-tiling array, revealed that first patient to have a breakpoint at Xq28 lying within the *FMR2* gene and the second to have a small deletion at Xq28 including part of *FMR2* together with the *IDS* gene responsible for MPS II. In Patient 1, X-chromosome inactivation predominantly occurred in the normal X in her

lymphocytes, suggesting that her MR might be explained by a disruption of the *FMR2* gene on der(X)t(X;15) concomitant with the predominant inactivation of the intact *FMR2* gene in another allele. We compared phenotypes of Patient 2 with those of MPS II cases with deletion of the *IDS* gene alone reported previously, suggesting that the early-onset MR might be affected by the additional deletion of *FMR2*.

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INTRODUCTION

FRAXE fragile site-associated mental retardation (OMIM 309548) is the cause of a non-syndromic X-linked mental retardation (XLMR), affecting 1 in 23,423 males [Youings et al., 2000]. The disorder is a consequence of either a hypermethylation of the FRAXE CpG island, caused by an expansion of a CCG repeat in the 5'-untranslated region (UTR) of exon 1 of the *FMR2* (*AFF2*; *AF4/FMR2* family, member 2) gene and resulting in a reduction of *FMR2* expression [Knight et al., 1993; Brown, 1996], or internal *FMR2* deletion(s) [Gecz, 2000]. The FRAXE phenotype is primarily characterized by mild-to-borderline mental

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retardation (MR), accompanied by a number of inconsistent symptoms, including a long, narrow face, mild facial hypoplasia, a high-arched palate, irregular teeth, hair abnormality, angiomas, clinodactyly, thick lips, and nasal abnormalities [Hamel et al., 1994; Knight et al., 1994, 1996; Mulley et al., 1995; Carbonell et al., 1996; Murgia et al., 1996]. Some FRAXE patients also have behavioral deficits, such as attention deficit, hyperactivity, and autistic-like behavior. Two patients with internal deletions of the *FMR2* gene had similar phenotypes [Gedeon et al., 1995] and mice lacking *FMR2* showed a condition similar to human MR with impaired learning and memory performance and increased long-term potentiation [Gu et al., 2002], supporting the notion that *FMR2* is solely responsible for MR in FRAXE. In addition, *FMR2* was reported as a potential gene responsible for epilepsy as well [Timms et al., 1997; Moore et al., 1999].

In this article, we report on two patients with non-mutational aberrations of *FMR2*: a female having mild MR with a balanced translocation, 46,XX,t(X;15)(q28;p11.2), whose breakpoint at der(X)t(X;15)(q28;p11.2) lying within the *FMR2* gene, and a male having mucopolysaccharidosis type II (OMIM 309900) with atypical early-onset MR and submicroscopic deletion at Xq28 which harbors part of *FMR2* together with the *IDS* gene responsible for MPS II.

SUBJECTS AND METHODS

Clinical Report

Patient 1. The patient is a 3-year-old girl with mild MR. She was born as the fourth child by vaginal delivery at 40 weeks to healthy parents. Her mother had had a miscarriage of the first child at 8 weeks of gestational age (GA) and the second child at 2 months of GA. The third child was healthy. Her birth weight was 3,075 g and occipitofrontal circumference (OFC) was 33 cm. Her only anomaly was a cleft soft palate which did not prevent normal breastfeeding. She revealed developmental retardation; at 6 months she could hold her neck up and at 22 months she could sit alone. At 19 months she started taking valproic acid for frequent febrile convulsion. At present, 36 months, she can stand with support. She shows a tendency of an auto-synnoia and speaks no words. Her muscular tonus and reflex are normal. She has not shown growth retardation. No abnormality was detected by a general blood examination. Brain MRI revealed a mild enlargement of lateral ventricles and the fourth ventricle.

Patient 2. The patient is a 23-month-old boy diagnosed with severe MPS II (Hunter syndrome). He was born as the second child at 38 weeks 2 days by vaginal delivery to healthy parents. His birth weight was 3,984 g. His elder brother was normal. At

5 months he was diagnosed as a floppy infant and was examined intimately. He presented with coarse face, slight macroglossia, abdominal distention and muscular hypotonia, but neither hepatomegaly nor splenomegaly. A routine biochemical examination did not reveal any obvious aberration. At this time his diagnosis was not established, but at 8 months he was suspected of having a mucopolysaccharidosis because his hepatomegaly and splenomegaly had become obvious. An analysis of urinary mucopolysaccharide revealed a high level of uronic acid (263 mg/g creatinin), and showed a very low level (0.5 nmol/mg protein/4 hr) of iduronate 2-sulfatase activity of peripheral leukocytes leading to a diagnosis of Hunter syndrome. He demonstrated developmental retardation including early onset MR; at 8 months he could hold his neck up, at 11 months he could roll over, and at 20 months he could sit alone. At present, 23 months, he cannot yet stand alone and makes only bubbling sounds, and up to now, has not suffered from seizures. His clinical features have shown changes characteristic of Hunter syndrome (Table I).

Samples and CGH-Array Analysis

A lymphoblastoid cell line (LCL) was established by infecting lymphocytes of the patient with Epstein-Barr virus as previously reported [Saito-Ohara et al., 2002].

Among recently constructed our in-house bacterial artificial chromosome (BAC) arrays [Inazawa et al., 2004], we employed MCG Whole Genome Array-4500, which contains 4,523 BAC clones cover-

TABLE I. Comparison of Clinical Features in Our Case and Other Reported Cases With Hunter Syndrome

Patient number	Our case	Karsten et al. [1998]		
	#2 ^a	#3	#20	#21
Mental retardation	+	+	+	+
Coarse facial features	+	+	+	+
Macroglossia	+	+	+	+
Hepatomegaly	+	+	+	-
Splenomegaly	+	+	+	-
Adenoids	+	+	+	+
Short stature	+	+	+	-
Platyspondylia	+	+	+	+
Muscular hypotonia	+	-	-	-
Hypothyroidism	+	-	-	-
Gastroesophageal reflux	+	-	-	-
Age at diagnosis	2	5	5	7
Gender	Male	Male	Male	Male
<i>FMR2</i> abnormality	del	None	None	None
<i>IDS</i> abnormality	del	del	del	del

Gray lane indicates phenotype originating from Hunter syndrome in case 2. +, feature present; -, feature absent; trans, translocation; del, deletion.

^aExamined at 23 months.

ing the entire genome at intervals of approximately 0.7 Mb, and MCG X-tiling array, which contains 1,001 BAC/PACs throughout the X-chromosome other than pseudoautosomal regions in an array-CGH analysis to screen for submicroscopic chromosomal aberrations. Hybridization was carried out as described previously [Sonoda et al., 2004; Hayashi et al., 2005; Takada et al., 2005] with minor modifications. Briefly, test and reference genomic DNA from the LCL and sex-matched normal lymphocytes were labeled with Alexa Fluor 555 and 647, respectively using a BioPrime Plus Array CGH Indirect Genomic Labeling system (Invitrogen, Carlsbad, CA), and co-hybridized to array slides in a hybridization machine, HYBRIMASTER HS-300 (ALOKA, Tokyo, Japan). After hybridization, slides were scanned with a GenePix 4000 B (Axon Instruments, Foster City, CA). Acquired images were analyzed with GenePix Pro 6.0 imaging software (Axon Instruments). Fluorescence ratios were normalized so that the mean of the middle third of \log_2 ratios across the array was zero. The thresholds for copy-number gain and loss were set at \log_2 ratios of 0.4 and -0.4 , respectively.

Fluorescence In Situ Hybridization (FISH) Analysis

Metaphase chromosomes were prepared from normal male lymphocytes and from each of the patient's lymphocytes using the standard method. FISH analyses were performed as described previously [Hayashi et al., 2005], using BAC clones located around the region of interest as probes.

Assays for X-Chromosome Inactivation

The pattern of X-chromosome inactivation in the female was first evaluated using the *androgen receptor (AR)* X-inactivation assay described by Kubota et al. [1999] with minor modifications. Briefly, DNA was modified with sodium bisulfite and amplified with primers specific for a methylated or unmethylated DNA sequence at the human *androgen receptor (HUMARA)* locus where methylation correlates with X-inactivation. Two different sized products, which were gained from the paternal and maternal alleles because of the polymorphism of the triplet repeat, were analyzed on a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA), and peak images of each PCR product were measured by GeneMapper Software v4.0 (Applied Biosystems). An imbalance of X-chromosome inactivation (skewing) was judged from the ratio between the amount of PCR product from paternal and maternal alleles. These ratios were corrected using a calculation described previously [Kubota et al., 1999].

In the X-chromosome revealed an imbalance of inactivation by *HUMARA*, X-inactivation was then

evaluated in metaphases from LCL using the bromodeoxy-dUTP (BrdU) labeling technique in late S-phase developed according to methods described previously [Panasiuk et al., 1997]. These chromosomes were used for FISH analysis to show the affected X-chromosome.

PCR Amplification

Genomic PCR to determine the deleted region in Patient 2 was carried out with primers designed to amplify genomic sequences around exons 8, 9, 10, and 11 of the *IDS* gene (Table II). The PCR products were electrophoresed, and analyzed with a LAS-3000 (Fujifilm, Tokyo, Japan).

RESULTS

Patient 1

A G-banded chromosomal analysis of peripheral blood lymphocytes of the patient revealed 46,XX,t(X;15)(q28;p11.2) (Fig. 1A). A chromosomal analysis was also carried out on the proband's parents, whose karyotypes were found to be normal (data not shown), indicating that the chromosomal abnormality observed in the proband is *de novo*.

In order to precisely map the breakpoint involved in the der(X)t(X;15) at Xq28, we performed FISH analyses with 5 BACs (RP11-75A14, 55B1, 105P24, 262K13, and 662A14; Fig. 1C). RP11-55B1 signals were detected on normal X, der(X), and der(15), suggesting that the break point at Xq28 occurred within this BAC (Fig. 1B), whereas RP11-105P24 signals were detected on normal X and der(15), but not on der(X), indicating that this BAC is distal to the der(X) breakpoint (Fig. 1B). Taken together, our FISH indicates that *FMR2* is probably disrupted between exons 3 and 4.

Since Patient 1 is female, we investigated the X-inactivation status of the patient based on methylation-specific PCR evaluating the (CAG) n polymorphism at the androgen receptor locus. As shown in Figure 1D, PCR products of different sizes respectively from different X-chromosomes in methyl-specific and unmethyl-specific PCR showed a non-random X-inactivation pattern (skewed

TABLE II. PCR Primers Used for Genomic PCR

Primer set	Primer sequence, 5'-3'	Size (bp)
FMR2_8_F1	GAGTGGAAATGACCCCAACCAC	121
FMR2_8_R1	AGCATTGAGGCACTGTTTCC	
FMR2_9_F1	CCTGAAGCTGAGCAGTGATG	145
FMR2_9_R1	AAGCCATTCCAGTTCCTCT	
FMR2_10_F1	CACCCTGTTTCGGTTAGCTC	145
FMR2_10_R1	GGTTTGCCTTTTCAACAGC	
FMR2_11_F1	GAGCAGCTCTGAGTCGGATT	124
FMR2_11_R1	CAGGCAGATCATGGTTAGGG	

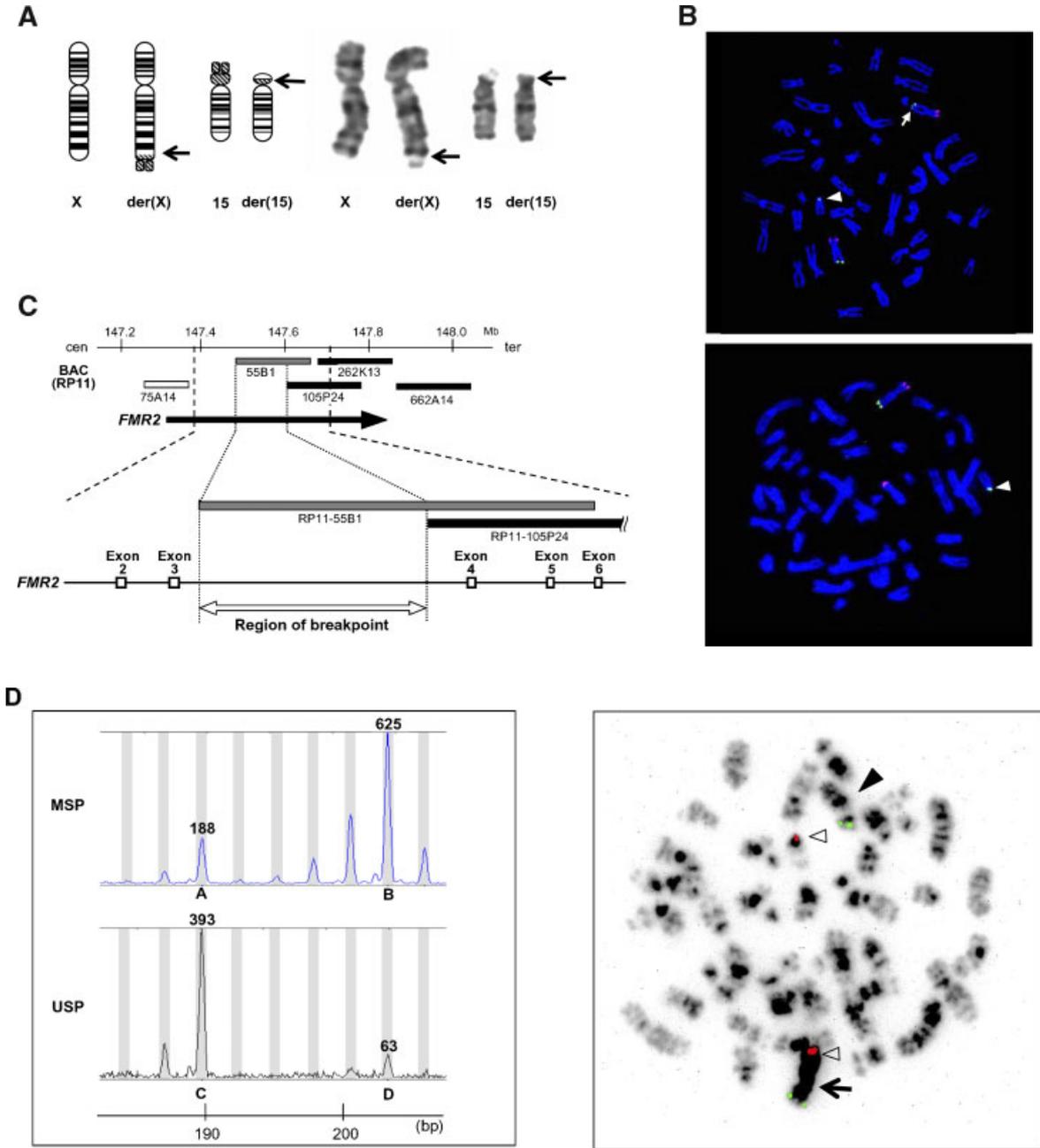


FIG. 1. **A:** Partial karyotype of Patient 1 with an apparently balanced translocation, 46,X,X,t(X;15)(q28;p11.2), as well as ideograms of chromosomes 15, X, der(15), and der(X). Arrows indicate break points. **B:** Representative FISH image of Patient 1. **Upper:** Signals of BAC RP11-55B1 (Xq28, green) were detected on chromosome X, der(X) (arrow), and der(15) (arrowhead). **Lower:** BAC RP11-105P24 (Xq28, green) were detected on X and der(15) (arrowhead). BAC RP11-115I10 (Xp21, red) was used as a control for the X-chromosome. **C:** Schematic presentation of the Xq28 translocation breakpoint in Patient 1. The filled bar indicates BACs translocated to chromosome 15, open bar indicates BAC located on the X-chromosome, and gray bar indicates BAC containing the Xq28 breakpoint region. **D (Left):** Representative result of a methylation-specific PCR-based androgen receptor (AR) X-inactivation assay in Patient 1. "A" and "C" indicate peak area of the inactive X (Xi) and the active X (Xa), respectively, from one parent, whereas "B" and "D" indicate the peak area of Xi and Xa, respectively from the other parent. Peak ratio was calculated from peak height (number on each peak) in each peak area according to the method described by Kubota et al. [1999], and was 95:5. **Right:** Representative results of a replicating study to determine which X-chromosome is late replicating (inactive) in Patient 1. FISH was performed using BACs specific for Xq28 (RP11-662A24, red, open arrowheads) and Xp22.2 (RP11-18M9, green) to discriminate the intact X and the der(X). In 42/50 cells analyzed, the normal X is late replicating (inactive, arrow) and the der(X) is early replicating (active, filled arrowhead), indicating a skewed X-inactivation pattern in the patient. Notably this result is consistent with the result obtained by the PCR-based method (left). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

X-inactivation): the distribution of the inactivated X-chromosome in this patient was 95:5. In order to determine which X-chromosome was inactivated, we carried out an X-replication study with

cytogenetic BrdU banding and FISH using a probe on Xq28 (RP11-662A24) containing the der(X)t(X;15) breakpoint and a probe on Xp22.2. (RP11-18M9) as the reference (Fig. 1D). In 42 of 50 cells analyzed, the

der(X)t(X;15) showed an early replicating pattern, whereas the normal X-chromosome showed a late replicating pattern. Since late replication is associated with the inactive (methylated) genomic DNA, this result indicates that X-inactivation predominantly occurred in the normal X-chromosome in LCL from Patient 1.

Patient 2

The patient was diagnosed with MPS II (Hunter syndrome) at 8 months. A G-banded chromosomal analysis of cultured peripheral blood lymphocytes revealed a normal male karyotype.

We first carried out an array-CGH using our MCG Whole Genome Array-4500, and identified a cryptic deletion, which was evident at clone RP11-130N6 (\log_2 ratio = -0.9), at Xq28 in the patient (Fig. 2A). FISH analysis with this BAC clone as a probe demonstrated that no signal was detected in either metaphase or interphase chromosomes from the patient (Fig. 2B), confirming the deletion around this BAC at Xq28.

In order to determine the precise region involved in the deletion at Xq28, we performed an array-CGH using the MCG X-tiling array. The ratio profile of array-CGH clearly showed that the ratio on each of the 6 BACs at 147.7–148.6 Mb from the telomere end of Xp was low (\log_2 ratio < -0.9), representing deletion (Fig. 2A). RP11-430I1, which is proximal to the deleted region, showed a ratio of -0.5 (\log_2 ratio), suggesting that this BAC is partially deleted in the patient, whereas RP11-722C13 showed a normal ratio. Altogether, the deletion seems to be approximately 0.9 Mb long. The region involved in the Xq28 deletion harbors at least six genes (<http://genome.ucsc.edu/>) including *FMR2*, *MAGEA8*, *MAGEA9*, *MAGEA11*, and *FAM11A* together with *IDS*, which is responsible for MPS II [Hopwood et al., 1993; Bondeson et al., 1995; Birot et al., 1996; Karsten et al., 1997]. To determine the deletion around *FMR2* more precisely, a genomic PCR was carried out using primer sets designed to amplify the sequence around each exon of the *FMR2* gene. The result of the PCR revealed the proximal border of the deletion to be between exons 9 and 10 (Fig. 2D).

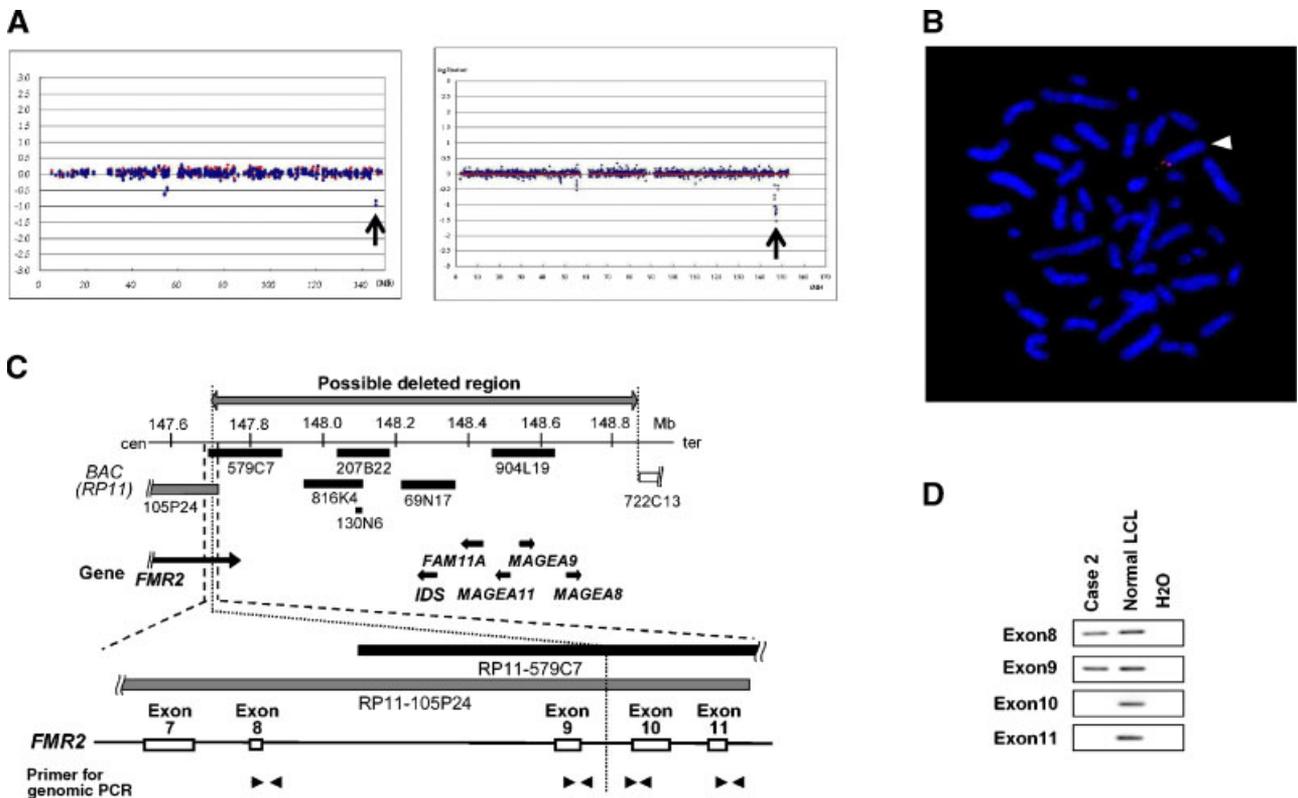


FIG. 2. **A:** The result of array-CGH analysis (left, MCG Whole Genome Array-4500; Right, MCG X-tiling array) in Patient 2 using genomic DNA from the patient and a normal female as a test and a reference, respectively. Fluorescence intensity on each BAC is normalized and plotted as described in Subjects and Methods. Each of the dots represents the \log_2 ratio of each BAC: blue dots represent results of the patient, whereas red dots show the average ratios from two independent healthy males. **B:** Representative result of FISH in Patient 2 using BACs specific for Xq28 (RP11-130N6, green) and the control BAC (RP11-802I24, red). A green signal could not be detected on the X-chromosome (arrowhead). **C:** Mapping of the Xq28 deletion region in Patient 2. BACs showed low (filled bar) and normal (open bar) ratios in the MCG X-tiling array, and BACs that showed a signal at Xq28 by FISH (gray bar) are listed. The gray closed arrow indicates the possible deleted region. Black thick arrows indicate genes around the deletion. Arrowheads in the bottom indicate primers for genomic PCR experiments. **D:** Representative image of genomic PCR experiments using the primer sets shown in Figure 2C. Note that this analysis showed that one of the borders of the deleted region was between exons 9 and 10. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

DISCUSSION

Here we described two atypical cases involving genomic aberrations at FRAXE, which were not caused by expansion of a CCG repeat in the 5'-UTR of exon 1 of the *FMR2* gene resulting in a hypermethylation of the FRAXE CpG island and reduction of *FMR2* expression.

Characterization of breakpoints in patients with apparently balanced constitutional chromosome rearrangements and phenotypic abnormalities has proved an invaluable strategy for identifying disease-related genes [Tommerup, 1993]. Indeed, several genes responsible for non-syndromic XLMR have been identified in this way [Billuart et al., 1998; Kutsche et al., 2000; Zemni et al., 2000; Vervoort et al., 2002]. Phenotypic abnormalities seen in patients with apparently balanced chromosome rearrangements have usually been explained by the disruption of a gene at the breakpoint causing a loss of gene function. In Patient 1, we have investigated a female patient with MR, who has an apparently balanced X;autosome translocation, 46,XX,t(X;15)(q28;p11.2), and successfully defined the breakpoint at Xq28 within the *FMR2* gene, which is known as one of the genes responsible for non-syndromic X-linked MR [Gecz et al., 1996; Gu et al., 1996]. Further examinations of the X-inactivation pattern demonstrated that the intact X-chromosome was preferentially inactivated in the girl, even in LCL, suggesting that her MR may be explained by a disruption of the *FMR2* gene in der(X)t(X;15) concomitant with a predominant inactivation of the intact *FMR2* gene in another allele, which has never before been reported.

MPS II or Hunter syndrome (OMIM 309900) is an X-linked recessive lysosomal storage disease caused by a deficiency of iduronate 2-sulfatase (IDS; EC3.1.6.13) that catalyzes the degradation of dermatan and heparan sulfate. The enzymatic defect results in an accumulation of the latter compounds in lysosomes and excretion of glycosaminoglycan fragments in urine [Neufeld and Muenzer, 2001]. Clinically, the phenotype of MPSII varies from severe to mild, depending on the different mutations or deletions at the *IDS* gene. As shown in Table I, we compared phenotypes of our Patient 2 with those of three cases of severe MPS II with a deletion of only *IDS* reported by Karsten et al. [1998]. However, even in patients with severe type of MPS II, MR manifests at approximately 2–4 years among various phenotypes of this syndrome [Jones, 2006]. As described above, on the other hand, Patient 2 could hold his neck at 8 months, roll over at 11 months, and sit alone at 20 months, suggesting that onset of his MR occurred earlier as compared to in severe type of MPSII. In Patient 2, our molecular cytogenetic analysis of the cryptic deletion within Xq28 using array-CGH together with subsequent FISH and genomic PCR

revealed that the deletion included *IDS* and a part of *FMR2* together with four other genes, *MAGEA8*, *MAGEA9*, *MAGEA11*, and *FAM11A*. Since functional significance of members of MAGEA family and FAM11A in neuronal development are unknown (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM>) and mRNA expression levels of them are extremely low in fetal or adult brain (<http://www.lsbm.org/database/index.html>), we excluded them from candidate genes related to the phenotype of this patient, and speculated that the early-onset MR in Patient 2 might have been affected by the additional deletion of the *FMR2* gene. In addition to early onset MR, Patient 2 showed muscular hypotonia as an atypical feature of Hunter syndrome, which might be affected by alteration(s) of neighboring gene(s) except *FMR2* and *IDS* at the Xq28 deletion. *MTM1*, responsible for X-linked myotubular myopathy (OMIM 310400) lies at Xq28 approximately 400 kb distal to the border of the deletion. Although *MTM1* is not included within the deleted region, it is possible that his phenotype may be affected with the functional aberrations of *MTM1* due to lack or rearrangement of genomic sequences associated with transcription of this gene. Further, Timms et al. [1997] investigated molecular and phenotypic variation in patients with severe MPSII. Since the occurrence of seizures in two individuals correlated with a deletion extending proximal of *IDS*, up to and including part of the *FMR2* locus, they reported that *FMR2* is a candidate causative gene for seizures, when mutated along with *IDS*. It will be necessary to carefully follow patients with this symptom, although Patient 2, currently 23 months old, has not shown seizures to date.

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