Consultations in Molecular Diagnostics

A Pseudo-Full Mutation Identified in Fragile X Assay Reveals a Novel Base Change Abolishing an EcoRI Restriction Site

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Diagnostic testing for the fragile X syndrome is designed to detect the most common mutation, a CGG expansion in the 5'-untranslated region of the fragile X mental retardation (FMR1) gene. PCR can determine the number of CGG repeats less than 100, whereas Southern analysis can detect large premutations, full mutations, and their methylation status. Bands larger than 5.8 kb observed via Southern analysis are usually considered a methylated full mutation, causing fragile X syndrome in males and varied clinical presentations in females. We observed a 10.9-kb band on a Southern blot assay from an autistic girl with language delay. Further investigation identified a novel G-to-A transition at an EcoRI cleavage site, upstream of the CGG repeat region of the FMR1 gene. This base change abolished the EcoRI restriction site, resulting in a 10.9-kb pseudo-full mutation. This G-to-A base change has not been previously reported and was not identified in a subsequent analysis of 105 male and 30 female patient samples. The clear 10.9-kb band detected on a Southern blot assay for fragile X syndrome mimics a large, methylated full mutation, which could result in a misdiagnosis without the benefit of family studies and further testing. (*J Mol Diagn* 2008, 10:469–474; DOI: 10.2353/jmoldx.2008.080059)

The fragile X syndrome is the most common inherited disorder associated with mental retardation, with an estimated prevalence of 1 in 4000 in males and 1 in 8000 in females. The syndrome in males is characterized by typical facial features including large ears, speech delay, autistic behavior,

macroorchidism, and mental retardation. Females with a full mutation exhibit a wide spectrum of phenotypes, ranging from no detectable learning or behavioral deficits to mild learning disabilities to effects as severe as those in a male fragile X patient with a full mutation.¹ Although mutations, such as deletions and point mutations, have been identified in fragile X patients, more than 99% of cases have been associated with an expansion of a segment of CGG repeats in the 5'-untranslated region (UTR) of the fragile X mental retardation (*FMR1*) gene. According to the Technical Standards and Guidelines for Fragile X Testing from the ACMG (<http://www.acmg.net/Pages/ACMG-Activities/stds-2002/tx.htm>, last accessed April 3, 2008), there are four allelic forms of the CGG repeat lengths: normal (5 to 44 repeats), intermediate (gray zone, 45 to 54 repeats), premutation (55 to 200 repeats), and full mutation (>200 to 230 repeats). Full mutations with a large CGG expansion in this region are associated with inhibition of transcription of the *FMR1* gene, causing deficiency or absence of the fragile X mental retardation protein. Therefore, standard laboratory techniques for fragile X syndrome diagnosis are designed to quantitate the number of CGG repeats in the 5'-UTR of the *FMR1* gene. These techniques include a PCR, which detects normal and premutation alleles (<100 CGG repeats), and a Southern analysis, to detect normal, large premutation (100 to 200 CGG repeats) or full mutation (>200 CGG repeats) alleles and their methylation status. In the Southern blot assay for fragile X syndrome, a normal male usually shows a 2.8-kb band, and a normal female, 2.8- and 5.2-kb bands. In females, a 2.8-kb band represents the active, unmethylated X chromosome, whereas a 5.2-kb band represents the inactive, methylated X chromosome. Methylated full mutations are not detectable by PCR and are often shown as

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bands larger than 5.8 kb on a Southern blot. Here, we report a 10.9-kb band identified with a Southern blot assay for fragile X syndrome from an autistic girl with language delay. However, this large band does not represent a methylated full mutation and is not associated with an expanded CGG repeat in the 5'-UTR in the *FMR1* gene.

Materials and Methods

Case Reports

A 7-year-old girl was evaluated for autistic features and language delay that became evident by age 2 years. The child was the product of a full-term pregnancy to a healthy 20-year-old primigravida and her 22-year-old partner. The mother was of Mexican ancestry, and the father, Salvadoran. There was no parental consanguinity and no first- or second-degree relatives of the proband with congenital anomalies or developmental delay. Both parents are physically and cognitively normal. The child had no siblings. Delivery took place by primary cesarean section for fetal heart rate decelerations. Birth weight was 3.2 kg, and length, 53 cm. The neonatal course was uneventful.

By age 2 years, delays in socialization and in expressive and receptive language led to a diagnosis of classical autism. The child has remained in good general health, with no seizures, hospitalizations, regression of milestones, or other serious health problems.

The child weighed 39 kg (98th percentile), was 132 cm tall (80th percentile), and had a 52.5-cm head circumference (75th percentile). Ear length of 6 cm was between the 50th and 75th percentiles. The patient exhibited no dysmorphism, and she was physically normal.

High-resolution chromosome analysis showed a normal female karyotype (46, XX). Molecular cytogenetic analysis using an LSI Prader-Willi/Angelman region probe (GABRB3; Abbott Laboratories, Des Plaines, IL) showed no deletion or duplication in 10 metaphase and 40 interphase cells. This analysis excluded the possibility that her autistic feature was due to a 15q duplication.²

Standard Molecular Diagnostic Testing for Fragile X

Molecular diagnostic testing was performed using both PCR and Southern blot analysis to determine the CGG repeat size at the 5'-UTR of the *FMR1* gene and its methylation status. Blood was drawn into EDTA tubes from the index patient, parents, and normal controls. DNA was isolated with the Puregene DNA isolation procedure (Qiagen, Valencia, CA). The primer set for PCR was from Fu et al,³ with a forward primer (5'-GACGGAGGCGCCGCTGC-CAGG-3') and a reverse primer (5'-GTGGGCTGCGGG-CGCTCGAGG-3') bracketing the CGG repeat region. Genomic DNA was amplified using the FailSafe PCR System (FSP995J premix) from Epicenter Biotechnologies (Madison, WI) according to the manufacturer's instructions. The reaction was first denatured for 10 minutes at 94°C, followed by 30 cycles of 20 seconds at 98°C and 3 minutes at 72°C. A final extension of 10 minutes was performed at 72°C. The PCR products were fractionated on a 2% agarose gel and detected with ethidium bromide using UV illumination (Quantity One; Bio-Rad, Hercules, CA). For the standard Southern analysis, 6 µg of genomic DNA was double-digested with NruI (a methylation-sensitive enzyme, cutting only unmethylated, active X chromosome) and EcoRI (100 U of each enzyme at 37°C for 6 hours), electrophoresed on a 0.7% agarose gel, transferred to a nylon membrane, and hybridized with digoxigenin-labeled probe pFXa1NHE (Chemicon International, Temecula, CA). This probe hybridizes to the region from a NheI site to an EcoRI site (GenBank accession no. L29074.1; Figure 1). After hybridization, the gel was visualized by immuno-chemiluminescence using a Sure Blot Chem Hybridization and Detection kit (Chemicon International) according to the manufacturer's instructions.

Determination of the Origin of the 10.9-kb Band

A 10.9-kb band was detected in the proband but not in her parents on the standard Southern blot assay as de-

Figure 1. Restriction map surrounding the *FMR1* gene illustrating the predicted fragments on Southern blots. Numbering refers to GenBank L29074.1. **a:** Probe used in all Southern blots (Chemicon International). **b:** Fragment size predicted with PstI digestion only. **c:** Fragment size predicted with EcoRI and NruI (a methylation-sensitive enzyme, cutting only unmethylated active X chromosome) double digestion, generating 2.8-kb (on active X chromosome) and 5.2-kb (on inactive X chromosome) fragments. **d:** EcoRI digestion only, generating either a 5.2-kb fragment (in a normal individual) or a 10.9-kb fragment if the 11,114 EcoRI cutting site is abolished. R, EcoRI restriction site; P, PstI restriction site; SF (special forward primer) and SR (special reverse primer), location of PCR primers designed to bracket the 11,114 EcoRI restriction site (from nucleotide position 10,993 to 11,292).

scribed above. Therefore, the following investigation was pursued to determine the origin of the 10.9-kb band.

Modified Southern Blot Analysis

Six micrograms of genomic DNA from the patient, parents, and controls was digested with PstI only, EcoRI only, and EcoRI/NruI (100 U of the enzyme at 37°C for 6 hours). Figure 1 shows the restriction map surrounding the *FMR1* gene and the predicted fragments after the digestions. The digested products were subjected to Southern blot analysis as described above.

PCR Analysis of the EcoRI Site

PCR primers were designed to bracket the region of the EcoRI site at the 11,114 position 5' of the *FMR1* gene (GenBank accession no. L29074.1; Figure 1). These PCR primers are: special forward primer (SF), 5'-TAGGCTTG-AGCAACGAAGT-3', and special reverse primer (SR), 5'-TGTGTCCCTGGCATTAAA-3', resulting in a 300-bp PCR product (from nucleotide position 10,993 to 11,292). The PCR reaction was performed using 200 ng of genomic DNA under the following conditions: 1 cycle at 95°C for 10 minutes, 35 cycles at 96°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute, with a final extension of 72°C for 10 minutes. PCR products were digested with 20 U of EcoRI, incubated at 37°C for 2 hours, separated on a 2.5% agarose gel, and visualized with ethidium bromide using UV illumination as described above. This test has been performed on DNA collected from 105 male and 30 female patient samples to determine the allele frequency.

Sequence Analysis

The PCR primers mentioned above (SF and SR) were also used for sequencing. The PCR products were purified using a DNA Clean & Concentrator kit (Zymo Research, Orange, CA) and sent to the DNA sequencing laboratory (City of Hope National Medical Center and Beckman Research Institute, Duarte, CA). The sequencing was done by capillary electrophoresis using Big Dye fluorescent sequencing reagents and a Hitachi AB model 3730 capillary DNA analyzer. The data were collected with AB DNA Sequencing Collection software V 3.0 and analyzed by Analysis software V 5.3.1.

Results

Standard Molecular Testing Results

The blood specimen from the index patient was subjected to fragile X testing due to her autistic features and language delay. Standard PCR analysis revealed two normal alleles with 23 of 32 CGG repeats from the proband, 18 of 23 CGG repeats from the mother, and a normal allele with 32 CGG repeats from the father (data not shown). Standard Southern blot analysis (EcoRI/NruI double digestion) showed that the index patient had a 10.9-kb band on the Southern blot,

Figure 2. Standard Southern blot of the family. Lanes 2, 5, and 9 are from the index patient (IP), mother (M), and father (F), respectively. Lanes 10 and 11 are the normal (NC) and abnormal controls (AC; Coriell GM07537A). Other lanes represent results from the same batch run from other patients. MW, the Lambda/HindIII MW marker (Chemicon International).

in addition to the normal 2.8- and 5.2-kb bands (Figure 2, lane 2). However, there were normal 2.8- and 5.2-kb bands for the mother (Figure 2, lane 5) and a normal 2.8-kb band for the father (Figure 2, lane 9).

Modified Southern Analyses

Southern blot using PstI digestion only was performed to search for or rule out the presence of a CGG expansion in the proband (Figure 3A). The index patient (Figure 3A, lane 2), her mother (Figure 3A, lane 3), and her father (Figure 3A, lane 4) all showed the predicted, normal-sized bands approximately 1.1 kb in size, same as the normal control (Figure 3A, lane 5). Figure 3A, lane 6, is an abnormal control (Coriell GM07537A) with a full mutation band (approximately 2.1 kb in size, with 336 CGG repeats). No extra band was observed in the index family, ruling out the possibility that the 10.9-kb band observed on the standard Southern blot assay was due to the large CGG expansion at the 5'-UTR of the *FMR1* gene. Because the size of 10.9-kb could result from an abolished EcoRI cutting site at the 11,114 position (see Figure 1d), a Southern blot after EcoRI digestion with and without NruI was analyzed for comparison (Figure 3B). In a standard Southern blot assay for fragile X syndrome with EcoRI/NruI double digestion, a normal female should have 2.8- and 5.2-kb bands (Figure 3B, lane 2), and a normal male, a 2.8-kb band (Figure 3B, lane 10). With EcoRI digestion only, both normal female and male should have a single 5.2-kb band (Figure 3B, lanes 3 and 11). As shown in Figure 3B, the proband's mother had the same banding pattern (Figure 3B, lanes 4 and 5) as a normal female (Figure 3B, lanes 2 and 3), and the father had a normal 2.8-kb band after EcoRI/NruI double digestion (Figure 3B, lane 8). However, when his DNA was digested with EcoRI only, the father showed one 10.9-kb

band (Figure 3B, lane 9) instead of a normal 5.2-kb band, indicating that the EcoRI cutting site at the 11,114 position is absent. Thus, the proband inherited this 10.9-kb band from her father and the 5.2-kb band from her

mother (Figure 3B, lane 7, with EcoRI digestion only). As a result, it is not surprising to observe the 10.9-kb band in the standard Southern analysis (EcoRI/NruI double digestion) from the index patient (Figure 3B, lane 6), when some of her father's X chromosome is fully methylated (inactivated), abolishing the NruI site.

PCR Analysis of the EcoRI Site

A PCR analysis was designed to bracket the 11,114 EcoRI site. In a normal individual, the PCR product should be 300 bp in size without EcoRI digestion and should show a 122/178-bp banding pattern after digestion. Figure 3C reveals that the proband's mother had a 300-bp band without EcoRI digestion (Figure 3C, lane 4) and a 122/178-bp banding pattern after digestion (Figure 3C, lane 5), as observed in a normal control (Figure 3C, lane 2 and 3). The father had only a 300-bp band with and without EcoRI digestion (Figure 3C, lane 8 and 9), indicating the abolished EcoRI cutting site. The index patient had one 300-bp band without EcoRI digestion (Figure 3C, lane 6). After digestion, three bands of 122, 178, and 300 bp were observed, confirming her heterozygous status (Figure 3C, lane 7) at the 11,114 EcoRI restriction site. The same PCR/restriction digestion test was performed on 165 alleles (105 male and 30 female patient samples from our laboratory). All showed the same digestion pattern as the normal control (Figure 3C, lanes 2 and 3; data not shown).

Sequence Analysis

Sequence analysis surrounding the 11,114 EcoRI cutting site (GenBank accession no. L29074.1; Figure 1) from both directions confirmed a G→A transition at position 11,114 in the father, changing GAATTC to AAATTC and eliminating the EcoRI restriction site (Figure 4a). The index patient inherited the G→A base change from her father and a normal allele from her mother, showing both G and A on DNA sequencing (Figure 4b). The mother had a G at position 11,114, as did the normal control (Figure 4c). No other sequence variation was detected in the 300-bp region bracketing the 11,114 EcoRI cutting site in the index patient, parents, and normal control (data not shown).

Discussion

We report here a 10.9-kb pseudo-full mutation band observed on a Southern blot analysis for fragile X testing from

Figure 3. Determination of the origin of the 10.9-kb fragment from the index patient. **A:** Southern blot using PstI digestion only showing bands of 0.99, 1.03, and 1.08 kb (the largest band approximately 1.1 kb in size). An abnormal control (AC, lane 6) shows a full mutation (Coriell GM07537A), with an additional 2.1-kb band (336 CGG repeats). No CGG expansion was noted in the family study (lanes 2 to 4) or in a normal control (NC, lane 5). MW, the Lambda/HindIII MW marker (Chemicon International). **B:** Southern analysis of the family digested with EcoRI, with (+) and without (−) NruI. MW, molecular marker as in **A**; NFC, normal female control; M, mother; IP, index patient; F, father; NMC, normal male control; AC, abnormal control as in **A**. Note that father and the index patient both showed the 10.9-kb fragment (lanes 6, 7, and 9). **C:** PCR products with (+) and without (−) EcoRI digestion. MR, 100-bp Molecular Ruler (Bio-Rad); NC, normal control; M, mother; IP, index patient; F, father.

Figure 4. Confirmation of a base change at EcoRI cutting site by sequencing. **a:** Arrow points to the position 11,114, where a G→A transition occurred in the father. **b:** The index patient is heterozygous, with both G and A. **c:** Mother has a normal G at the same position.

an autistic girl with language delay. In clinical laboratory practice, a real full mutation band for fragile X syndrome usually accompanies with variable size or methylation mosaic bands or with a smear pattern. However, the 10.9-kb band detected in the index patient is a very clean band (Figure 2, lane 2). Therefore, a parental study was requested. This report demonstrated that this 10.9-kb band observed in the index patient was inherited from her father and was the result of a G→A transition upstream of the CGG repeats in the 5'-UTR of the *FMR1* gene, abolishing the 11,114 EcoRI restriction site. The G→A base change has not been identified from 165 alleles (105 males and 30 females), suggesting that this is not a common polymorphism. The father, who carried the transition on his X chromosome, was physically and cognitively normal, indicating that this base change may not be clinically significant. Therefore, the final report for the index patient was interpreted as "the 10.9-kb band was not the result of a large CGG expansion in the *FMR1* gene." Interestingly, a pseudo-deletion case resulting from an A→G transition at position 14,744, which created an EcoRI cutting site downstream of the 5'-UTR of the *FMR1* gene, was reported in 2000.⁴ The authors showed that this rare base change was a novel polymorphism that does not cause an *FMR1* gene deletion.

Molecular diagnostic testing for fragile X syndrome has been available since 1991.³ The basic techniques used are PCR and Southern analysis. Usually, PCR is used to determine the number of CGG repeats less than 100, whereas Southern analysis is used to detect large pre-mutations (100 to 200 CGG repeats), full mutations (>200 CGG repeats), and their methylation status. These tests are reported with more than 99% sensitivity, and positive results are 100% specific (<http://www.acmg.net/Pages/ACMG-Activities/stds-2002/fx.htm>, last accessed April 3, 2008). In practice, some laboratories would screen CGG repeats using PCR analysis to exclude males with a normal CGG repeat or females showing two normal al-

Figure 5. DNA sequence alignment around the 11,114 EcoRI restriction site with 18 species showing conservation status of the base G (derived from University of California Santa Cruz Genome Bioinformatics, <http://genome.ucsc.edu/cgi-bin/hgTracks>, last accessed April 10, 2008).

les.⁵ To detect a larger repeat expansion, the Southern blot assay is usually used only if no PCR products are detected in a male patient and if females show only one band on PCR analysis. In this report, the index patient and her mother both showed two normal alleles (23 of 32 and 18 of 23 CGG repeats, respectively) and the father had a normal 32 CGG repeat. Therefore, the laboratories that use PCR as the first screening method would not have run a Southern analysis in this case. On the other hand, if this 10.9-kb fragment were identified for the proband without additional family studies, it could have been misinterpreted as a methylated full mutation. Furthermore, if the father's X chromosome is preferentially activated (without methylation) during his daughter's embryological development, the 10.9-kb fragment may not be observed (or in a trace amount) on a standard Southern assay with EcoRI/NruI double digestion. These may be some of the reasons that this G→A base change has not been reported after 17 years of routine fragile X testing.

The *FMR1* gene is highly conserved at the sequence and amino acid levels, as evidenced by its presence in the human, mouse, *Caenorhabditis elegans*, *Xenopus laevis*, *Drosophila melanogaster*, and chicken,⁶ but the base G at the position 11,114, upstream of the 5'-UTR of the *FMR1* gene, seems not well conserved. The conservation score is approximately 10% of the maximal, based on the PhastCons package.⁷ As shown in Figure 5 (derived from University of California Santa Cruz Genome Bioinformatics, <http://genome.ucsc.edu/cgi-bin/hgTracks>, accessed April 10, 2008), this base is conserved in the chimpanzee, Rhesus monkey, bushbaby monkey, Guinea pig, dog, cat, cow, elephant, and tenrec but not in the rabbit. Treeshrew, mouse, rat, shrew, hedgehog, horse, and armadillo have one or more unalignable bases in the gap region, due to either an excessive evolutionary distance between the species or an independent insertion/deletion in the aligned region among these species. This may explain why the father who carries this G→A change is clinically normal.

Although the father has not presented any measurable symptoms, the significance of the 10.9-kb band will not be clear until additional individuals with this G→A base change have been identified and clinically evaluated. Further studies of

the *FMR1* gene and its expression in this family may help to resolve the issue. Nevertheless, whenever a clean 10.9-kb band is observed on a Southern blot analysis in a fragile X assay, family studies and further testing should be undertaken for appropriate clinical interpretation.

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