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Novel Polymorphism in the *FMR1* Gene Resulting in a "Pseudodeletion" of *FMR1* in a Commonly Used Fragile X Assay

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The fragile X syndrome is the most commonly inherited cause of mental retardation. Genetic diagnosis of this disease relies on the detection of triplet repeat expansion in the *FMR1* gene on the X chromosome. Although the majority of disease in fragile X patients is due to mutations involving triplet repeat expansion, deletion of various portions of *FMR1* has also been described in association with the fragile X syndrome. Here we describe a rare polymorphism in the noncoding region of *FMR1* that mimics detection of a deletion in a commonly used assay for fragile X syndrome, which can result in misdiagnosis of the disease. *(J Mol Diag 2000, 2:128–131)*

Fragile X mental retardation is the most common cause of inherited mental retardation, with an incidence of 1 in 4000 in males and 1 in 6000 in females.[1] This syndrome is characterized by mental retardation coupled with characteristic physical features, such as long facies, large ears, and macroorchidism.[2] At the molecular level, this disease is associated with expansion of a CGG repeat in the 5′ UTR of the *FMR1* gene on the X chromosome. This expansion creates dynamic instability in the *FMR1* gene, with the size of the repeat often increasing in size during female meiosis in succeeding generations.[3] When this expansion reaches a critical size, hypermethylation of both the repeat and the adjoining promoter region of *FMR1* leads to the decreased transcription of *FMR1*. Although the vast majority of patients with fragile X disease show this pattern of expanded repeats, a small number of patients have been described in whom partial deletions of *FMR1* result in a disease phenotype.[4]

Molecular diagnosis of fragile X is based on the demonstration of expansion of the CGG repeat in patient samples. Traditionally, this diagnosis was made by cytogenetic detection of the fragile site after culture of patient cells in medium depleted of folic acid and thymidine.[5] After the discovery of the *FMR1* gene and its role in disease, most clinical laboratories adopted DNA-based strategies to test for fragile X syndrome. These include methods based on both polymerase chain reaction (PCR) and Southern analysis of the *FMR1* locus. In the genomic Southern hybridization method of direct mutation testing used in our laboratory,[6] genomic DNA isolated from peripheral white blood cells of the patient is digested with *Eco*RI and *Sac*II (a methylation-sensitive enzyme). This digest is then probed with an *FMR1* fragment (StB12.3)[6] to determine both the methylation status and relative length of the trinucleotide repeat. A normal male will show a single band of 2.8 kb, and a normal female will have an additional 5.2-kb band representing the inactive, methylated allele (Figure 1A). Premutations (between 50 and 200 CGG repeats) and full mutations (>200 copies of the triplet) are detected by increases in the size of these bands.

In the course of clinical testing in our laboratory, we discovered a male patient who appeared to have a deletion of the *FMR1* gene, as evidenced by the absence of the 2.8-kb band described in the above assay. Here we describe the work-up of this patient and his family members and a novel polymorphism that produces this unusual result.

Materials and Methods

Fragile X Analysis

Patient DNA was isolated from anticoagulated peripheral blood with the "Puregene" DNA isolation procedure (Gentra Systems, Minneapolis, MN). Five micrograms of genomic DNA was digested with 50 U each of *Eco*RI and *Sac*II at 37 degrees for 2 hours. The digested DNA was separated by electrophoresis on a 1.5% vertical agarose gel (Hoefer Scientific Instruments, San Francisco, CA) without ethidium bromide for 16 hours at 45 volts. Gels were subsequently stained with ethidium bromide (EtBr) and photographed to visualize DNA, followed by alkaline Southern transfer to nylon membranes (Zeta Probe; Biorad, Hercules, CA). Blots were probed with a [^{32}P]dCTP-

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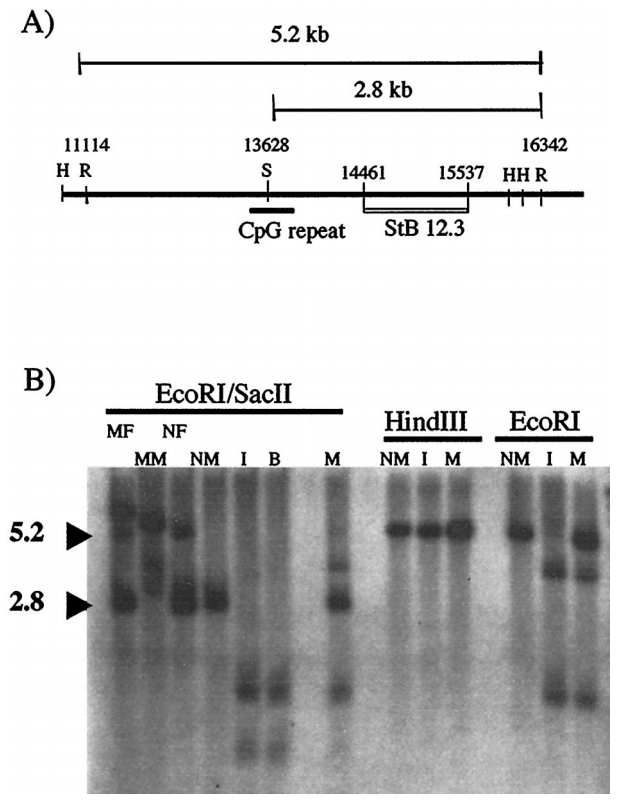

Figure 1. Southern analysis of index patient. **A:** Patient DNA is digested with *Eco*RI (R) and *Sac*II (S), then probed with the cloned fragment StB12.3. Normal males generate a 2.8-kb *Sac*II/*Eco*RI fragment, whereas normal females generate an additional 5.2-kb *Eco*RI/*Eco*RI fragment from the methylated allele. Additional restriction sites used in this paper (*Hin*dIII (H)) are shown. **B:** Lanes 1–4 show the patterns seen after digestion with *Eco*RI/*Sac*I in control patients with full mutations (MM, mutant male; MF, mutant female) as compared to normal controls (NM, normal male; NF, normal female). Both the index patient (I) and his brother (B) show a loss of this normal pattern and the appearance of smaller bands. The mother (M) has a more complex pattern. Digestion with *Hin*dIII produces identical bands in a normal male, the index patient, and his mother. In contrast, digestion with *Eco*RI alone demonstrates the presence of a new *Eco*RI site in both the index patient and his mother, when compared to the normal male control.

radiolabeled (*redi*vue; Amersham Pharmacia Biotech, Piscataway, NJ) *FMR1* probe (StB12.3) that hybridizes to the region from bp 14461 to 15537 in *FMR1*.

FMR1 *PCR*

The following PCR primers were designed to bracket regions of the StB12.3 probe fragment region of *FMR1* (numbers indicate positions in the *FMR1* sequence as denoted in GenBank, reference L29074): FMR1: CCTAAACATCATCTCCCAGCG (14373–14393); FMR2: TTAGACGCTGAAGCATGTGC (14775–14755); FMR3: GAGGGAAGGACTGGACTTGG (14153–14173); FMR4: CAGTTGCCATTGTGATTTGG (14604–14584); FMR5: GTAGTAAGAAGCGGTAGTCG (14562–14582); FMR6: CCAGCAGTGCATTGAAGAAG (14680–14700); FMR7: CAGCCTTCCTTCCACACGCA (15240–15220). Final primer concentrations were 0.2 μmol/L each. PCR was performed using 500 ng of genomic DNA with the following conditions: 30 cycles of 94°C for 45 seconds, 60°C for 1 minute, 72°C for 90 seconds, with a final extension of 72°C for 5 minutes. After PCR, 10 U of *Eco*RI was added directly to the PCR mixture. After a 15-minute incubation at 37°C, reactions were stopped by adding bromophenyl blue and visualized on a 3% agarose gel.

Sequence Analysis

Direct sequencing of PCR fragments amplified from primers FMR1 and FMR7 was performed by the Sanger method, using Big Dye fluorescent sequencing reagents and an ABI 373 analyzer (Applied Biosystems, Foster City, CA).

EcoRI Screening of Patient Samples

Stored genomic DNA from previous samples submitted to our laboratory for analysis were anonymously screened using the PCR/*Eco*RI digestion protocol described above.

Results

Fragile X Analysis of Index Patient Sample by Southern Blot Analysis

Figure 1B shows the results of Southern blot analysis of the index patient and family members, using the StB12.3 probe described above. After the *Eco*RI/*Sac*II digestion used for routine clinical testing in our laboratory, the index patient (I) showed a loss of the 2.8-kb fragment expected in male subjects, suggesting a deletion within *FMR1* in the area of the StB12.3 probe. (The smaller bands seen in Figure 1B were not seen in the original clinical gel. Presumably these shorter fragments were eluted off the gel, because of the longer electrophoretic times used in clinical testing to provide adequate separation of the premutation fragments.) Further analysis of family members showed that the patient's brother (B) also had a loss of the 2.8-kb fragment. The mother (M) of the index patient showed a more complex abnormal pattern, with two additional bands seen in addition to the expected 2.8-kb and 5.2-kb fragments.

Because deletions in the *FMR1* gene are rare and have variable fragile X phenotypes, we decided to use additional restriction enzymes that flank the StB12.3 probe site to determine the extent and location of the deletion. Surprisingly, digestion with *Hin*dIII (Figure 1B) and *Bgl*II (data not shown) produced a fragment in the index patient and his mother that was identical to that seen in normal control samples, indicating that there was no large-scale deletion in this area of *FMR1*. This observation suggested that the "deletion" of the 2.8-kb band seen in the index patient might be due to a novel *Eco*RI site in *FMR1*. Subsequent analysis showed that digestion with *Eco*RI alone produced bands in both the index patient and his mother that were not seen in normal control patients (Figure 1B).

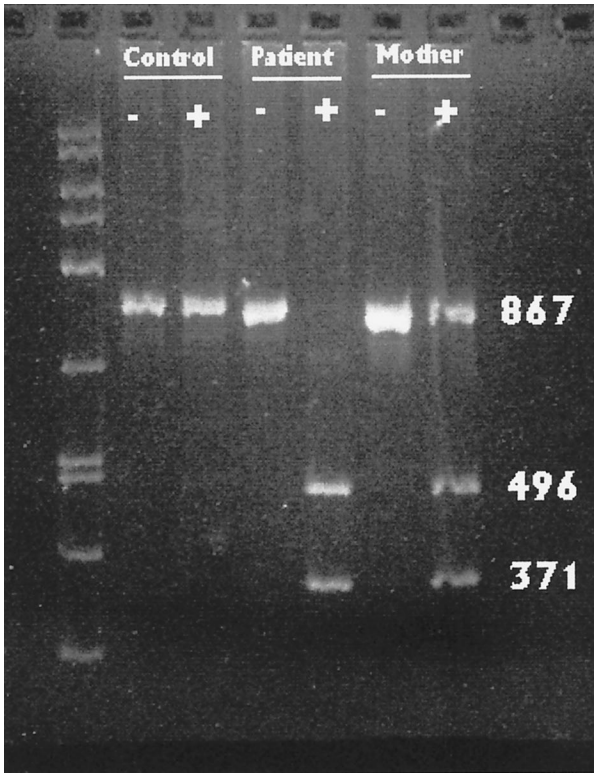

Figure 2. PCR-restriction fragment length polymorphism analysis for an *Eco*RI site. Genomic DNA was amplified using primers FMR1 and FMR7 to produce a 867-bp fragment spanning bases 14373 to 15240 in the *FMR1* gene. Lanes marked + have been digested with *Eco*RI. PCR using control DNA from a normal subject produces a fragment that does not cut with *Eco*RI. Digestion of the PCR product from the index patient's DNA produces fragments of 371 and 496 bp. DNA from the mother shows that she is heterozygous for the *Eco*RI site.

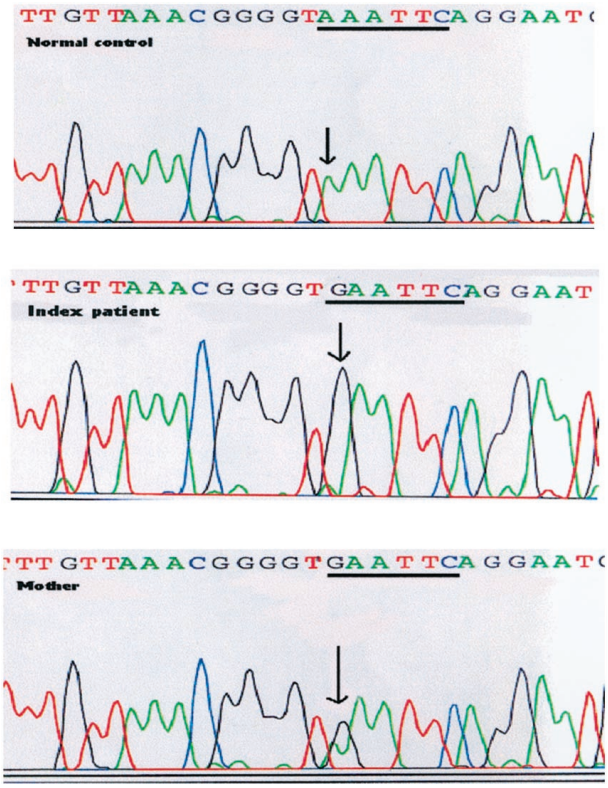

Figure 3. Sequence analysis of patient DNA. The PCR fragment amplified using primers FMR5 and FMR7 was sequenced from both the index patient and his mother. Patient DNA shows an A → G transition at bp 14744. The mother is heterozygous for this mutation.

Restriction Fragment Length Polymorphism Analysis of the StB12.3 Fragment

Analysis of the published sequence of the *FMR1* gene revealed six potential sites in the StB12.3 fragment where a single base change would introduce a new *Eco*RI site. We constructed a series of PCR primers to span these areas, amplified each region, and digested the resulting PCR fragments with *Eco*RI. PCR fragments generated from control DNA, using primers FMR1 and FMR7, did not cut with *Eco*RI (Figure 2, lane 2). In contrast, DNA from the index patient generated a PCR fragment that was cleaved by *Eco*RI into two smaller fragments (Figure 2, lane 4). Genomic DNA isolated from the mother of this patient showed that she was heterozygous for this mutation (lane 6), and a brother also carried the mutation (data not shown). Sequence analysis of this region confirmed that an A → G transition at position 14744 had produced a new *Eco*RI site in both the index patient and his mother (Figure 3). No other novel *Eco*RI sites were detected using the other FMR primer combinations.

Frequency of Polymorphism

Because this polymorphism has not previously been reported, we used the PCR/*Eco*RI assay to rapidly screen DNA samples from our patient stores to estimate the prevalence of this polymorphism in the general population. Seventy-two patient samples (40 male, 32 female, 104 chromosomes) were analyzed by PCR restriction fragment length polymorphism analysis. No additional examples of this polymorphism were found, suggesting that the frequency of this polymorphism in the general population is less than 1%. In addition, a retrospective review of more than 900 fragile X assays performed in our laboratory over the past 5 years with the *Eco*RI-*Sac*II genomic Southern hybridization protocol revealed no evidence of similar "pseudodeletions" in past testing.

Conclusions

We have described a novel polymorphism in the *FMR1* gene, where an A → G transition produces a new *Eco*RI site. For the diagnostic method discussed here, the presence of this polymorphism results in cleavage of the normal unmethylated 2.8-kb fragment into two smaller fragments of 1.6 and 1.2 kb. These smaller fragments may run off the gel under the conditions used for clinical testing, as gels are run for prolonged periods on high-percentage agarose to optimize separation of the larger fragments necessary for detection of premutations. The loss of the normal 2.8-kb band or the presence of smaller fragments generated by this new *Eco*RI site

can give the appearance of a deletion in *FMR1*, as was seen in this case.

It is not clear whether this polymorphism has functional significance for the mental retardation phenotype. The mutation is located outside both the coding and promoter regions of *FMR1* and does not appear to produce a new splice site. However, it is interesting to note that the mother shows preferential methylation of the mutant allele, as evidenced by the pattern seen with Southern analysis after the *Eco*RI/*Sac*II digestion. The presence of four bands in the mother's sample is consistent with heterozygosity for the mutation, and comparison of the fragments generated from the maternal allele without the polymorphism shows that the 5.2-kb fragment (methylated allele) is much less intense than the 2.8-kb fragment (unmethylated allele), suggesting a skewed pattern of X chromosome inactivation. However, it is not clear whether this shift in methylation is associated with the polymorphism or is a separate phenomenon.

For the purposes of clinical diagnostic testing, this patient did not have a triplet repeat expansion typically associated with clinical symptoms of the fragile X syndrome. The final report for the patient described in this study was "Triplet repeat expansion not detected," and consultation with the ordering physician was provided to describe the results more fully. Although the direct phenotypic influence(s) of this unusual polymorphism with respect to the fragile X syndrome is unknown, from a diagnostic standpoint, this newly described polymorphism can affect the ability of laboratories to correctly diagnose clinical samples. Laboratories that use the method of detection described here could incorrectly identify the patient as having a deletion of part of the *FMR1* gene. Because the deletions of *FMR1* that have been described show variable association with the mental retardation phenotype, misclassification of a patient as carrying a *FMR1* deletion could result in the misdiagnosis of a genetic basis for the patient's mental retardation.

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