Methylation-Specific Triplet-Primed PCR and Melting Curve Analysis as a Rapid Screening Tool for Identifying Actionable \(FMR1\) Genotypes

To the Editor:

Full mutation (FM)\(^1\) and premutation (PM) expansions of the fragile X mental retardation 1 (\(FMR1\)) CGG repeat are the underlying causes of 3 \(FMR1\)-related disorders: fragile X syndrome (FXS), fragile X–associated primary ovarian insufficiency (FXPOI), and fragile X–associated tremor/ataxia syndrome (FXTAS) (1). PM and FM females are also at risk of conceiving FXS-affected offspring.

Molecular diagnosis of \(FMR1\)-related disorders involves both repeat length and methylation state determination. The advantages and barriers to newborn, early childhood, and carrier screening have been debated at length, and recommendations have been proposed (2), but high test costs present a practical barrier to widespread implementation. We recently described a single-step, closed-tube, and readily scalable strategy for rapid large-scale screening detection of \(FMR1\) expansion mutations by melting curve analysis (MCA) of triplet-primed PCR products from unmethylated genomic DNA (3). However, the assay does not discriminate between males and females or between PM and FM expansions, thus potentially identifying nonactionable conditions. We have developed a modified strategy that can differentiate between actionable and nonactionable \(FMR1\) expansion genotypes.

We optimized the assay on cell line genomic DNA (Coriell Cell Repositories) and previously characterized reference DNAs (4). DNA samples were pretreated with sodium bisulfite (EZ-DNA Methylation Gold kit, Zymo Research), and then subjected to methylation-specific triplet-primed PCR (mTP-PCR) of the modified antisense strand. Each 50-\(\mu\)L reaction contained 0.2 mmol/L dNTP (deoxynucleotide triphosphate), 2.5 U HotStarTaq DNA polymerase in 1× supplied PCR buffer and 1.8×Q solution (Qiagen), 4 \(\mu\)L bisulfite-modified DNA, and methylation-specific primers sets. A 15-min denaturation at 95 °C was followed by 40 cycles of 98 °C for 1 min, 60 °C for 1 min, and 72 °C for 2 min, followed by MCA as previously described (3). The gray zone (GZ) 54-repeat allele was used to establish a threshold temperature (TT) that distinguishes normal (NL) and GZ individuals from PM and FM individuals.

For every sample tested, melt peak profiles were obtained using either the 5′ or 3′ mTP-PCR assay (Fig. 1). NL, GZ, and PM males carry only an unmethylated \(FMR1\) allele and thus produce only an unmethylated allele triplet-primed PCR (uTP-PCR) peak. Conversely, an FM male will generate a right-shifted methylated allele triplet-primed PCR (mTP-PCR) peak from its fully methylated expansion, with a down-slope intersecting the TT. Females generate both uTP-PCR and mTP-PCR melt peaks, due to Lyonization of their 2 X chromosomes. In NL and GZ females, the uTP-PCR and mTP-PCR melt peaks do not exceed their respective TTs. Females with a PM and/or an FM allele have uTP-PCR and/or mTP-PCR melt peaks that are right shifted and exceed their respective TTs. For a PM female with random X inactivation, both melt peaks are right shifted beyond their \(TTs\), because the PM allele is present in both unmethylated and methylated states. For a constitutional FM female, only the mTP-PCR melt peak exceeds its TT, because an FM allele is present only in the methylated state. Results were highly reproducible for both mTP-PCR assays, based on the analysis of 10 replicates of the same sample for each assay. Also, using mixtures of NL and FM male DNA, low-level mosaicism of the expanded allele was also detected down to 5%. A blinded validation screen of the DNA of 6 NL males, 5 NL females, 1 PM male, 6 PM females, 14 FM males, and 9 FM females correctly classified all samples (data not shown).

The mTP-PCR MCA assay may be an ideal tool for targeted FXS screening among males with intellectual disabilities. It has a short turnaround time and is less labor-intensive than Southern blotting or even the newer PCR and capillary electrophoresis–based tests. With prompt referral by physicians for FXS testing, this assay may facilitate early detection of FM-affected males and potentially shorten the diagnostic pathway for families of affected children, thereby allowing parents to consider measures to avoid the birth of another affected child. This assay may also be technically ideal for population-based newborn or early childhood screening of FXS, although the ethical issue of incomplete penetrance of FXS in FM females remains to be resolved. The assay may also be useful in voluntary screening programs for preconception females to identify PM or asymptomatic FM carriers, who would be at risk for FXPOI and/or having FXS-affected offspring. Both \(FMR1\) genotype classes are actionable, because early identification enables genetic counseling and preemptive action to mitigate risks for FXPOI and FXS-affected offspring.

Either the 5′ or the 3′ mTP-PCR assay can be used, although a combined analysis will further

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\(^1\) Nonstandard abbreviations: FM, full mutation; PM, premutation; FXS, fragile X syndrome; FXPOI, fragile X–associated primary ovarian insufficiency; FXTAS, fragile X–associated tremor/ataxia syndrome; MCA, melting curve analysis; mTP-PCR, methylation-specific triplet-primed PCR; GZ, gray zone; TT, threshold temperature; NL, normal; uTP-PCR, unmethylated allele triplet-primed PCR; mTP-PCR, methylated allele triplet-primed PCR.
Fig. 1. FMR1 msTP-PCR and MCA.

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Fig. 1. Continued
The 5’ and 3’ assays utilize methylation-specific primers targeted to the bisulfite-modified antisense strand, paired to opposing triplet-primed primers (top). Tail primers anneal only to PCR amplicons. NL, GZ, and PM males display only the uTP-PCR melt peak, whereas FM males display a right-shifted mTP-PCR melt peak. Female samples generate both uTP-PCR and mTP-PCR peaks. In NL and GZ females, both peaks are clearly left shifted with respect to the TT, with the exception of the sample carrying the largest GZ allele size (asterisks). PM and FM females will generate clearly right-shifted uTP-PCR and/or mTP-PCR peaks. Primers: 5uF (5’ uTP-PCR 5’ flanking forward primer), CAAACAACAACTACAAAAACACCTACCAA (0.2 μmol/L); 5uTPR (5’ uTP-PCR triplet-primed reverse primer), CGACTGGCTACCTATGTTGACGCAAC (0.02 μmol/L); 3uR (3’ uTP-PCR 3’ flanking reverse primer), TGTTTTGAAAGGTTGTGGGTGTTT (0.2 μmol/L); 3uTPF (3’ uTP-PCR triplet-primed forward primer), CGACTGGCTACCTATGTTGACGCAAC (0.02 μmol/L); 5mF (5’ mTP-PCR 5’ flanking forward primer), GCGCTACAAACAGTACAGACAC (0.2 μmol/L); 5mTPR (5’ mTP-PCR triplet-primed reverse primer), ATTCATCCAGTTCACGAC (0.02 μmol/L); 3mF (3’ mTP-PCR 3’ flanking reverse primer), GTTGCAGGCGGTAGGTATG (0.2 μmol/L); 3mTPF (3’ mTP-PCR triplet-primed forward primer), ATTCATCCAGTTCACGAC (0.02 μmol/L); 5mR (primer identical to the 5’ tail of 5uTPR and 3uTPF), CGACTGGCTACCTATGTTGACGCAAC (0.02 μmol/L); 3mTPR (3’ mTP-PCR triplet-primed reverse primer), GTTGCAGGCGGTAGGTATG (0.2 μmol/L); 3mR (3’ mTP-PCR 3’ flanking reverse primer), GTTGCAGGCGGTAGGTATG (0.2 μmol/L); rpt, repeat.

References

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Tolerance of Droplet-Digital PCR vs Real-Time Quantitative PCR to Inhibitory Substances

To the Editor:

Real-time quantitative PCR (qPCR) is a rapid and sensitive method that forms the foundation for many clinical diagnostic tests. Droplet digital PCR (ddPCR) shares these qualities with qPCR, but owing to reaction partitioning, ddPCR is proposed to exhibit increased tolerance to interfering substances, making it an attractive alternative to qPCR for diagnostic applications.

Nonstandard abbreviations: qPCR, real-time quantitative PCR; ddPCR, droplet digital PCR; CMV, cytomegalovirus; IC50, half-maximal inhibitory concentration.