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*Short Communication*

# DIAGNOSIS OF THE FRAGILE X SYNDROME IN MALES USING METHYLATION-SPECIFIC PCR OF THE *FMR1* LOCUS

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## ABSTRACT

We have developed a non-isotopic technique based on methylation-specific PCR (MSP) of the *FMR1* promoter for the identification of fragile X full mutations among men. DNA samples were first treated with sodium bisulfite to convert unmethylated, but not methylated, cytosines to uracil, followed by PCR amplification with oligonucleotide primers specific for methylated versus unmethylated DNA. We designed two primer pairs: one produced a 142-bp fragment from the bisulfite-treated methylated CpG island, while the other generated an 84-bp product from the treated non-methylated promoter. In normal males only the 84-bp fragment was seen, while the diagnosis of *FRAXA* was doubly indicated by the appearance of a 142-bp product together with absence or weak visualization of the 84-bp band. As an indispensable internal control for the efficiency of the sodium bisulfite treatment, we used a primer pair specific for the imprinted maternal methylated version of the CpG island of the *SNRPN* gene on human chromosome 15. Using the methylation-specific PCR we identified with 100% sensitivity and accuracy eight previously diagnosed *FRAXA* male patients mixed with 42 normal controls. Because of its simplicity and high efficiency, methylation-specific PCR may become the method of choice for the diagnosis of the fragile X syndrome in mentally retarded males.

## INTRODUCTION

The fragile X syndrome (*FRAXA*) is the most common inherited form of mental retardation in man. The molecular pathogenesis of the disease generally involves expansion of a $(CGG)_n$ trinucleotide repeat located on the 5' region of the *FMR1* gene, leading to hypermethylation of the promoter and shutdown of gene expression (reviewed by Nelson, 1998; Kaufmann and Reiss, 1999). The molecular diagnosis of *FRAXA* has depended on simultaneous Southern blot analysis of $(CGG)_n$ length and methylation status. However, this is a complex procedure and, to screen for *FRAXA* among mentally retarded children, we need simpler and cheaper PCR-based diagnostic tests.

The first PCR-based test that we developed was based on direct PCR amplification of the $(CGG)_n$ trinucleotide repeat with primers flanking the microsatellite, with a product of 557 bp for the $(CGG)_{29}$ allele (Haddad *et al.*, 1996). Conditions were established so that full mutations failed to amplify. To produce an internal control we added to the reaction a third primer, internal to this fragment, allowing the multiplex amplification of a monomorphic band corresponding to a CG-rich stretch 147 base pairs upstream the polymorphic region. In blind trials the PCR-based test showed specificity of more than 98.6%, accuracy of 99% and a sensitivity of 98%. The test had two main disadvantages. Firstly, a normal $(CGG)_n$ allele was preferentially amplified by PCR due to its smaller size and thus the PCR technique could not be used for the diagnosis of *FRAXA* in females, because they are heterozygous and would be scored as normal. For the same reason, mosaic patients with a normal sized allele might yield a false negative result. That is why the test was not 100% sensitive. The second drawback resulted from the "failure-to-amplify" characteristic of the test that thus could not provide a definitive diagnosis of fragile X syndrome. Although not quite suitable for medical diagnosis, the PCR test proved to be a useful tool for fragile X syndrome screening in populations of mentally retarded males (Haddad *et al.*, 1999).

Recently Herman *et al.* (1996) developed an elegant PCR assay for methylation status of CpG islands. DNA samples are first treated with sodium bisulfite to convert unmethylated, but not methylated, cytosines to uracil, followed by PCR amplification with oligonucleotide primers specific for methylated versus unmethylated DNA. We wish to report the successful application of this methylation-specific PCR (MSP) for the study of the *FMR1* promoter. This led to a much-improved method for PCR diagnosis of the fragile X syndrome in affected males.

## MATERIAL AND METHODS

### Patients

We used DNA from eight patients with fragile X syndrome, all confirmed by Southern blot analysis: five of these were ascertained in a screening study of mentally retarded boys in Brazil (Haddad *et al.*, 1999), two were patients diagnosed at GENE and the other (NA06852) was obtained from the Coriell Mutant Cell Repository (Camden, NJ, USA). DNA samples from 42 normal controls were obtained from paternity testing cases at GENE.

### PCR primers

For development of primers we used the data of

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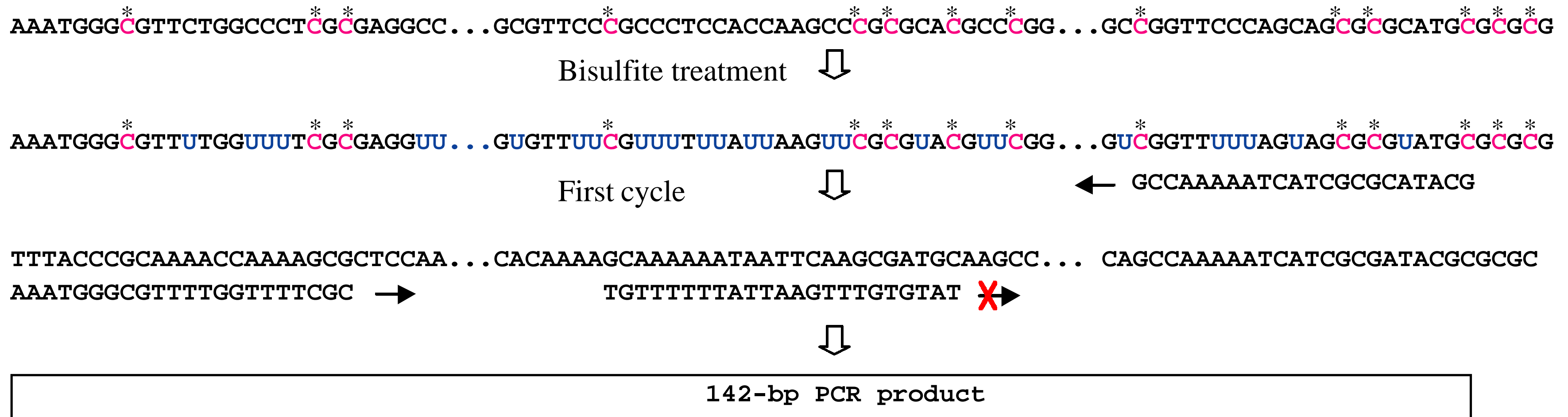


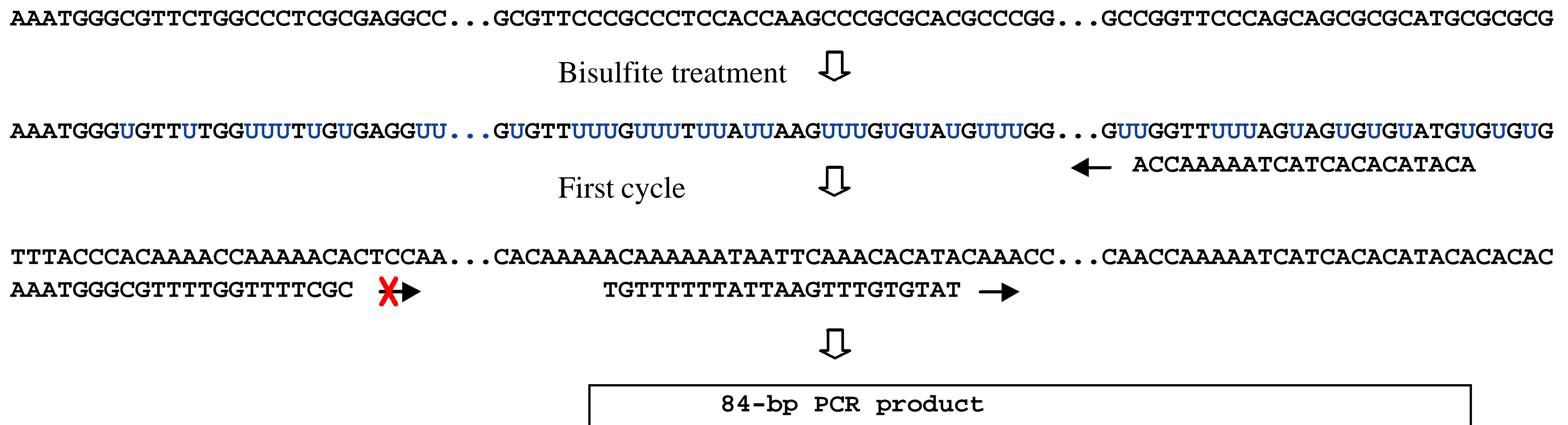


**Figure 1 -** Strategy for the methylation-specific PCR based on the methylation pattern of the *FMR1* CpG island as described by Stöger *et al.* (1997). The methylated cytosines are shown in red and marked as asterisks. The non-methylated cytosines are converted into uracil residues (shown in blue) by the bisulfite treatment. This diagram is based on the one published by Zeschnigk *et al.* (1997) for the diagnosis of Angelman and Prader-Willi syndromes by MSP.

Stöger *et al.* (1997) who described the pattern of cytosine methylation at the CpG island of the *FMR1* gene. We designed two primer pairs: the first, 5'-AAATGGGCGTTTTGGTTTTCGC-3' and 5'-GCCAAAAATCATCGCGCATACG-3', produces a 142-bp fragment from the bisulfite-treated methylated CpG island, while the other, 5'-TGTTTTTTATTAAGTTTGTGTAT-3' and 5'-ACCAAAAATCATCACACATACA-3', generates an 84-bp product from the treated non-methylated promoter. The strategy behind the primer development is shown schematically in Figure 1.

### Methylation-specific PCR

DNA samples were treated with sodium bisulfite as described by Herman *et al.* (1996). They were then submitted to PCR amplification in separate tubes with primers specific for the methylated (M) or non-methylated (N) versions of the CpG island of the *FMR1* gene. In the reaction with the M primers we also included primers specific for the methylated version of the *SNRPN* gene (Kubota *et al.*, 1997). PCR was performed in a final volume of 13 µl using 0.65 µ AmpliTaq Gold (Perkin Elmer, Foster City, CA, USA) in the manufacturer's recommended buffer, 200 µM of each dNTP, 0.4 µM of each primer and 100 ng of human genomic DNA. Thermal cycling conditions were: initial denaturation at 95°C for 5 min, followed by 35 cycles of 1 min of annealing at 53°C for the M reaction and 43°C for the N reaction, 1 min of extension at 72°C and denaturation at 95°C for 1 min. Afterwards, the PCR reaction products were separated by electrophoresis in a 6% polyacrylamide gel and visualized by silver staining.

## RESULTS AND DISCUSSION

In normal males only the 84-bp fragment was seen (Figure 2), while the diagnosis of *FRAXA* was doubly indicated by the appearance of a 142-bp product together with visualization of a much weaker 84-bp band (Figure 2). The probable reasons that the 84-bp product did not disappear as could be expected are that methylation is generally not complete (Stöger *et al.*, 1997) and that somatic mosaicism occurs in the length of the $(CGG)_n$ repeat in complete mutations. As an indispensable internal control for the efficiency of the sodium bisulfite treatment, we used a primer pair specific for the imprinted maternal methylated version of the CpG island of the *SNRPN* gene on human chromosome 15 (Kubota *et al.*, 1997) generating a fragment of 174 bp (Figure 2).

Using the methylation-specific PCR we identified with 100% specificity, sensitivity and accuracy, eight previously diagnosed *FRAXA* male patients mixed with 42 normal controls. In theory the test should not be prone to producing false positive results and should also be very sensitive, permitting diagnosis even in mosaics with normal-sized alleles. Indeed, we have found that we can still obtain a clear methylated product even when DNA from

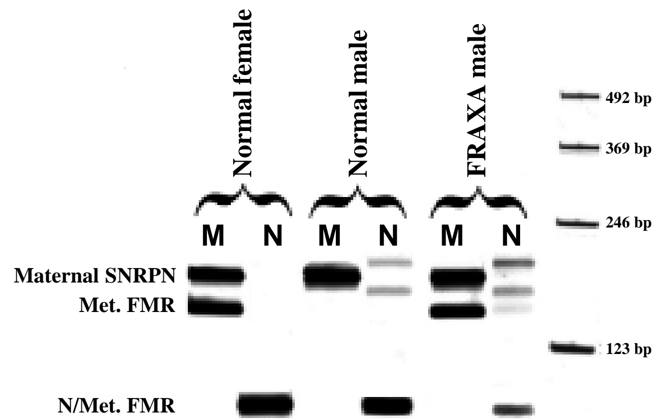


**Figure 2 -** Polyacrylamide gel electrophoresis of the products of methylation-specific PCR of the human *FMR1* gene on the X chromosome. **Met. FMR** is the 142-bp fragment from the bisulfite-treated methylated CpG island, while **N/Met. FMR** is the 84-bp product from the treated non-methylated promoter. **Maternal SNRPN** is the internal control 174-bp product of amplification of the imprinted maternal methylated version of the CpG island of the *SNRPN* gene on human chromosome 15 (Kubota *et al.*, 1997). DNA samples from a normal woman, a normal man and a patient with the fragile X syndrome diagnosed by chromosomal studies and Southern blot analysis were treated with sodium bisulfite as described by Herman *et al.* (1996). On the rightmost lane of the gel are the 123-bp ladder molecular size standards (Life Technologies, Gaithersburg, MD, USA).

*FRAXA* patients is diluted 20-fold with normal male DNA. If needed, sensitivity could be further increased by the use of fluorescently labeled primers and detection in an automatic DNA sequencer.

Apparently the pattern of methylation in the promoter region of the FMR1 is identical in full mutations of *FRAXA* and in X inactivation in normal females (Stöger *et al.*, 1997). Thus, the MSP test cannot be used to diagnose the fragile X syndrome in affected females, since they already have, in virtue of X inactivation, a methylated *FMR1* promoter region.

In summary, methylation-specific PCR emerges as a simple and efficient method for assessing methylation in the *FMR1* CpG island. Indeed, it may become the method of choice for diagnosis of the fragile X syndrome in mentally retarded males.

## RESUMO

Nós desenvolvemos uma técnica não-isotópica baseada na PCR para a identificação de mutações completas da síndrome do X-frágil em homens. O método é baseado na PCR específica para metilação do promotor do gene *FMR1*. Amostras de DNA são tratadas com bissulfito de sódio para converter citosinas não-metiladas para uracilo, seguindo-se amplificação por PCR com oligonucleotídeos iniciadores específicos para DNA metilado versus não-metilado. Desenhamos dois iniciadores: um produz um fragmento de 142 pb da ilha CpG metilada tratada com bissulfito de sódio, enquanto o outro gera um produto de 84 pb do promotor tratado não-metilado. Em homens normais apenas

o fragmento de 84 pb é visto, enquanto o diagnóstico da síndrome do X-frágil é indicado duplamente pela aparição do produto de 142 pb e pelo desaparecimento ou visualização muito fraca da banda de 84 pb. Como um controle interno indispensável para avaliação da eficiência do tratamento com bissulfito de sódio, nós usamos iniciadores específicos para a versão materna metilada da ilha CpG do gene *SNRPN* no cromossomo 15 humano. Com a PCR específica para metilação nós identificamos com sensibilidade e acerto de 100% oito pacientes previamente diagnosticados como tendo a síndrome do X-frágil misturados com 42 controles normais. Dada a sua simplicidade e alta eficiência, a PCR específica para metilação pode tornar-se o método de escolha para o diagnóstico da síndrome do X-frágil em homens com retardo mental.

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