Identification of Males with Cryptic Fragile X Alleles by Methylation-Specific Quantitative Melt Analysis

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BACKGROUND: FMR1 full mutations (FMs) (CGG expansion >200) in males mosaic for a normal (<45 CGG) or gray-zone (GZ) (45–54 CGG) allele can be missed with the standard 2-step fragile X syndrome (FXS) testing protocols, largely because the first-line PCR tests showing a normal or GZ allele are not reflexed to the second-line test that can detect FM.

METHODS: We used methylation-specific quantitative melt analysis (MS-QMA) to determine the prevalence of cryptic FM alleles in 2 independent cohorts of male patients (994 from Chile and 2392 from Australia) referred for FXS testing from 2006 to 2013. All MS-QMA–positive cases were retested with commercial triplet primed PCR, methylation-sensitive Southern blot, and a methylation-specific EpiTYPER-based test.

RESULTS: All 38 FMs detected with the standard 2-step protocol were detected with MS-QMA. However, MS-QMA identified methylation mosaicism in an additional 11% and 15% of patients in the Chilean and Australian cohorts, respectively, suggesting the presence of a cryptic FM. Of these additional patients, 57% were confirmed to carry cryptic expanded alleles in blood, buccal mucosa, or saliva samples. Further confirmation was provided by identifying premutation (CGG 55–199) alleles in mothers of probands with methylation-sensitive Southern blot. Neurocognitive assessments showed that low-level mosaicism for cryptic FM alleles was associated with cognitive impairment or autism.

CONCLUSIONS: A substantial number of mosaic FM males who have cognitive impairment or autism are not diagnosed with the currently recommended 2-step testing protocol and can be identified with MS-QMA as a first-line test.

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Fragile X syndrome (FXS)5 is the most common single-gene cause of intellectual disability and comorbid autism (1). It is almost always associated with an unstable CGG triplet expansion within FMR1 (fragile X mental retardation 1), termed full mutation (FM) (>200 CGGs) (2). The incidence of FM in the general population is estimated to be approximately 1 in 4000 males and 1 in 8000 females (3). FM alleles induce complete methylation of the FMR1 promoter, leading to absence of mRNA transcription and loss of the fragile X mental protein (FMRP), which is essential for normal brain development (4). Rare FMR1 loss-of-function mutations involving deletions (small to large), macro duplication, and point mutation can also cause FXS without promoter hypermethylation (5–7).

Smaller CGG expansions, including premutation (PM) expansions (8) ranging from 55 to 199 repeats and gray-zone (GZ) expansions ranging from 45 to 54 repeats, have an unmethylated FMR1 promoter and mild to moderate reduction in FMRP translation (9, 10). PM and GZ alleles have also been associated with increased FMR1 gene expression, related to the increased size of the CGG expansion and RNA gain-of-function toxicity observed in FMR1-related late-onset disorders (11, 12). Normal size (NS) alleles are <45 CGG repeats, with 31 CGG repeats being the most common allele size in the general population (13).

Methylation mosaicism or PM/FM CGG size mosaicism have been previously reported in 12% to 41% of FM males identified (14, 15) with the conventional

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5 Nonstandard abbreviations: FXS, fragile X syndrome; FM, full mutation; FMRP, fragile X mental protein; PM, premutation; GZ, gray zone; mSouthern, methylation-sensitive Southern blot; HRM, high-resolution melting; FREE2, fragile X-related epigenetic element 2; MS-QMA, methylation-specific quantitative melt analysis; WISC-IV, Wechsler Intelligence Scale for Children; WAIS-IV, Wechsler Adult Intelligence Scale; ADOS, Autism Observation Scale.
2-step testing protocol (16). The phenotype of these males is milder than in FM males with a completely methylated FMR1 promoter, presumably because a proportion of cells in these individuals express FMRP (17). Variability in phenotype may also be due to a variable degree of mosaicism in different cells or tissues, at different CpG sites, at different ages, and in active and inactive X-chromosomes (18–21).

The current conventional 2-step testing protocol for FXS uses a relatively low-cost first-line CGG triplet-repeat sizing PCR assay to exclude individuals with a normal or GZ allele from reflexed FM testing with methylation-sensitive Southern blot (mSouthern) or a triplet primed PCR test (16, 22, 23). Because the standard CGG sizing PCR does not amplify alleles with >130 repeats, only PM positives, “no product” samples (e.g., males with FM) or females “apparently” homozygous for the same NS allele are reflexed for second-line testing. It is therefore likely that individuals who are mosaic for normal or GZ alleles with a cryptic FM remain unidentified (16). The incidence of such cryptic FM alleles is unknown.

We recently developed a high-throughput FXS test on the basis of a combination of outputs from high-resolution melting (HRM) and quantitative real-time PCR that targets a novel methylation biomarker region proximal to the FMR1 promoter, known as the fragile X-related epigenetic element 2 (FREE2). We named this test methylation-specific quantitative melt analysis (MS-QMA) (24) and report here a study of the prevalence of cryptic FM, which were undetected in first-line screening, in 2 independent cohorts referred for FXS testing in Chile and Australia. The study also included a direct comparison of MS-QMA results on exactly the same samples with the 3 most widely used triplet primed PCR commercial kit tests (AmplideX, FastFraX, and X Sense), mSouthern, and Epityper-based FMR1 methylation analysis.

**Materials and Methods**

**PARTICIPANT SAMPLES**

Of the combined cohort of 3588 cases referred for routine FXS testing between 2006 and 2013, 1172 were from Chile and 2416 were from Australia. For the Chilean referrals, FXS molecular diagnosis was made for approximately 8% [92 males (84 probands and 8 cascade referrals), whereas for the Australian cohort, it was approximately 1.4% [35 males (21 probands, 5 cascade referrals) and 9 unknown provenance)]. For these cohorts, 178 Chilean and 24 Australian cases were not tested by MS-QMA because there was insufficient DNA available. The remaining samples tested in this study included 994 (967 NS, 27 FM) DNA samples from the Centre for Diagnosis and Treatment of Fragile X Syndrome, INTA University of Chile, and 2392 (2381 NS, 11 FM) from the Cyto-molecular Diagnostic Laboratory, Victorian Clinical Genetics Services, Australia. Participants from the Chilean cohort were age 2 months to 60 years and referred for routine FXS testing between 2006 and 2013. Conventional karyotyping analysis to rule out other gross genetic abnormalities was performed on 475 DNA samples from the Chilean cohort. Participants from the Australian cohort were age 1–61 years and referred for routine FXS testing between 2011 and 2013. Routine chromosome microarray testing was performed on all Australian samples to rule out copy number abnormalities. This study was approved by the Royal Children’s Hospital and INTA Research Ethics Committees.

**SAMPLE COLLECTION**

Blood samples from participants were collected at the time of diagnosis (t1) as part of standard testing. For confirmatory testing, blood, buccal brushes, and saliva samples were obtained from 10 participants and their mothers on a second occasion (t2). Further details are described in Supplemental Data 1, which accompanies the online version of this article at http://www.clinchem.org/content/vol62/issue2.

**ROUTINE FXS TESTING WITH PCR AND mSOUTHERN**

For the Chilean and Australian cohorts, routine FXS testing involved first-line PCR-based assessment of CGG repeat size (precision within 1 repeat) with a validated PCR amplification assay, with the upper limit of detection 330 CGG repeats for the Chilean cohort (25) and 170 CGG repeats for the Australian cohort (23). Blood DNA samples from all males who showed a CGG size in the PM range or failed to show a PCR product, as well as blood DNA samples from their mothers, were referred for second-line confirmatory testing by mSouthern, performed as described (26, 27).

**BISULFITE CONVERSION, DNA POOLING SCREEN, AND MS-QMA**

Three-microliter aliquots from each of 6 samples collected from the 3386 cohort were pooled for the initial HRM screen. This created 560 DNA pools that were treated with sodium bisulfite with EZ-96 DNA Methylation-GoldTM (Zymo Research), as previously described (28). The bisulfite-converted DNA pools were then assayed with real-time PCR followed by HRM with MeltdoctorTM reagents in 10-μL reactions on the ViiaTM 7 Real-Time PCR System, according to manufacturer’s instructions (Life Technologies), with primers targeting specific CpG sites within the FREE2 region (Fig. 1A), as previously described (24). The real-time PCR component used 65°C as the annealing temperature for 40 cycles. The HRM step after real-time PCR used the HRM Software Module for the ViiaTM 7 System to identify DNA pools that had higher aligned fluorescence at the melting temperature of 78°C than NS.
controls (Fig. 1D). Samples from these HRM-positive pools were then retested separately with the same primers, and the methylation ratio was determined with the MS-QMA, as described earlier (24). The methylation ratio was then multiplied by 100 to be expressed as a methylation percentage. Briefly, quantitative measurement of DNA methylation for each sample was based on MS-QMA analysis of 4 serial dilutions of the converted
DNA at the melting temperature of 78 °C. The methylation ratio was then determined from the real-time PCR and HRM data generated by Viia7 system with Q’Max software (Curve Tomorrow) developed to automate previously described MS-QMA–based analysis (24).

SECOND-LINE TESTING OF MS-QMA–POSITIVE SAMPLES
All MS-QMA–positive DNA samples were retested with 3 triplet primed PCR commercial kits (AmplideX, Asuragen; FastFraX, BioFactory; and X Sense, Abbott Molecular), as previously described (29–31). Further details are provided in online Supplemental Data 2. FMRI CpG island and FREE2 methylation testing were also performed on the same MS-QMA positive samples with mSouthern and the EpiTYPER system, respectively, as previously described (17).

CLINICAL MEASURES
Neurobehavioral testing was performed for 12 MS-QMA–positive males missed by the standard first-line PCR CGG-based sizing protocol. These males ranged in age from 5 to 25 years and were recruited through the INTA University of Chile. Clinical assessments included an intelligence quotient test performed with the Wechsler Intelligence Scale for Children (WISC-III) and Wechsler Adult Intelligence Scale (WAIS-IV) (32, 33). The presence of autism spectrum disorder was determined with the Autism Observation Scale (ADOS) (34).

DATA ANALYSIS
MS-QMA diagnostic sensitivity was determined as a measure of the probability of correctly identifying the presence of an FM allele, confirmed with ≥1 of the comparator tests. MS-QMA diagnostic specificity was determined as a measure of the probability of correctly identifying the absence of an FM allele, confirmed with ≥1 of the comparator tests. The positive predictive value was expressed as the percentage of MS-QMA–positive samples confirmed by ≥1 comparator tests from the total number of MS-QMA–positive results. The negative predictive value was expressed as the percentage of MS-QMA-negative samples confirmed by ≥1 comparator tests from the total number of MS-QMA–negative results (see online Supplemental Fig. 1). Comparator tests included routine PCR CGG sizing in combination with mSouthern, FREE2 EpiTYPER methylation analysis, AmplideX, FastFraX, and X Sense CGG-based analyses.

Results

INTERASSAY COMPARISON WITH ARTIFICIALLY SPIKED DNA SAMPLES
To determine the lower limit of detection for each assay used in this study, artificial NS/FM mosaic samples were created from high-quality DNA extracted from lymphoblast cell lines used in previous studies (17, 28). Fully methylated FM (530 CGG) male DNA was mixed with NS (30 CGG) male DNA at different ratios. MS-QMA showed the lowest lower limit of detection (1%) of all tests examined. For the EpiTYPER system, the lower limit of detection was 10%. On the basis of manufacturer instructions for what should be considered a positive signal, the lower limit of detection for FastFraX was 25%, X Sense 20%, and AmplideX 5% (see online Supplemental Fig. 2). mSouthern showed a lower limit of detection of 20% for both FM and NS alleles (Fig. 1B).

MS-QMA PERFORMANCE ON CLINICAL SAMPLES
From 3386 male FXS referrals tested, MS-QMA and mSouthern blot showed 100% concordance for detection of typical FMRI FM (no NS allele present) in 27 Chilean and 11 Australian samples. In addition, MS-QMA identified methylation mosaicism in another 18 samples (14 from Chile and 4 from Australia) (Table 1, Figs. 1–3, and online Supplemental Fig. 3). All of these cases had NS alleles identified with routine CGG repeat sizing PCR as part of standard testing and thus had not been previously reflexed to a second-line FM test. Two of these samples (males 2 and 3) were from the same individual, referred for FXS testing on 2 different occasions over a 1-year period.

Sixteen of the additional methylation-positive cases had higher aligned fluorescence at the melting temperature of 78 °C than NS controls, indicating the presence of abnormal methylation (see online Supplemental Fig. 3). Nine of the additional methylation-positive results by MS-QMA were confirmed to have PM or FM alleles with ≥1 CGG-based reference methods (Table 1, nos. 1–8 and 10). An extra case (Table 1 and online Supplemental Fig. 3), male 9, who had a normal CGG sizing assay result, appeared to have abnormal methylation as determined by EpiTYPER analysis.

There was sufficient DNA for 9 of the MS-QMA–positive probands (Table 1, nos. 1, 2, 5, 6, 8, 9, 10, 11, and 13) to be retested by mSouthern. mSouthern identified methylated FM alleles in nos. 1, 2, and 6 (Fig. 1C). Mothers of these probands were also recruited, as well as the mother of 1 other male (Table 1, male 4) for whom there was insufficient DNA available for confirmatory mSouthern testing. The following PM alleles were identified in these 4 mothers by a combination of mSouthern blot and standard CGG sizing PCR (mother of male 1: 180 CGGs; mother of male 2: 75 CGGs; mother of male 4: 85 CGGs; and mother of male 6 was a rare compound heterozygote with 60 and 100 CGG alleles and no NS allele) (Fig. 1C). Expanded FMRI alleles were not detected in males 9, 10, 11 and 13, or in their mothers. However, it is important to note that ≥2 of these mSouthern-negative cases harbored abnormally methylated FMRI alleles missed by mSouthern, potentially owing to its lower limit of detection of only 20% (Fig. 1B).
Male 9 was confirmed to have abnormal methylation by EpiTYPER analysis, whereas male 10 was confirmed to harbor an FM allele in saliva by the AmplideX test (Fig. 3). The remaining 8 samples (4 from Australia and 4 from Chile) with abnormal methylation were not confirmed to have an expanded allele by another reference method. If these were considered to be false positives, then MS-QMA showed a diagnostic sensitivity of 100% for the separate Chilean and Australian cohorts and the combined cohort, with a diagnostic specificity of 99.8% for the Australian cohort, 99.6% for the Chilean cohort, and 99.8% for the combined cohort. The positive predictive value was 86% for the Australian cohort, 95.8% for the Chilean cohort, and 93.5% for the combined cohort.

There was a substantial discordance among comparator methods for true positives (cases positive for the cryptic alleles by /H113502 methods), especially for cases with low-level methylation, between t1 and t2 collection points and between tissues (see online Supplemental Data 4).

**CRYPTIC FM PREVALENCE IN TWO COHORTS**

For the Australian cohort, 35 typical FM males were identified of 2416 referrals with the conventional protocol, which equated to 1.4% prevalence among individuals referred because of clinical suspicion of FXS. In this population, inclusion of MS-QMA as a first-line test...
would have increased the number of positive cases identified by 11% (Table 2). For the Chilean cohort, 92 typical FM males were identified of 1172 referrals with the conventional protocol, which equated to 7.9% prevalence among individuals referred because of clinical suspicion of FXS. If the MS-QMA true-positive samples were only those confirmed by \(11350\) comparator methods, then the estimate of prevalence of a cryptic FM allele in males referred for fragile X testing in Chile would be approximately 1.0% (10 of 994), increasing the yield of FM cases identified by 15% (Table 2).

### NEUROCOGNITIVE ASSESSMENTS

Standardized neurocognitive assessments were performed for 8 of the 9 cases of cryptic FM. These indicated that 75% of them (6 of 8) were cognitively impaired (IQ <70), and 50% (4 of 8) had comorbid autism (Table 1 and online Supplemental Table 2). Interestingly, the 3 males (1, 3 and 6) with both cognitive impairment (IQ <70) and comorbid autism had the highest MS-QMA methylation output at t1 and t2, ranging from 65% to 84%. In contrast, 25% (2 of 9) confirmed positive cases with higher cognitive function (IQ >70) had MS-QMA output approaching 1% methylation.

### Discussion

Mosaicism for a NS, GZ, or GZ/PM borderline allele together with an FM expansion has previously been reported in 26 FXS cases (25 males and 1 female) tested by mSouthern (online Supplemental Data Table 3). Of these 26 FXS cases, 3 case reports of males (35–37) suggest that this cryptic mosaic pattern is associated with a severe FXS phenotype of mental retardation, behavioral anomalies, learning disabilities, and poor attention. To estimate the prevalence of cryptic FM, >3000 males recruited from 2 countries were systematically studied. All patients were previously tested with a CGG triplet repeat

### Table 1. Samples identified by MS-QMA as positive that were missed using the conventional testing protocol.\(^a\)

<table>
<thead>
<tr>
<th>Sample from male patient</th>
<th>Methylation, %</th>
<th>CGG repeat number</th>
<th>Clinical measures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MS-QMA(^c)</td>
<td>EpiTYPER</td>
<td>FastFraX</td>
</tr>
<tr>
<td>1</td>
<td>&lt;10</td>
<td>&gt;200</td>
<td>&gt;200</td>
</tr>
<tr>
<td>2</td>
<td>&lt;10</td>
<td>&gt;200</td>
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<td>&lt;10</td>
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<td>5</td>
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<tr>
<td>14</td>
<td>&lt;10</td>
<td>&gt;200</td>
<td>&gt;200</td>
</tr>
</tbody>
</table>

\(^a\) Samples in venous blood of 3386 children with developmental delay or autism spectrum disorder referred for FXS testing.

\(^b\) Males 1–8 were confirmed to carry methylated FM or PM alleles as well as a normal-size allele in blood using ≥1 alternative approach. Low-level mosaic males 5–9 had discordant percentages of methylation between MS-QMA and the EpiTYPER system. This may be explained by the EpiTYPER system being more sensitive to DNA quality, with poor-quality DNA producing an artificially higher methylation output, as previously described (24). Alternatively, whereas both assays target the same DNA region, MS-QMA provides information on methylation of 3 extra sites that cannot be analyzed by the EpiTYPER system because of their larger fragment size.

\(^c\) MS-QMA (24), Sequenom® EpiTYPER system of the FREE2 region (17), FastFraX™ (29), X Sense® (30), AmplideX (29).

\(^d\) t1, blood DNA collected at the time of diastasis; t2, blood DNA obtained for confirmatory testing on a second occasion.

\(^e\) VIQ, verbal intellectual quotient; PIQ, performance IQ; FSIQ, full-scale IQ (33); ADOS, autism spectrum disorder diagnosis (34); NA, results pending.

\(^f\) Samples from the same individual collected on different occasions.

\(^g\) Poor-quality DNA: abnormal aligned fluorescence detected, but did not pass QC filter process to be quantified by MS-QMA.

\(^h\) Mean methylation below the limit of detection of 10%; however, one of the CpG units targeted was above the 10% threshold.

\(^i\) Saliva DNA sample provided for confirmatory testing after being re-approached following an MS-QMA positive result in blood. Presence of an FM allele was confirmed in saliva using both the MS-QMA and AmplideX CGG sizing assay (negative in blood) (Figure 3).
first-line test for FXS as per the standard protocol. Samples from these males were retested with MS-QMA to detect abnormal FMR1 methylation, with follow-up confirmation testing with other methods. Compared with the previously applied testing protocols, use of MS-QMA as a first-line test detected 15% additional abnormally methylated male samples. This estimate is consistent with findings from 2 earlier smaller studies with mSouthern as a first-line test. These smaller studies identified 1 in 236 and 3 in 300 cryptic FMs in cognitively impaired males referred for FXS testing. Considering that 0.6–6.5% of this referral population have been reported to have FXS with standard testing, identification of 1% additional abnormal methylated male samples is highly significant. Furthermore, detection of PM alleles in the mothers of these probands, as demonstrated in this study, has clear implications for provision of cascade testing and genetic counseling to other family members at risk of having affected children.

Cognitive impairment was confirmed in most of these cases, although there was no comorbid autism at low-level methylation (<5%) in 60% of cases. Cryptic FM males are likely to have NS alleles that express normal levels of FMR1 transcript and FMRP, whereas traces of RNA toxicity and decreased FMRP may be evident from cells harboring PM alleles in FM/PM mosaic males. These differences and the molecular and clinical consequences should be further characterized in larger studies.

We detected an additional 9 individuals (also positive by ≥1 reference methods) with a confirmed cryptic FM expansion not detected previously with the conventional FXS testing protocol. The higher analytical and diagnostic sensitivity of the MS-QMA test is highlighted by the results for 1 of the MS-QMA positive males in whom abnormal methylation was detected in all tissues tested, a cryptic FM allele confirmed in a saliva sample with the AmplideX CGG-based test. However, the AmplideX test did not identify FM in blood or buccal samples from this individual. This 11-year-old male had normal intelligence and no comorbid autism; the reason for his referral for FXS testing included facial features consistent with FXS, attention deficit hyperactivity disorder, and language and social disorder.

Although other causes of this phenotype have not been definitively ruled out, this study supports the notion that methylation mosaicism as low as 1% might have clinical significance, although such low levels would require detection with tests of higher diagnostic sensitivity (Fig. 4). First-line testing with mSouthern blot is not suitable for this, as it is a low-throughput, laborious, and
costly technique. Furthermore for reflex testing, a spiking experiment demonstrated that mSouthern blot is not particularly analytically sensitive compared to MS-QMA or AmplideX triplet primed PCR used in this study, with the lower limit of detection of only 20% for methylated FM or unmethylated NS alleles. This precludes detection of the type of low-level (<20%) mosaicism for either NS or FM alleles exemplified in this study.

Table 2. Cryptic FM prevalence in the Chilean and Australian cohorts.

<table>
<thead>
<tr>
<th>Group</th>
<th>Chilean cohort (n)</th>
<th>Australian cohort (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventional protocol*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total tested</td>
<td>1172</td>
<td>2416</td>
</tr>
<tr>
<td>FM males identified</td>
<td>92</td>
<td>35</td>
</tr>
<tr>
<td>Normal CGG size males</td>
<td>1080</td>
<td>2381</td>
</tr>
<tr>
<td>FM males identified by the conventional protocol not tested by MS-QMA</td>
<td>65</td>
<td>24</td>
</tr>
<tr>
<td>MS-QMA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total tested</td>
<td>994</td>
<td>2392</td>
</tr>
<tr>
<td>Not tested (insufficient DNA)</td>
<td>178</td>
<td>24</td>
</tr>
<tr>
<td>FM males identified by MS-QMA, positive by conventional protocol</td>
<td>27</td>
<td>11</td>
</tr>
<tr>
<td>MS-QMA negative, normal CGG size males</td>
<td>967</td>
<td>2381</td>
</tr>
<tr>
<td>Extra methylation positive cases identified by MS-QMA</td>
<td>14</td>
<td>4</td>
</tr>
<tr>
<td>Extra methylation positive samples from the total FM males identified by the conventional protocol, % (n)†</td>
<td>15 (14/92)</td>
<td>11 (4/35)</td>
</tr>
</tbody>
</table>

* Conventional protocol for the Australian cohort and Chilean cohort included first-line CGG sizing PCR performed as described by Khaniani et al. (2) and Saluto et al. (3), respectively. The second-line Southern blot test was performed on all positive cases in the 2 cohorts as described in Francis et al. (26) and Alliende et al. (27), respectively.
† The percentage calculation is potentially a prevalence underestimate because it assumes that there were no extra methylation-positive cases in the 178 Chilean and 24 Australian samples with insufficient DNA for MS-QMA testing.

Fig. 4. Two proposed FXS testing work flows to enable improved detection of males mosaic for cryptic fragile X alleles. (A), MS-QMA incorporated as a first-line test. (B), MS-QMA incorporated as a second-line test.
MS-QMA identified the largest number of confirmed cystic FM male cases in a single study to date. It should be remembered that in regard to interpreting the clinical significance of cystic FM mosaicism, there is an element of ascertainment bias in selecting patients referred for FXS testing. Thus, the main limitations of this study are not only the small number of positive cases for comprehensive assessment of the epigenotype-phenotype relationship, but also the unknown prevalence of cystic FM in the normal population. Unresolved is the failure to confirm a small proportion of the MS-QMA–positive cases by any of the reference methods used in this study or by absence of PM alleles in their mothers. This could be due to the comparator tests not having the analytical sensitivity to detect low levels of NS/PM mosaicism or X chromosome aneuploidy mosaicism (<5%), or even that they are genuine MS-QMA false positives.

In summary, MS-QMA was used to estimate the prevalence of cystic FM alleles in individuals who also carry an NS or GZ allele. Although MS-QMA did not detect unmethylated PM alleles, its diagnostic and analytical sensitivity was higher than that of any of the triplet-primed PCR or Southern blot second-line confirmatory methods. Together, this emphasizes the accepted limitations inherent in all current FXS tests. The identification of PM alleles in a proportion of the mothers of cystic FM carriers not only provides independent validation of the MS-QMA test results, but also demonstrates the importance of identifying FXS families through this method. The clinical significance of cystic FM alleles requires further epigenotype-phenotype studies. IFMS-QMA were to become a standard first-line test, other more analytically sensitive methods, such as pyrosequencing and droplet digital PCR with reported lower analytical sensitivity was higher than that of any of the triplet-primed PCR or Southern blot second-line confirmatory methods. Together, this emphasizes the accepted limitations inherent in all current FXS tests. The identification of PM alleles in a proportion of the mothers of cystic FM carriers not only provides independent validation of the MS-QMA test results, but also demonstrates the importance of identifying FXS families through this method. The clinical significance of cystic FM alleles requires further epigenotype-phenotype studies. IFMS-QMA were to become a standard first-line test, other more analytically sensitive methods, such as pyrosequencing and droplet digital PCR with reported lower limit of detection <5% in other applications (41, 42), would need to be developed for second-line testing. Testing of multiple tissues and more extensive CGG triplet repeat size testing of other family members, especially mothers, could also be used for confirmation of MS-QMA positive results. Finally, features that make MS-QMA an attractive first-line FXS test for males are: (a) turnaround time of only 5 h per run, (b) high throughput (200 samples per run), (c) closed-tube format minimizing risk of contamination (no post-PCR sample handling or capillary electrophoresis-based analysis), (d) automated Q’Max-based analysis (sample number independent) that provides real-time PCR QC information on bisulfite conversion efficiency in each reaction, and (e) use of standard low-cost HRM reagents and real-time PCR equipment that is available in most diagnostic laboratories. Finally, the diagnostic specificity of MS-QMA for methylated FM alleles and not unmethylated NS, GZ, or PM alleles makes it ethically acceptable for screening of young children because of the issue of presymptomatic identification of alleles that predispose to increased risk of developing late-onset FMR1 PM-related disorders.

References