alternative explanation may be that assays based on mutant-enriched PCR result in a higher rate of K-ras-positive samples. This suggestion is further supported by a study by Kopreski et al., in which 27% of 105 individuals with negative colonoscopy results had positive results for mutant K-ras in plasma samples tested with an assay based on mutant-enriched PCR (16).

To estimate the potential of the proposed assay for screening, an important question is whether it enables identification of individuals who currently bear colorectal or pancreatic neoplasms or who are at increased risk of developing neoplasms at these organs in the future. The lack of associations between important risk factors for the respective cancer sites and mutant K-ras in stool provides more rebutting than supporting evidence regarding the value of the proposed assay to identify high-risk individuals. In regard to colorectal disease, none of the individuals diagnosed with advanced adenomas or CRC within 2 years after stool collection tested positive. In the most important study for comparison, 16% of CRC cases (95% confidence interval, 5%–34%) and ~4% of advanced adenoma cases (95% confidence interval, 3%–7%) tested positive for mutant K-ras in stool at the time of diagnosis (15). According to these figures, 2 positive findings would have been expected among the 31 cases with advanced adenomas/CRC in our study. Although the analytical sensitivity of the assay used in our study (i.e., the probability of detecting mutant K-ras in stool if present) could be improved through stool quantity and storage conditions, these measures are expected to go along with a decrease in diagnostic specificity (i.e., the probability that healthy people test negative). Given that for CRC, K-ras has been proposed for parallel testing in combination with further markers, the validity of such a multiple marker panel is highly vulnerable to decreased specificity of any component.

Mutant K-ras in stool may also play a role for the detection of pancreatic cancer. Sequence testing in combination with additional markers has been suggested to distinguish patients with pancreatic cancer from patients with benign pancreatic disease (8, 17). In this context, the high detection rate of the proposed assay may be a minor problem or even be advantageous provided that the right specific combination of markers is found. Whether the combination of fecal pancreas elastase 1 as a marker of exocrine pancreatic insufficiency and mutant K-ras, which are both independent predictive factors for pancreatic cancer development in patients with chronic pancreatitis (17), may be useful remains to be clarified by further studies.

In conclusion, our study results do not support the use of the proposed assay for CRC screening. Its potential for early detection of pancreatic cancer (in combination with other markers) requires further investigation.

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References


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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 differentiate 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 female and 10 male donors with normal numbers of CGG trinucleotide repeats, 9 male donors who were premutation carriers, 4 male donors who carried both a premutation and a full mutation, and 25 patients with fragile X syndrome.

Results: Samples from all 20 male patients 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. Of 24 samples from affected males, 9 (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 fragile X syndrome in male patients.

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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 the alleles in 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).

Because 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 that their specificity for detecting full-mutation and large premutation alleles is only moderate, owing 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 prescreening 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 increase 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 be used to differentiate 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 indicates molecular mosaicism, in which 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 2 healthy persons, a male and a female, and in vitro-methylated DNA (CpGenome Universal Methylated DNA, Chemicon). The primers were 5’-GTG AAG TGG TTT TAG TGT TTA TAT T-3’ (2541) and 5’-TAC CCT CCA CC-3’ (2645R), 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, 1X 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 ($T_m$) of $-77^\circ$C (Fig. 1A), in vitro-methylated DNA showed a single melting peak with a $T_m$ $-4^\circ$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 sample donors, 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 male and 5 female patients with fragile X syndrome. These samples had previously been examined by Southern blot analysis. The samples were donated for investigation with informed consent from the patients/parents. All samples from healthy females showed both low and high melting peaks, corresponding to the unmethylated and fully methylated FMR1 alleles, respectively. Samples from healthy male donors showed a single low melting peak, whereas samples from all 24 male patients with fragile X syndrome showed a high melting peak corresponding to fully methylated FMR1 (Fig. 1C). The 4 samples from male donors who carried both full-mutation and premutation alleles showed both low and high melting peaks (Fig. 1C). In addition, of the 20 samples from fragile X male patients who carried a full mutation, 5 samples showed 2 melting peaks but were not identified as mosaics by Southern blot analysis.

In our study, all 5 samples from female patients with fragile X syndrome showed both low and high melting peaks and thus could not be distinguished from samples from healthy female donors (Fig. 1D). This is in agreement with other studies showing that blood lymphocytes from female patients with fragile X syndrome hold both methylated and unmethylated FMR1 alleles (14, 15). We found that samples from male premutation carriers had a single low melting peak similar to that seen in samples from those of healthy males in previous studies (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 $T_m$s, suggesting that molecular mosaicism at the FMR1 locus is rare and, accordingly, the distribution of FMR1 promoter methylation is bimodal (18).

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 po-

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 differentiate 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 $T_m$s of $-81^\circ$C and $-77^\circ$C, respectively. Also shown are melting peaks for DNA from (B) a healthy female donor; (C) 2 male patients with fragile X syndrome, including 1 whose DNA shows a mosaic pattern (thick line); and (D) a female patient with fragile X syndrome (thick line) and a male donor whose DNA shows a premutation carrier pattern.
Nevertheless, the unmethylated FMR1 donors because the methylation patterns were similar to female donors or permutation carrier status in the male not enable us to detect fragile X syndrome in DNA from male donors. On the other hand, the assay did simultaneously, mosaicism was also readily identified in assay reads both methylated and unmethylated alleles tifying fragile X syndrome in male patients. Because the potential problem associated with MCA is that resolution of Tm differences using the LightCycler may be reduced when more samples are melted at the same time (20). Nevertheless, considering that the main indication to test for FMR1 abnormalities is mental retardation in male patients, the MS-MCA assay could find widespread use as an initial screening procedure due to its low cost, simplicity, and closed-tube format.

In summary, closed-tube resolution of methylated and unmethylated FMR1 alleles on the basis of differences in Tm after bisulfite conversion was 100% effective in identifying fragile X syndrome in DNA from female donors or permutation carrier status in the male donors because the methylation patterns were similar to those of healthy individuals. In addition, for some male patients who had fragile X with mosaicism, DNA patterns were indistinguishable from those of healthy females. Nevertheless, considering that the main indication to test for FMR1 abnormalities is mental retardation in male patients, 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