

ORIGINAL

# Single-strand conformation polymorphism analysis of the *FMR1* gene in autistic and mentally retarded children in Japan

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**Abstract :** Fragile X syndrome is one of the most common causes of mental retardation in males, and patients with fragile X syndrome occasionally develop autism. It is usually caused by an expansion of the trinucleotide repeat in the 5'-untranslated region of the *FMR1* gene, but in a small number of patients deletions and point mutations have been identified. We screened all 17 exons of the *FMR1* gene for mutations in 90 autistic or mentally retarded children using polymerase chain reaction (PCR)-single strand conformation polymorphism (SSCP) analysis. No mutations were found in 76 male patients. However, one female patient was heterozygous for a normal allele and a mutant allele with an A to C substitution at nucleotide 879 in exon 9. This mutation was not found in 50 controls. Reverse transcription-PCR revealed that a large proportion of the mutant transcripts were spliced aberrantly, causing premature termination of the protein synthesis. Although uncommon, point mutations in the *FMR1* gene may be a cause of autism and mental retardation in Japanese patients. *J. Med. Invest.* 51 : 52-58, February, 2004

**Keywords :** *FMR1*, SSCP analysis, screening, mental retardation, point mutation

## INTRODUCTION

Fragile X syndrome is one of the most common forms of familial mental retardation. Population surveys have established that the prevalence in males is one in 4,000 and in females approximately one in 6,000 (1). Typical characteristics in male patients include long face, prominent chin, large floppy ears, macroorchidism, retarded language development, hyperactivity and seizures. In most patients the disease is caused by an expansion of the CGG trinucleotide repeat located in the 5'-untranslated region of the *FMR1* gene (2-4). The subsequent methylation of the expanded CGG repeat region and adjacent CpG island results in a lack of expression of the *FMR1* gene (5-7). Normal individuals have approximately 6-52 CGG repeats, and smaller expansion

of 53-200 CGG repeats called a premutation can be detected in carriers. Most patients with fragile X syndrome have full expansion with more than 200 CGG repeats. However a small number of patients have been found with deletion of all or part of the *FMR1* gene (8). Furthermore, three types of intragenic small mutations in the *FMR1* gene have been found ; one nucleotide substitution, (9), one nucleotide deletion and two nucleotide substitution (10).

The *FMR1* gene, located on chromosome Xq27.3, spans 38 kb and consists of 17 exons (11). *FMR1* codes for a protein designated as FMR protein (FMRP). FMRP is an RNA-binding protein, possessing two RNA-binding motifs, two KH domains and an RGG box (12, 13). It has been shown that FMRP indeed binds to RNA *in vitro* (14). Several alternatively spliced forms of *FMR1* mRNA are known to exist (11, 15-17) and the gene products vary in size from 75 to 85 kDa (18-20).

In many patients with fragile X syndrome, the symptoms are indistinguishable from autism (21, 22). Moreover, a triplet repeat expansion of the *FMR1* has been found in several patients previously diagnosed as autism

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or “non-specific” mental retardation. The possibility thus exists that autism and mental retardation are the result of mutations in the *FMR1* gene other than expansion of the CGG repeat. To test this hypothesis, we screened 76 males and 14 females for mutations in the *FMR1* gene using polymerase chain reaction (PCR)-single strand conformation polymorphism (SSCP).

**MATERIALS AND METHODS**

*Patients*

We screened 90 patients (76 male and 14 females) who were referred to our department for autism or “non-specific” mental retardation. An informed consent was obtained according to a guideline from the Ethics Committee of the University of Tokushima. All patients were negative for the CGG triplet repeat expansion.

*PCR-SSCP analysis*

Genomic DNA was isolated from the peripheral white blood cells of each patient. Using genomic DNA as a template, all 17 exons were amplified by PCR in a 20-μl reaction volume. The primers were designed so that the splice site regions were also analyzed. The sequences of the primers used for amplification are shown in Table I. All exons aside from exon 1 were amplified using 50 ng

genomic DNA with 20 pmol of each primer (forward and reverse) in a total volume of 20 μl, containing 1.5 mM MgCl<sub>2</sub>, 0.2 mM dNTP, and 0.5 units of *Taq* polymerase (Promega) in 1x PCR buffer (Promega). Amplification was performed during 40 cycles of denaturing at 94 for 20 sec followed by annealing at 53 for 1 min and extension at 72 for 1 min. Exon 1 was amplified in a total volume of 20μl, containing 50 ng of genomic DNA, 10 pmol of each primer (forward and reverse), 1.5 mM MgCl<sub>2</sub>, 0.2 mM dNTP, and 0.5 units of *Taq* polymerase in the 1x buffer. Amplification was carried out during 40 cycles of denaturing at 94 for 20 sec, annealing at 63 for 1 min, and extension at 72 for 1 min. SSCP analysis was carried out according to the method of Oto *et al* (23). One microliter of the PCR product was combined with 9 μl of 95% formamide loading dye containing 10 mM NaOH, 0.05% of bromophenol blue and 0.05% of xylene cyanol, was boiled for 5 min and immediately chilled on ice. Five microliters of the denatured DNA was applied onto 10 or 12% polyacrylamide gel depending on its length (see Table I), and electrophoresed both at 4 and at 20 in 0.5x TBE buffer (45mM Tris-HCl, pH 8.0, 45 mM borate, and 1 mM EDTA). Following electrophoresis, the gels were stained using silver staining system (Promega).

Table 1. Primer sequences sizes of the PCR product and concentrations of the acrylamide gel used for SSCP analysis

Exon	Forward	Reverse	Size (bp)	Acrylamide gel concentrations (%)
1	GCGCTAGCAGGGCTGAAGAG	AGGGAAGGAAGGGCGAAGAT	118	14
2	CACAAGTTAATTTAACGTTTTTCTTACA	CCTCATCATTAAAATTATATAACGAGACAC	119	14
3	CATGTTAAATAATTGTATGTTTGCTTATTT	CAGTGCTAAATGACTTTATGGCAG	163	12
4	GAAATATTCTGTGTTGTAATTTTTGTGT	GAAAAAAATTTAAATAGGCATTTTCCT	131	12
5	TTGTGATTAGAAGTGACTTTTATTTATTTT	TCAGCATTTCTGTGTATCAACTT	209	10
6	CTGCATTTATTTATGTGAGTAGTTGGTAA	ACTAAAAATAAACAACCTCAAAAATGCTC	187	10
7	GCCTTGATAATAATGTTGTTAATTTAAATC	TAGGTACCTTACAAGCAGTGCTA	192	10
8	TATTCATCAGACGTCCATTTCTCT	GGGAAAAAAAACCTATGCAGTAAAATA	231	10
9	TGTCTTAAAATGTTTCCCTTTTA	TGTTGTAATATATGTCAACAGATACTTCTG	143	10
10	TTTTAAAACCAACTTGATTTATTTATTTT	CACAAAATATTCGCACTGTAACAT	170	12
11	GAGCTAAATAAAGTCTTAAATTGGTCCTT	TTGTAATTCAAAGTGACAAGTAATTCTT	228	10
12	GACATCCCTTGCACTTCTTATACT	GTGTATTTCTCAAGGAATGTTTTCTT	122	12
13	GATCATTGTTGCAATTTCTTTTTT	AGGATTAGAAGTTAAAGTTCTTTCTGTTT	146	12
14	AATGTCAAATTTTTACTGTTATCTTGT	TAAAGTACTGAGTAAAATGCTTAGTTTGT	271	10
15	CAGACAATGGTATATAACTTTTAACTCTCG	AGCTGTTACAATTCACCTTTGATTTCTT	261	10
16	TCAGAATCAGTAACTGTTGAACCTTT	TAAGATGAAAAGAGGCAGACAGGT	155	12
17	CAGTAGGATATGGTCTGTGTATATAACAAC	GGTATAGGAAATATAACTTCAGAATTATGC	240	10

### Sequence Analysis

DNA fragments showing abnormal electrophoretic mobility were purified using PCR Preps DNA Purification System (Promega), and directly cloned into a pGEMT-Easy vector (Promega). Multiple clones were randomly picked up and sequenced using Thermo Sequenase Cycle Sequencing Kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

### Confirmation of 879 A to G mutation by *AhdI* digestion

A 273-bp genomic DNA fragment encompassing nucleotide 34514 in the genomic sequence (847 in the cDNA) in exon 9 and nucleotide 34786 in intron 9 was amplified by PCR. The sequences of the forward and reverse primers were 5'-aagatgtaatacaagttccaagacacttag and 5'-caagttagtggtgagcagcgaccagcat, respectively. In the forward primer, two nucleotides shown in bold were substituted so that the mutant DNA was cleaved into two fragments (246 bp and 27 bp) by *AhdI* while the normal DNA was not digested. PCR was performed in a total volume of 50  $\mu$ l containing 10 pmol of forward and reverse primers, 50 ng of genomic DNA, 0.2 mM dNTP, 1.5 mM MgCl<sub>2</sub>, and 0.5 units of *Taq* DNA polymerase in 1 x PCR buffer. After purifying the PCR product, the amplified DNA was digested with *AhdI* and analyzed on a 1.5% agarose gel.

### Reverse-transcription (RT)-PCR and sequencing

Total RNA was isolated from the patient's peripheral white blood cells using SV Total RNA Isolation System (Promega). Human brain mRNA (OriGene Technologies) was used as a control. Using these RNAs as a template, reverse transcription was carried out in a total volume of 20  $\mu$ l containing 2  $\mu$ g of total RNA or 0.1  $\mu$ g of mRNA, 0.25  $\mu$ g of random hexamer, 0.5 mM NTP, 2.5 mM MgCl<sub>2</sub>, 10 mM DTT, 200 units of Super Script II RT (Lifetech), and 40 units of RNase inhibitor in 1 x first-strand buffer. The mixture was incubated at 25  $^{\circ}$ C for 10 min and then at 42  $^{\circ}$ C for 50 min. First-strand cDNA was directly used for PCR to amplify a segment encompassing nucleotides 592 and 1219 of the *FMR1* cDNA. After column purification, the PCR product was subcloned en mass into a plasmid and sequenced as detailed above.

## RESULTS AND DISCUSSION

Using PCR-SSCP analysis, we screened all 17 exons of the *FMR1* gene in 90 children (76 males and 14 females) who were referred to us for autism or mental

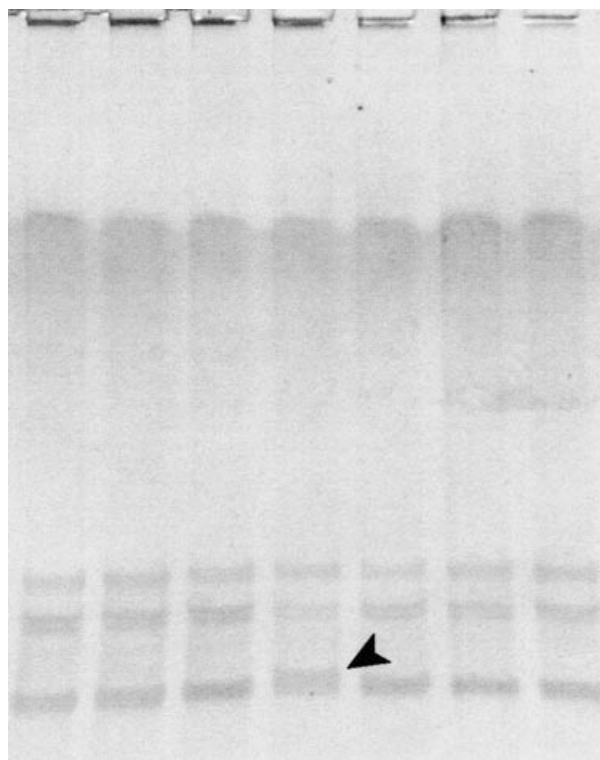


Fig. 1. PCR-SSCP analysis of exon 9 of the *FMR1* gene. The samples were run on a 10% agarose gel at 4  $^{\circ}$ C. Arrowhead indicates the band that showed an anomalous migration.

retardation. In order to enhance the sensitivity of detecting mutations, the amplified exons were run at two different temperatures, 4  $^{\circ}$ C and 20  $^{\circ}$ C. No mutations were found in male individuals, suggesting that mutations of the *FMR1* are not a common cause of autism or mental retardation. However, abnormal electrophoretic mobility was observed in one sample, exon 9 from an autistic female (Fig.1). This abnormality was detected on the gel run at 4  $^{\circ}$ C, but not detected on the gel run at 20  $^{\circ}$ C.

The patient in whom the abnormality was found was a 15-year old female. Her mother had of a normal pregnancy and she was delivered without complication. Her developmental milestones were normal until one year of age, when a psychomotor delay was first noted. At three years of age she was referred to our hospital for delayed speech. She presented as a remote child with poor eye contact. At nine years of age, focal spikes were noted in the mid-frontal region on electroencephalogram and carbamazepine therapy was initiated. At 11 years of age, she met the International Classification of Diseases criteria for autism. When she was examined at 14 years of age, she had severe mental retardation with an IQ below 20, and her Childhood Autism Rating Scale (CARS) was 34.5 suggesting that she was moderately autistic.

To characterize the mutation found in exon 9 of the

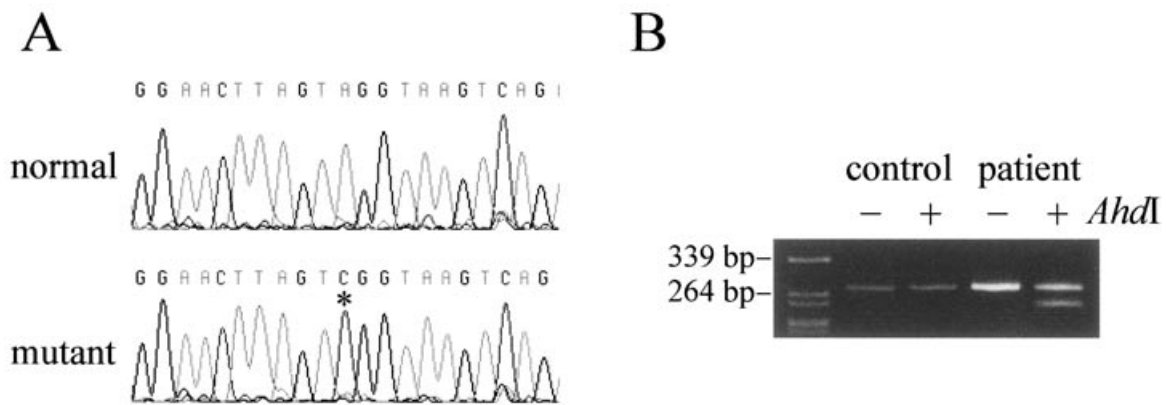


Fig. 2. A : Sequence analysis of exon 9 showing abnormal migration in PCR-SSCP analysis. Two types of sequence were detected normal and 879 A to C transversion (asterisk). B : *AhdI* digestion of exon 9 in the control and patient. The control fragment (273 bp) was not cut by *AhdI* whereas approximately half of the fragment amplified from patient's genomic DNA was digested into two segments (246 bp and 27 bp). The shorter 27 bp segment was not visible on the gel.

patient, the fragment was amplified by a new round of PCR and the amplified fragment was cloned into a vector. Seventeen clones carrying exon 9 were sequenced. Ten clones had an A to C transversion at nucleotide 879 (Fig. 2A), whereas the remaining seven had a normal sequence, suggesting that the patient was a heterozygote of the normal sequence and 879 A to C transversion. This was confirmed by *AhdI* digestion of the PCR product (Fig. 2B). This mutation was not found in 50 normal controls. Although this nucleotide substitution did not result in any change of the amino acid sequence, the possibility exists that this mutation affected splicing of the *FMR1* pre mRNA, given that 879 A is the second last nucleotide in exon 9. To explore this possibility, total RNA was isolated from the patient's peripheral blood cells. Total RNA from human brain was used as a control. The RNAs were reverse-transcribed, and a segment encompassing exon 7 (nucleotide 592) and exon 13 (nucleotide 1219) was amplified by PCR. As shown in Figure 3, two distinct bands were observed in normal controls. When these products were cloned and sequenced, three clones out of 12 had a normal sequence, whereas in the remaining nine clones, the entire exon 12 was skipped. This alternative splicing has been found in past studies, and it is not considered to be pathogenic (15, 17). The electrophoretic pattern of the DNA fragments amplified from the patient's total RNA were markedly different from that of the normal control. At least three distinct bands with different sizes were visible on as 1% agarose gel. After purifying the DNA using a column, this PCR product was directly subcloned en mass into a vector, and 36 clones were randomly picked up and sequenced. The results are shown in Figure 4. Among 36 clones, six clones had an A at nucleotide 879 and the remaining 30 clones

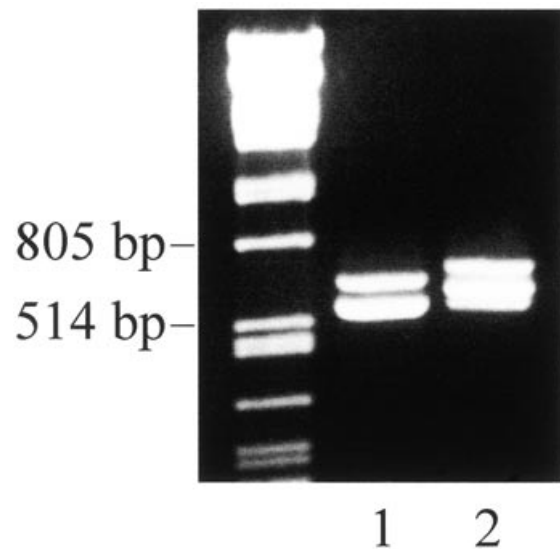


Fig. 3. RT-PCR products from control human brain mRNA and patient's total RNA. Lane 1 human brain mRNA; lane 2 total RNA from the patient.

had a C at 879, suggesting that the mutant allele is preferentially transcribed in the patient's white blood cells. In six clones with a normal sequence, only one clone had exon 12. In 30 clones which had an 879 A to C transversion, two different sequences were observed, aside from the difference caused by the alternative splicing of exon 12. In seven clones out of 30, an 879 A to C transversion was the only mutation (only two clones had exon 12). This type of mRNA resulted in FMRP with a normal amino acid sequence. In 23 clones, however, the first 47 nucleotides of intron 9 were inserted between exon 9 and exon 10 (only five clones had exon 12). In all clones with the 47-bp insertion, an 879 A to C transversion was found. Since 879 A was the second last nucleotide of exon 9, which is highly conserved

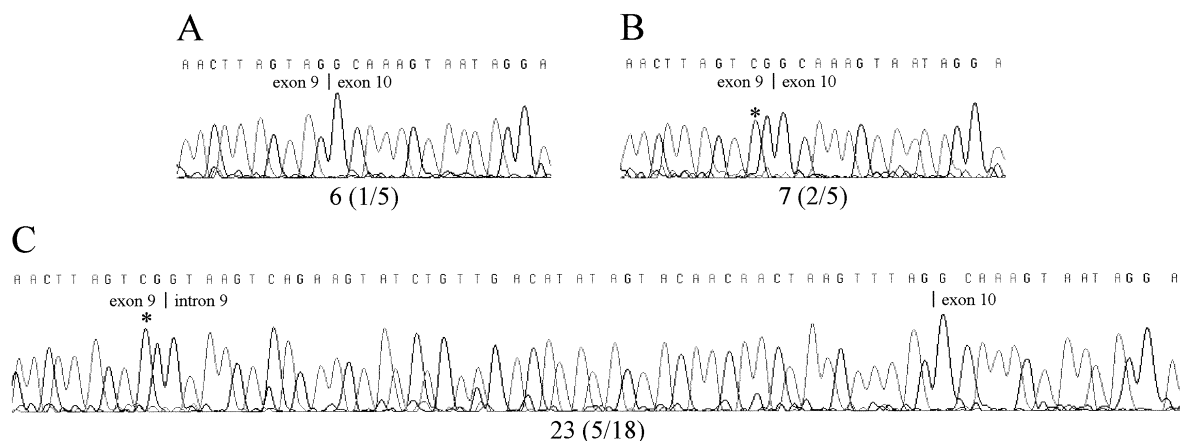


Fig. 4. Sequence analysis of the RT-PCR products in the patient. Thirty-six clones were sequenced and three different types were observed; normal (A) 879 A to C transversion (B) and 879 A to C transversion + insertion of first 47 bp of intron 9 between exon 9 and exon 10 (C). In addition exon 12 was either present or absent in all three species due to alternative splicing. Numerals below the electropherogram indicate ; total number of clones (number of clones with exon 12/number of clones without exon 12).

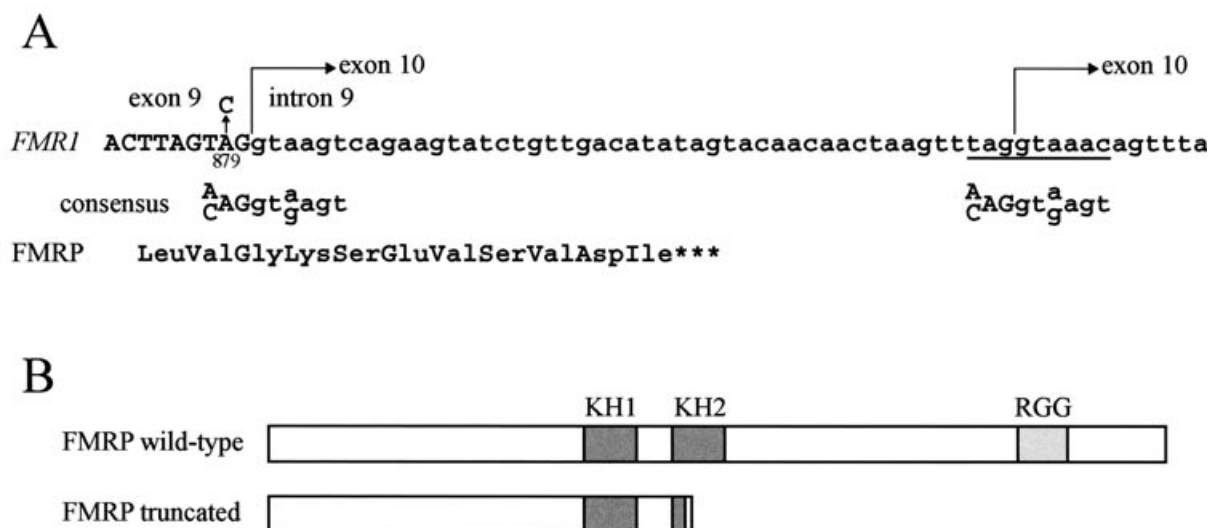


Fig. 5. A : Possible mechanism of aberrant splicing caused by 879 A to C transversion. Due to the mutation, original splicing donor site was "inactivated" and as a result a cryptic splicing donor site in intron 9 (underlined) was "activated." Consensus donor site sequence is shown underneath the *FMR1* sequence. The predicted amino acid sequence translated from aberrantly spliced *FMR1* mRNA is shown at the bottom (FMRP). B : Schematic representation of the normal full-length *FMR1* protein (FMRP wild-type) and the truncated *FMR1* protein (FMRP truncated) deduced to be expressed in the patient. Two KH domains and an RGG domain which play critical role in RNA binding are shown in gray.

in the splice donor site (24, 25), the likelihood was high that the original splice donor site was inactivated and the cryptic splice donor site was in turn activated (Fig. 5A). When this type of mutant mRNA is translated, a premature termination of the protein synthesis occurs and the product protein lacks the second KH domain and RGG box (Fig. 5B). Given that KH domains and RGG box are essential for RNA binding of the FMRP (12, 14, 26), we assumed that RNA binding of the truncated protein was impaired. Supporting our hypothesis, a patient in a previous study was found to have a missense mutation in the second KH domain (9), and *in vitro* studies have shown that the mutated FMRP has a reduced

RNA binding capacity (14).

We cloned the total amplified transcripts en mass into a cloning vector and sequenced 36 randomly picked clones. It has been shown that the number of clones of each splicing isotope reflect the abundance of each type of transcript (27). Judging from the number of clones obtained after RT-PCR of *FMR1* mRNA, the mutant allele was predominantly transcribed, and a large proportion of the transcripts from the mutant allele were aberrantly spliced. Approximately 64% of the total *FMR1* transcripts underwent aberrant splicing. It has been suggested that molecular findings in white blood cells may not accurately represent the picture in the

brain of patients with fragile X syndrome (28), but it is likely that the amount of normal FMRP is decreased in the central nervous system which causes autistic features in the patient.

Symptoms of female heterozygotes who carry a normal number of CGG triplet repeats in one allele and a full expansion (more than 200) of the CGG repeat in another allele have been characterized (28-30). These patients present with a wide variety of symptoms ranging from normal to severe mental retardation. Between 25 and 50 % of females with the full mutation demonstrate mental retardation (IQ<70), whereas about 25% of these females present with a borderline IQ (between 70 and 84). The remaining 25% to 50% have a normal IQ ( $\geq 85$ ). A relationship between the ratio of active normal X chromosomes to normal inactive chromosomes and the severity of mental retardation has been reported (31, 32), whereas the relationship between the number of triplet repeats and IQ remains controversial (28, 33). Our patient had severe mental retardation and moderate autistic features, suggesting that relatively large proportion of the normal alleles were inactive in her neuronal cells.

In conclusion, mutations in *FMR1* are not commonly found in patients with autism or mental retardation. However, the possibility exists that *FMR1* mutations are a cause of autism or mental retardation in a small number of patients. Future studies need to be carried out on large number of individuals and should include female patients.

## ACKNOWLEDGEMENTS

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