A Homogeneous Assay for Analysis of FMR1 Promoter Methylation in Patients with Fragile X Syndrome, Christina Dahl, Karen Grønskov, Lars A. Larsen, Per Guldborg, and Karen Brendum-Nielsen (1 Institute of Cancer Biology, Danish Cancer Society, Copenhagen, Denmark; 2 The Kennedy Institute-National Eye Clinic, Glostrup, Denmark; and 3 Wilhelm Johannsen Centre for Functional Genome Research, Department of Medical Biochemistry and Genetics, University of Copenhagen, Denmark; * address correspondence to this author at: The Kennedy Institute-National Eye Clinic, Gl. Landevej 7, DK-2600 Glostrup, Denmark; fax 45-43-43-11-30, e-mail kbn@kiso.org)

Background: Fragile X syndrome is caused by the expansion of a CGG trinucleotide repeat at the 5' untranslated region of the fragile X mental retardation 1 gene (FMR1). When expanded to >200 repeats (full mutation), the repeat region and the adjacent promoter CpG island become hypermethylated, rendering FMR1 transcriptionally inactive. Conventional molecular diagnosis of fragile X syndrome involves determination of the CGG repeat number by Southern blot analysis.

Methods: A homogeneous methylation-specific melting curve analysis (MS-MCA) assay for methylation status of the FMR1 promoter region was developed on the LightCycler platform. Genomic DNA was treated with sodium bisulfite, and a region containing 8 CpG sites was amplified in the presence of SYBR Green I, using primers that do not discriminate between methylated and unmethylated FMR1 molecules. After amplification, the samples were melted at 0.05 °C/s, and fluorescence melting curves were recorded. We studied samples, previously characterized by Southern blot analyses, from 10 females and 10 males with normal numbers of CGG trinucleotide repeats, 9 male premutation carriers, 4 mosaic males carrying both a premutation and a full mutation, and 25 patients with fragile X syndrome.

Results: Samples from all 20 males with fragile X syndrome showed a high-melting peak corresponding to fully methylated FMR1, whereas samples from healthy males showed a single low-melting peak corresponding to unmethylated FMR1. Nine of the 24 affected-male samples (38%) showed 2 melting peaks, suggesting that cellular methylation mosaicism is common in fragile X syndrome.

Conclusions: MS-MCA allows rapid and reliable identification of males with fragile X syndrome.

Fragile X syndrome is the most common inherited cause of mental retardation, with a frequency of 1 in 4000 males and 1 in 6000 females (1). The mental impairment in affected individuals ranges from learning disabilities to autism and severe mental retardation and may be accompanied by a variety of physical and behavioral characteristics, making the clinical diagnosis difficult.

At the genomic level, fragile X syndrome is associated with the expansion of a naturally occurring CGG trinucleotide tandem repeat in the promoter region and 5' untranslated region of the fragile X mental retardation 1 gene (FMR1) at Xq27.3 (1). The number of CGG repeats is highly variable, and 4 allelic forms of FMR1 have been defined: normal alleles have <50 CGG repeats and are stable upon transmission from generation to generation; intermediate (or “gray-zone”) alleles have 50–58 CGG repeats and may show some instability; premutations have between 59 and ~200 repeats and are liable to further expand upon transmission; and individuals with fragile X syndrome have >200 repeats. The FMR1 allele with a CGG repeat number of >200 is known as a full mutation and is associated with hypermethylation of the repeat region and the adjacent promoter CpG island. It is generally recognized that promoter hypermethylation causes transcriptional silencing of FMR1 and that the pathophysiological background for disease manifestations is the absence or deficit of the fragile X mental retardation protein (FMRP) (1). The theory of methylation-coupled silencing is supported by rare reports of healthy individuals with FMRP expression from full-mutation FMR1 alleles without promoter hypermethylation (2).

Due to the lack of definitive clinical diagnostic criteria, molecular tests are important for detection of individuals with fragile X syndrome. The gold standard is Southern blot analysis, which requires large amounts of DNA and is labor-intensive and time-consuming. Numerous PCR-based tests have been developed for screening procedures, which estimate the number of CGG repeats (3, 4). The major disadvantage of these methods is their moderate specificity for detecting full-mutation and large premutation alleles due to the common failure of conventional PCR to amplify long repetitive regions with a very high G+C content. The Clinical Molecular Genetics Society (CMGS) best practice guidelines recommend pre-screening by PCR amplification of the CGG repeat and subsequent Southern blot analysis of samples that fail to amplify (males) or show a single allele (females). A disadvantage of this strategy is the inability to reliably distinguish between healthy males and males who are mosaic for normal and full-mutation alleles. Alternative PCR-based tests to determine FMR1 methylation status and/or repeat length have been developed (5–8); these rely on treatment of DNA template with bisulfite to convert unmethylated cytosine, but not methylated cytosine, into uracil before PCR amplification (9). However, these methods all require a 2-step procedure, comprising initial PCR amplification followed by product analysis, which implies the risk of carry-over contamination.

We have developed a homogeneous assay for fragile X syndrome on the LightCycler platform, based on detection of hypermethylated FMR1 alleles by methylation-specific melting curve analysis (MS-MCA). This method resolves differentially methylated DNA sequences on the
basis of differences in melting temperature after treatment of DNA with bisulfite (10). The basic steps, which can be integrated by means of a thermal cycler coupled with a fluorometer, involve nondiscriminatory amplification of methylated and unmethylated alleles in the presence of SYBR Green I, followed by step-wise elevation of the temperature under continuous fluorescence monitoring to examine the melting properties of the PCR products. The derived melting peaks provide a graphic profile of the entire pool of DNA molecules in the sample and can discriminate among 4 different methylation states: a) unmethylated alleles generate a single low-melting peak, b) fully methylated alleles generate a single high-melting peak, c) a mixture of unmethylated and fully methylated alleles generate both the low- and high-melting peaks, and d) “heterogeneously” methylated alleles generate a broadened melting top located between the low- and high-melting peaks. The latter state refers to molecular mosaicism where the content and distribution of methylated cytosine residues differ among different alleles in the same sample.

Genomic DNA was treated with sodium bisulfite, as previously described (10). To establish an MS-MCA assay for detection of FMR1 hypermethylation, we designed a primer set that spans 8 CpG sites within the FMR1 promoter and tested it on bisulfite-treated DNA from a healthy male, a healthy female and in vitro-methylated DNA (CpGenome Universal Methylated DNA, Chemi-con). The primers were 5’TAT GTG AAG TGG TTT TAG TGT TTA TAT T-3’ (2541) and 5’CTC AAA AAC TAC CCT CCA CC-3’ (2655R), which amplify a 105-bp region of the FMR1 promoter 5’-CpG island (GenBank Accession No. X61378). The underlined nucleotides indicate mismatches within the primer sequences representing CpG sites in the FMR1 promoter sequence. PCR was carried out using the LightCycler 1.0 instrument (Roche) in 10-μL reaction mixtures containing 5 pmol of each primer, 3 mmol/L MgCl2,1× FastStart DNA Master SYBR Green I (Roche), and bisulfite-modified DNA. Reactions were started by initial denaturation at 95 °C for 10 min, followed by 34 cycles at 95 °C for 5 s, 65 °C for 10 s, and 72 °C for 15 s. Melting curve analysis was performed immediately after amplification by measuring the fluorescence of SYBR Green I during a temperature transition from 60 °C to 95 °C at 0.05 °C/s. Fluorescence data were converted into melting peaks using the LightCycler Software 4.05. DNA from the healthy male showed a single melting peak with an apparent melting temperature (Tm) of ~77 °C (Fig. 1A), in vitro-methylated DNA showed a single melting peak with a Tm ~4 °C higher (Fig. 1A), and DNA from the healthy female showed both melting peaks because 1 allele was methylated with X-chromosome inactivation (Fig. 1B).

We blindly tested DNA from 58 individuals, including 10 females and 10 males with normal numbers of CGG trinucleotide repeats, 9 male premutation carriers, 4 mosaic males carrying both a premutation and a full mutation, and 20 males and 5 females with fragile X syndrome. These samples had previously been examined by Southern blot analysis. All healthy female samples showed both low- and high-melting peaks, corresponding to the unmethylated and fully methylated FMR1 alleles, respectively. Healthy males presented with a single low-melting peak, whereas samples from all 24 males with fragile X syndrome showed a high-melting peak corresponding to fully methylated FMR1 (Fig. 1C). The 4 males samples carrying both full-mutation and premutation alleles showed both low- and high-melting peaks (Fig. 1C). In addition, 2 melting peaks were observed in 5 of 20 fragile X males who carried the full mutation but were not identified as mosaics by Southern blot analysis. The size of the low-melting peak in the mosaic males showed some interrun variation, probably due to stochastic amplification. In total, 9 of the 24 samples from affected males (38%) contained both unmethylated and fully methylated FMR1 alleles, suggesting that cellular FMR1 methylation mosaicism is common in fragile X syndrome. Previous studies have shown highly variable frequencies of mosaic males, ranging from 12% (11) to 41% (12), which probably reflects differences in assay resolution and/or the tissues analyzed (13).

All 5 samples from fragile X females in this study showed both low- and high-melting peaks and thus could not be distinguished from normal females (Fig. 1D). This is in agreement with other studies showing that blood lymphocytes from fragile X females hold both methylated and unmethylated FMR1 alleles (14, 15). Male premutation carriers had a single low-melting peak similar to healthy males (Fig. 1D), consistent with previous studies showing that premutation FMR1 alleles are unmethylated (16, 17). None of the 58 samples showed melting peaks with intermediate Tm80, suggesting that molecular mosaicism at the FMR1 locus is rare and, accordingly, the distribution of FMR1 promoter methylation is bimodal (18).

Fig. 1. MS-MCA analysis of the FMR1 promoter region. Bisulfite-treated DNA was amplified in the presence of SYBR Green I using primers that do not discriminate between methylated and unmethylated FMR1 alleles. The melting characteristics of the PCR products were determined directly in the PCR tube by continuous fluorescence monitoring during a temperature transition at 0.05 °C/s. (A), the melting peaks for in vitro-methylated DNA (M) and DNA from a healthy male (U) have Tms of ~81 °C and ~77 °C, respectively. (B), a healthy female. (C), 2 males with fragile X syndrome, including 1 mosaic (thick line), (D), a fragile X female (thick line) and a male premutation carrier.
As with other methods relying on treatment of DNA with bisulfite, incomplete conversion of cytosine to uracil may be a problem and may cause false-positive results (19). However, MS-MCA of the FMR1 promoter in DNA from healthy males consistently showed a single low-melting peak, suggesting that the protocol used in this study led to complete bisulfite conversion. Another potential problem associated with MCA is that resolution of $T_m$ differences using the LightCycler may be reduced when more samples are melted at the same time (20). Nevertheless, the $T_m$ difference between methylated and unmethylated FMR1 sequences was so large ($\sim 4 ^\circ C$) that interpretation of the melting peaks was unproblematic even when a full rotor was analyzed.

In summary, closed-tube resolution of methylated and unmethylated FMR1 alleles on the basis of differences in $T_m$ after bisulfite conversion was 100% effective in identifying males with fragile X syndrome. Because the assay reads both methylated and unmethylated alleles simultaneously, mosaic males were also readily identified. On the other hand, the assay could not identify either fragile X female or male carriers of a premutation, as the methylation patterns were similar to those of healthy individuals. In addition, some mosaic fragile X males had a pattern indistinguishable from healthy females. Nevertheless, considering that the main indication to test for FMR1 abnormalities is mental retardation in males, the MS-MCA assay could find widespread use as an initial screening procedure due to its low cost, simplicity, and closed-tube format.

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References


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