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Extra Alleles in *FMR1* Triple-Primed PCR

Artifact, Aneuploidy, or Somatic Mosaicism?

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Triple-primed PCR assays have become the preferred fragile X syndrome testing method. Using a commercially available assay, we detected a reproducible extra peak(s) in 0.5% of 13,161 clinical samples. The objectives of this study were to determine the cause of these extra peaks; to identify whether these peaks represent an assay specific artifact, an underlying chromosome aneuploidy, or somatic mosaicism; and to ascertain their clinical relevance. The presence of an extra allele(s) was confirmed by a laboratory-developed PCR, with sequencing of the *FMR1* 5′ UTR or Southern blot for some samples. The laboratory-developed procedure detected the extra allele(s) in 57 of 64 samples. Thus, we confirmed an extra peak, typically of lower abundance, in approximately 0.4% of all samples. Of these samples, 5 were from males and 52 were from heterozygous or homozygous females. Six patients likely had X chromosome aneuploidies. In 82.3% of samples, the extra allele had fewer repeats than the predominant allele(s). Additional alleles detected by *FMR1* triple-primed PCR are not an assay-specific artifact and are likely due to X chromosome aneuploidies or somatic repeat instability. Additional normal alleles likely have no clinical significance for fragile X syndrome carrier or affected status. Extra alleles in individuals with normal karyotypes probably represent *FMR1* somatic variation. *(J Mol Diagn 2014, 16: 689–696; http://dx.doi.org/10.1016/j.jmoldx.2014.06.006)*

Fragile X syndrome (FXS) is the most common inherited form of intellectual disability, affecting 1:3000 to 1:4000 males and 1:6000 to 1:8000 females.[1] Affected males have moderate to severe intellectual disability, behavioral difficulties, macro-orchidism, and characteristic facial dysmorphism. Females are usually more mildly affected than males, with normal to mildly impaired intellect, learning difficulties, and emotional problems, including depression and anxiety disorders.[2]

FXS is caused by loss of expression of the *FMR1* gene located on chromosome Xq27.3.[3–5] *FMR1* encodes the fragile X mental retardation protein (FMRP), a RNA-binding protein highly expressed in neurons.[6] The stability, subcellular localization, and translation of several mRNAs involved in synaptic structure and function are regulated by FMRP.[7–9] Greater than 98% of FXS cases result from a CGG repeat expansion in the 5′ untranslated region (UTR) of *FMR1*.[3,4] Full mutations have >200 CGG repeats and are hypermethylated, resulting in transcriptional silencing of *FMR1*.[5,10] Normal alleles have ≤44 CGG repeats. Gray zone or intermediate alleles have 45 to 54 repeats.[11] In the United States,

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approximately 1:35 females and 1:42 males carry a gray zone allele.[12] Although gray zone alleles have no associated phenotype, these alleles have been reported to expand to a full mutation in two generations.[13]

Premutation alleles (55 to 200 CGG repeats) are particularly unstable in female meioses, with repeats as small as 56 and 59 CGGs expanding to full mutations in one generation.[13,14] Approximately 1:151 to 1:178 females and 1:468 males in the United States are premutation carriers.[12,15] In addition to the significant risk of having a child with a full mutation, premutation carriers are also at risk of developing other *FMR1*-related disorders. Up to 20% of female carriers will develop fragile X primary ovarian insufficiency, defined as cessation of menses by the age of 40 years. Fragile X−associated tremor ataxia syndrome is a late-onset progressive cerebellar ataxia with intention tremor that affects 30% of male and 8% to 16.5% of female premutation carriers older than 50 years.[16]

Over the years, FXS diagnostic testing has evolved from a cytogenetic assay to a molecular genetics test. Currently, the American College of Medical Genetics and Genomics recommends a combination of traditional PCR and Southern blotting or methylation-specific PCR to determine CGG repeat size and methylation status.[17] Traditional laboratory-developed PCR assays allow for accurate sizing of alleles up to 100 to 150 CGG repeats. Although time-consuming and requiring greater amounts of genomic DNA, Southern blotting permits detection, size estimation, and determination of methylation status of expanded alleles. Newly designed triple-primed PCR assays amplify alleles ranging from normal to full mutations.[18−23] These newer assays require less genomic DNA and are higher throughput because only samples with premutations or full mutations need to be reflexed to Southern blot or methylation-specific PCR.[17]

During a 2-year period, we used a commercially available triple-primed *FMR1* PCR assay for 13,161 FXS carrier screens and diagnostic tests.[20,21,23] In this assay, females homozygous for the same sized allele and males have a single peak, whereas females heterozygous for different sized alleles have 2 peaks. However, in 64 samples we detected a reproducible extra peak(s), typically of lower abundance. Because this frequency is much greater than the reported population incidence of X chromosome aneuploidies,[24−26] we initially hypothesized that the extra alleles may be an artifact of triple-primed PCR. To confirm the presence of these extra peaks, we repeated testing using a laboratory-developed FXS PCR method. For one sample, sequencing of the *FMR1* 5′ UTR and Southern blotting were also used as confirmatory methods.

Materials and Methods

Genomic DNA Isolation

Blood was obtained from 13,161 patients for fragile X testing. Indications for testing were carrier screening or diagnostic testing. DNA was extracted from peripheral whole blood with the QuickGene DNA Whole Blood Kit S (Fujifim, Tokyo, Japan) or Gentra Puregene DNA isolation kit (Qiagen, Venlo, the Netherlands) according to the manufacturer's recommendations. Genomic DNA was isolated from amniotic fluid with the Gentra Puregene DNA isolation kit.

Triple-Primed Fragile X PCR

Forty nanograms of genomic DNA were amplified with the AmplideX *FMR1* PCR assay (Asuragen, Austin, TX) as previously described.[23] This assay is not approved by the Food and Drug Administration. All samples with additional alleles, save two, were reamplified at least once. Instead, these 2 samples (Table 1 from patients 12 and 24) were immediately confirmed by the laboratory-developed test. Fifty-two samples were reamplified from a second DNA isolation. For three patients (patients 1, 4, and 57), a second blood sample was obtained and the test was repeated.

FXS Laboratory-Developed Procedure

A total of 250 ng of genomic DNA were amplified in 15-μL reactions that contained 10 μmol/L forward (5′-FAM-GACGGAGGCGCCGCTGCCAGG-3′; NG_007529.1:5025-5045) and reverse (5′-TCCTCCATCTTCTCTTCAGCCCT-3′; NG_007529.1:5175-5197) primers (Life Technologies, Carlsbad, CA); 0.35 mmol/L deaza-dGTP (Roche Applied Science, Basel, Switzerland); 0.32 mmol/L deoxyadenosine triphosphate, deoxythymidine triphosphate, and deoxycytidine triphosphate (Roche Applied Science); 2% dimethyl sulfoxide; 2.5 mol/L betaine; 10 mmol/L Tris HCl pH 8.3; 50 mmol/L KCl; 2 mmol/L $MgCl_2$; 0.001% (w/v) gelatin; and 0.5 U of AmpliTaq DNA polymerase (Life Technologies). Thermocycling conditions were an initial denaturation at 95°C for 5 minutes; 40 cycles of 95°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute; and final extension at 72°C for 10 minutes. All amplicons were evaluated on a 3130xl Genetic Analyzer (Life Technologies).

FMR1 5′ UTR Sequencing

Genomic DNA (250 ng) was amplified in 30-μL reactions as above except the reactions contained 250 nmol/L of M13 tagged forward (5′-CCCAGGCCACTTGAAGAGAG-3′; NG_007529.1:4961-4829) and reverse (5′-CACCACCAGCTCCTCCTATCT-3′; NG_007529.1:5188-5207) primers (Life Technologies). PCR products were sequenced with BigDye Terminator (Life Technologies) chemistry on a 3130xl Genetic Analyzer (Life Technologies).

Southern Blotting

Five micrograms of genomic DNA were digested by EcoRI and EagI and electrophoresed on a 0.8% agarose Tris-acetate-EDTA gel. After transfer of DNA, charged nylon membranes