A Novel Polymorphism in the FMR1 Gene
Implications for Clinical Testing of Fragile X Syndrome

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Fragile X syndrome is the most common cause of inherited mental retardation among males. Most cases, the molecular basis of fragile X syndrome is the expansion and subsequent methylation of a CGG trinucleotide repeat in the 5’ untranslated region of the fragile X mental retardation 1 (FMR1) gene. Laboratory diagnosis usually relies on a combination of Southern blot and polymerase chain reaction analyses. In this case report we describe an unusual Southern blot result in a patient who presented with developmental delay and had a normal CGG repeat number by polymerase chain reaction analysis. Further investigation revealed a novel G3310C transversion in the FMR1 gene resulting in a new recognition site for the BssHI restriction enzyme. This novel restriction site could potentially mimic a partial deletion of the FMR1 gene on Southern blot analysis and thus represents a possible pitfall in the diagnosis of fragile X syndrome.

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Case Reports

The patient is a 9-year-old Somali boy who presented with a history of developmental delays and autistic behavior. He reportedly developed seizures following a head trauma between the ages of 3 and 4 years. As the patient only recently immigrated to the United States, details about his developmental history are difficult to document precisely. The patient’s mother is deceased and his father remains in Africa. Therefore, family studies could not be undertaken in this case. Fragile X testing was requested because of the history of developmental delays and autistic behaviors.

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MATERIALS AND METHODS

Southern Blot Analysis

Three micrograms of genomic DNA isolated from peripheral blood using QIAGEN DNA extraction kits (QIAGEN Inc, Valencia, Calif) was incubated overnight at 37°C with 18 units of EcoRI and 15 units of BssHII. Southern blot analysis was performed as described earlier using a denatured pE5.1 probe.8,9

PCR CGG Repeat Number Analysis

The number of CGG repeats was determined using PCR containing 200 ng of genomic DNA template, 12.5 pmol of each primer, 1.75mM MgCl₂, 12% DMSO, 2.35mM of each of the dNTPs (dATP, dCTP, dTTP, and 7-deazadGTP), and 2.5 U of Taq polymerase in a 15-µL reaction. Forward and reverse primers used in the PCR were as follows:

F primer (25 pmol/µL)
5’-FAM-GCTCAGCTCGGTTTCACTTCCGTGT-3’

R primer (25 pmol/µL)
5’-AGCCCCGCACCTCCACACGCCACGCCTCCCA-3’

Polymerase chain reaction conditions included a denaturation step at 95°C for 10 minutes, an amplification step with 34 cycles of 95°C for 90 seconds, an annealing step at 58°C for 60 seconds and 72°C for 2 minutes, and an elongation step at 72°C for 7 minutes. The PCR products were analyzed by capillary electrophoresis on an ABI 3100 Genetic Analyzer using GeneScan software (Applied Biosystems, Foster City, Calif). CGG repeat copy number was analyzed using the formula (PCR product size – 220)/3.

Polymerase chain reaction conditions included a denaturation step at 95°C for 10 minutes, an amplification step with 45 cycles of 95°C for 15 minutes, an amplification step with 34 cycles of 95°C for 90 seconds, annealing at 54°C 72°C for 2 minutes, and an elongation step at 72°C for 7 minutes. The PCR products were analyzed by agarose gel electrophoresis.

PCR Conditions for Amplification of the Fragment Containing Suspected Mutations

A 532-base pair (bp) fragment in the FMR1 gene containing the sequence with the suspected mutations was amplified in a PCR containing 200 ng of the genomic DNA template, 12.5 pmol of each primer, 1.5mM MgCl₂, and 2.5 U of Taq polymerase in a 50-µL reaction. The following primers were used in the PCR:

Forward primer: 5’-AATCGCGGCTAAGTGACG-3’
Reverse primer: 5’-TAATACCTGAAGTTTCACTGGC-3’

Polymerase chain reaction conditions included a denaturation step at 95°C for 10 minutes, an amplification step with 34 cycles of 95°C for 90 seconds, annealing at 54°C for 60 seconds and 72°C for 2 minutes, and an elongation step at 72°C for 7 minutes. The PCR products were analyzed by agarose gel electrophoresis.

Thirty-one Somalian immigrants were also analyzed to determine frequency of the G3310C mutation using the Sequenom method.8,9

Sequencing Methodology

The PCR products obtained from a normal control and the patient, using primers described previously, were separated using 1.5% agarose gel electrophoresis and extracted from the agarose gel using a QIAquick column (QIAGEN). Subsequently, 2 µL of each purified PCR product was added to 4 µL of the Terminator Ready Reaction Mix and 1.4 pmol of sequencing primer in a 20-µL reaction. The following primers were used in the sequencing reactions:

Forward sequencing primer:
5’-AATCGCGGCTAAGTGACG-3’
Reverse sequencing primer:
5’-CCGACTACCCTTCTTACTACT-3’

Sequencing reaction conditions included a denaturation step at 94°C for 5 minutes and 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes. The extension products were then precipitated with ethanol, air dried, and dissolved in Hi-Di formamide, denatured at 95°C for 5 minutes, and then sequenced using an ABI 3100 Genetic Analyzer. The resulting sequences were analyzed using the SEQUENCHER software (Genes Codes Corporation, Ann Arbor, Mich).

RESULTS

Polymerase chain reaction analysis on the patient sample demonstrated a single normal allele, estimated to contain 42 trinucleotide repeats (data not shown). Initially, the Southern blot showed a 1.8-kb fragment and a 2.3-kb fragment. A 1-kb fragment was also identified on repeat Southern blot (Figure 1). Southern blot on a normal female DNA sample showed 3 fragments (5.1 kb, 2.3 kb, and 2.8 kb).
Figure 2. Schematic representation of a portion of the FMR1 gene showing the location of the repeat segment, the fragment sizes generated from the commonly used restriction enzymes, and the pE5.1 probe.

Figure 3. Sequencing results from the index case and a normal control are shown. Both the forward and reverse sequences from the index case show a C at position 3310, whereas the normal control shows a G at the same position in both the forward and reverse sequences.
single digest of the patient’s DNA with EcoRI revealed an expected 5.1-kb fragment (data not shown), suggesting that the observed 1.8-kb and 1-kb fragments were not the result of a novel EcoRI restriction site (Figure 2). These observations suggested that a previously undescribed mutation in the 2.8-kb fragment (routinely produced by BssHII digestion of the EcoRI-generated 5.1-kb fragment and having the recognition sequence: G^CGCGC) created a novel restriction site for the BssHII restriction enzyme.

Based on sequence information, 2 potential regions were identified within intron 1 of the FMR1 gene where sequence variation could create a novel restriction site for the BssHII enzyme: a G3310C transversion or a deletion of 3667T (based on GenBank accession No. 31502). Polymerase chain reaction amplification of a 532-bp portion of intron 1 of FMR1 (see previous description of primers) followed by digestion with BssHII revealed bands of 100 and 432 bp (data not shown), consistent with the G3310C transversion. We confirmed this result using sequence analysis, which showed that the DNA sample was hemizygous for the G3310C mutation (Figure 3).

COMMENT

We identified a novel G3310C mutation in the noncoding region (intron 1) of the FMR1 gene that creates a new restriction site for the BssHII enzyme. The additional cut site lies within the 2.8-kb fragment observed in Southern blot analysis and results in 2 bands: a 1-kb fragment and a 1.8-kb fragment. This mutation has clinical significance as gel conditions in clinical laboratories performing fragile X testing via Southern blot analysis are often optimized to provide maximum separation of larger fragments. Under these conditions, a smaller size fragment such as the 1-kb fragment, as produced in our patient’s test, may run off the gel. The presence of a single 1.8-kb fragment can give an appearance of a deletion in the FMR1 gene. Although rare, deletions in the FMR1 gene have been associated with cases of fragile X syndrome, and so the G3310C mutation may give the false impression that a clinically significant deletion has been detected. Furthermore, an earlier report by Chiurazzi et al. has reported that mutations in the region of the FMR1 gene are more common in people of African descent as compared with those of other ancestries. However, screening for the G3310C polymorphism showed no mutant alleles among 31 Somalian participants (18 women and 13 men; 49 X chromosomes) that were tested indicating that this mutation has a frequency of 2% or less in the Somalian population. In addition, the 1.8-kb and 1-kb fragments found on Southern blot were not observed in about 1500 (predominantly white) individuals screened for expansion of FMR1 gene at the University of Minnesota during the past 6 years. Thus, this particular mutation appears to be uncommon in both the African and the white population and may not contribute significantly to error in the laboratory diagnosis of fragile X syndrome.

At present, clinical significance of this mutation for this patient is unclear. The G3310C mutation is present in intron 1 of the FMR1 gene and not associated with changes in either the promoter region or the coding region of the FMR1 gene. Hence, it seems unlikely that this G-to-C transversion would lead to any significant changes in the FMR1 protein or its level of expression in cells. Further, analysis of surrounding sequence did not suggest that this mutation would create a consensus splice sequence. Unfortunately, this patient’s clinical and family situation did not permit family studies or extensive follow-up. The limited clinical information available did not suggest fragile X syndrome as a likely clinical diagnosis for this patient. At present, it is our interpretation that this mutation represents a rare, but likely benign, variation in intron 1 of the FMR1 gene. However, the possibility that this variation may be biologically significant cannot be definitively excluded.

An earlier report describes the various point mutations in a limited flanking region around the trinucleotide repeat that could possibly result in abnormal band sizes in Southern blot analysis when making use of commonly used methylation-specific restriction enzymes such as NruI, Eagl, BssHII, and SacII. The loss of small bands on Southern analysis (ie, the 1-kb band) may be misinterpreted as deletions. For purposes of clinical laboratory testing, it is important to recognize that the presence of rare mutations may create novel restriction sites and therefore abnormal patterns on Southern blot analysis. This may potentially lead to a laboratory error such as reporting the presence of a deletion in the FMR1 gene, thereby misdiagnosing the genetic defect underlying the patient’s mental retardation.

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References