Consultations in Molecular Diagnostics

A Single Nucleotide Variant in the *FMR1* CGG Repeat Results in a “Pseudodeletion” and Is Not Associated with the Fragile X Syndrome Phenotype

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The molecular diagnosis of fragile X syndrome relies on the detection of the pathogenic CGG repeat expansion in the *FMR1* gene. Deletions and point mutations have occasionally been reported. Rare polymorphisms might mimic a deletion by Southern blot analysis, leading to false-positive results. We describe a novel rare nucleotide substitution within the CGG repeat. The proband was a woman with a positive family history of mental retardation. Southern blot analysis showed an additional band consistent with a deletion in the region detected by the StB12.3 probe. Sequencing of this region revealed a G>C transversion that interrupts the CGG repeat and introduces an *EagI* site. The same variant was observed in both the healthy son and father of the proband, supporting the hypothesis that the nucleotide substitution is a silent polymorphism, the frequency of which we estimated to be less than 1% in the general population. These findings argue for a pathogenic role of nucleotide variants within the CGG repeat and suggest possible consequences of unexpected findings in the molecular diagnostics of fragile X syndrome. Thus, although the sequence context of a single nucleotide substitution may not predict possible effects on mRNA or protein function, a specific change in the higher order structures of DNA or mRNA may be functionally relevant in the pathological phenotype. (*J Mol Diagn* 2008, 10:272–275; DOI: 10.2353/jmoldx.2008.070163)

The fragile X syndrome, one of the most common causes of inherited mental retardation, is caused in almost all cases by an expansion of the polymorphic CGG trinucleotide repeat located in the 5' untranslated region of the *FMR1* gene. Large CGG expansions (>200 CGG repeats) in this region are associated with hypermethylation of the proximal CpG islands and inhibition of transcription, leading to the loss of the protein product FMRP.¹ Limited expansions (called permutations; 50–200 repeats) are not associated with the disease phenotype but trigger meiotic instability with increase of the repeat length in succeeding generations. Deletions and point mutations of *FMR1* associated with a complete lack of FMRP are very rare. A few patients have been described in whom deletions, or mosaics of a deletion and a full mutation, were associated to the fragile X syndrome phenotype.²

Molecular diagnosis of fragile X syndrome mainly relies on Southern analysis and polymerase chain reaction (PCR) by using primers flanking the CGG repeat. Southern hybridization allows the simultaneous detection of large expansions and methylation status as well as mosaic patterns.³ PCR allows an accurate sizing and is crucial to identify premutation alleles.¹ In most laboratories the Southern hybridization protocol is based on the double digestion of genomic DNA with *EcoRI* and a methylation-sensitive enzyme, such as *EagI* or *SacII*. The restriction product is probed with a labeled *FMR1* fragment (commonly StB12.3).³ In normal males this procedure results in the detection of a single band of 2.8 kb, whereas normal females show an additional 5.2-kb band corresponding to the methylated allele in the inactivated X chromosome. Premutations and full mutations are detected through a band shift with respect to the normal

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pattern. The detection of shorter fragments may reveal a deletion, but "pseudodeletions" due to rare nucleotide variants within the StB12.3-probed region have been occasionally reported.^{4,5} Interestingly, Tarleton and co-workers⁵ found a single base substitution within the CGG tract in a male child with mild speech and developmental delay. Their experiments suggested that such CGG>CCG variant (at the 26th CGG of a 31-repeat-long tract) reduces the FMRP expression by 24% and consequently may exert a pathogenic effect.

Here we describe a rare single nucleotide substitution within the CGG repeat that mimics a deletion in Southern blot analysis, found in a female with positive family history for mental retardation. We also report on the phenotype associated with this variant in two male carriers and discuss the possible pathogenic role of single nucleotide polymorphisms in the CGG repeat.

Materials and Methods

Clinical Report

The proband was a healthy woman with two male maternal first cousins affected with mental retardation of unknown origin. After informed consent, she underwent molecular analysis for fragile X syndrome, which revealed the pseudodeletion (see Results).

The proband's father, aged 76 years, declined consent to clinical examinations but provided a detailed personal history and consented to blood sampling. He regularly worked as craftsman and retired at 60 years of age. He never complained about neurological disturbances, and neither behavioral nor cognitive dysfunctions were noticed. No dysmorphic features of the face were apparent during the interview. His three brothers never demonstrated signs of intellectual dysfunction and died after 70 years of age.

The proband's son showed normal development and growth until the age at examination (7 years). His speech development was regular (first words at 12 months). He attended educational activities with good proficiency and never revealed any adaptive disturbance. No dysmorphic features were noticed. The mother gave consent to blood sampling.

Fragile X Analysis

Patient DNA was isolated from peripheral blood using an automated DNA extractor, Geno-M6 (Genovision, QIAGEN). The FRAXA locus was analyzed with conventional Southern analysis of genomic DNA (7 µg) digested with the restriction enzymes *EcoRI* and *EagI*. Blots were probed with a [³²P]dCTP-labeled (Redivue; GE Healthcare Europe) StB12.3 probe that hybridizes to the region from nucleotide 14461 to 15537 in *FMR1* (GenBank reference sequence L29074).³

FMR1 Sequencing

PCRs were performed using 50 ng of genomic DNA in a 25-µl reaction mix including 10X PCR buffer (Invitrogen), 0.75 µl of MgCl₂ 50 mmol/L (Invitrogen), 200 µmol/L deoxynucleoside-5'-triphosphates, 0.4 µmol/L primers, and 0.5 U of Taq Platinum (Invitrogen), applying the following thermal conditions: 94°C for 4 minutes, followed by 40 cycles of 94°C for 30 seconds, 58–60°C for 30 seconds, 72°C for 30 seconds, and a final extension at 72°C for 7 minutes.

The *FMR1* region spanning the CGG repeat was analyzed using the GC-Rich PCR System kit (Roche Applied Science, Indianapolis, IN) following the manufacturer's instructions (C and F primers shown in Table 1). The amplicon containing the CGG repeat (10 µl of PCR product) was digested with *EagI* and resolved on a 3% Metaphor agarose gel (Biospa Division, Milan, Italy). Direct sequencing of PCR fragments was performed on a 3130xl capillary sequencer (Applied Biosystems, Foster City, CA). Primer sequences are shown in Table 1.

A cohort of 235 normal unrelated individuals (47 males, 188 females) was examined to estimate the frequency of the nucleotide variant. Exact binomial confidence intervals were calculated from observed frequency using Stata 9 (StataCorp, College Station, TX).

Results

Molecular Analysis

EcoRI/EagI Southern analysis in the proband did not reveal any band in the range corresponding to CGG ex-

Table 1. Primer Sequences

Primer	Sequence	Position in <i>FMR1</i> (GenBank L29074)
C ¹	5'-GCTCAGCTCCGTTTCGGTTTCACTTCCGGT-3'	13712-13741
F ¹	5'-AGCCCCGCACTTCCACCACAGCTCCTCCA-3'	13992-13963
FMR1	5'-GCTCGGCGGGATGTTGTTGG-3'	14133-14152
FMR2	5'-GTAGTAAGAAGCGGTAGTCG-3'	14562-14581
FMR3	5'-CGACTACCGCTTCTTACTAC-3'	14581-14562
FMR4	5'-AGTAGCAGCGCTGCTAATGG-3'	14798-14789
FMR5	5'-CCTACATACCAACAACGCAC-3'	15120-15100
FMR6	5'-CAGCCTTCCTTCCACACGCA-3'	15239-15220
FMR7	5'-TGAAATTGTTTCAGTTGACTCTGC-3'	15513-15536
FMR8	5'-GAAGGTGAATGAAGAATAGGTTGG-3'	15937-15960
FMR9	5'-TTCTTCCATCAGTCAGACC-3'	16089-16070
FMR10	5'-GAGCTGTCCTTGCAAATATGG-3'	16394-16374

Figure 1. A: Southern blot analysis. DNA was double-digested with *EcoRI* and *EagI* and then probed with StB12.3. Family individuals were denoted as proband (P), proband's father (F), and proband's son (S). Proband shows the expected 2.8- and 5.2-kb bands and an extra band of 2.5 kb. The profile is compared to a normal female control (NF). The 2.8- and 2.5-kb bands show the same signal intensity, accounting for a random X inactivation. A premutated female (PF) showing a faint 2.9-kb premutation band attributable to a skewed X inactivation was presented as control. Both proband's father and son demonstrate loss of the normal 2.8-kb band and the appearance of a 2.5-kb smaller band. No expanded bands were detected in the family members. Images were taken from two experiments. B: Sequence analysis. The electropherograms show the heterozygous G>C substitution in the proband and the variant C allele in her father and son. The nucleotide substitution is marked by arrows. The *EagI* site introduced by the G>C substitution is underlined. C: The agarose gel shows the *EagI*-digested PCR products encompassing the GGC repeat. The proband shows three bands, one corresponding to the undigested normal allele (338 bp, detected in a normal female control) and two corresponding to the variant allele (*EagI* digestion products: 198 bp and 140 bp). The digested products

were detected in both male hemizygous carriers of the pseudodeletion, the proband's son and the proband's father.

pansions. A band of approximately 2.5 kb was detected, in addition to the expected 2.8-kb and 5.2-kb bands (Figure 1A, lane P). The presence of this additional fragment led us to suspect a heterozygous deletion spanned in the *EcoRI* restricted fragment.

As deletions within the *FMR1* gene are associated with variable fragile X phenotypes, a set of experiments was designed to: 1) evaluate the presence of mosaicism with an expanded allele; 2) confirm the presence of a deletion and determine its extension and localization; and 3) evaluate the segregation pattern and possible association with mental retardation. To accomplish these tasks, the father and the son of the proband were included in subsequent analyses.

EcoRI/EagI Southern hybridization revealed a novel 2.5-kb fragment in both the father and son of the proband (Figure 1A, lanes F and S), excluding the association of the unusual pattern with mental retardation, which was reported in the maternal side of the family. No major effect on X inactivation can be attributed to the rare allelic variant, as the additional band showed the same intensity with respect to the one corresponding to the wild-type X chromosome (Figure 1A, lanes P and PF). PCR analysis performed with primers C and F detected two alleles corresponding to 30 and 39 CGG repeats, respectively.

Southern blot analysis was then performed using *EcoRI* only. The three members of the family demonstrated the normal restriction pattern and no novel band. Conversely, *HindIII/EagI* digestion revealed the same profile produced by *EcoRI/EagI* restriction (data not shown). Therefore, a modification of the *EagI* restriction map in the *EcoRI* restriction fragment (from nucleotide 111109 to 116341 in GenBank reference sequence L29074) appeared a likely explanation of these findings.

To test this hypothesis, we screened the region for nucleotide variants introducing novel *EagI* sites. Direct sequencing of the region including the CGG repeat detected in the proband a heterozygous G>C transversion at the eighth CGG repeat (CGG>CCG), as shown in Figure 1B. The substitution introduces a novel *EagI* site (CGGCGG>CGGCCG) resulting in a fragment of about 2.5 kb overlapping the StB12.3 probe. No other variant introducing a *EagI* site was found. The C allele was demonstrated by direct sequencing in the proband's father and son (Figure 1B). To confirm this result, a PCR product of 338 bp, containing the G>C substitution at nucleotide 144, was examined in the proband, her family members, and a negative control male. After *EagI* digestion, three bands were detected in the proband, one corresponding to the wild-type allele (338 bp, undigested) and two corresponding to the variant allele (140 bp and 198 bp, *EagI* digested); the proband's father and son showed only the two bands indicating the variant allele (Figure 1C).

A series of unrelated control individuals was analyzed by direct sequencing to estimate the frequency of the G>C variant in the general population. The G>C substitution was not found in 423 normal X chromosomes, resulting in an estimated frequency <<1% (observed frequency: 0.0; 95% exact confidence interval: 0.0000-0.0087). Interestingly, a normal female individual carried a heterozygous G>T substitution (CGG>CTG) at the eighth repeat. Including this finding, the frequency estimation for all nucleotide variants detected within the CGG tract increased to 0.23% (observed frequency: 0.0023; 95% exact confidence interval: 0.0001-0.0131). In addition, a retrospective review of more than 4000 fragile X assays (a total of approximately 5100 X chromosomes) performed in our laboratory with the *EcoRI/EagI* Southern

protocol provided no evidence of pseudodeletions in the hybridization pattern.

Discussion

We described a novel variant in the CGG repeat region of the *FMR1* gene that introduces a *EagI* site in the restriction fragment used to detect the repeat expansion. The presence of this variant resulted in the absence of the normal unmethylated 2.8-kb fragment, replaced by a shorter fragment of about 2.5 kb. This restriction profile mimics the presence of a deletion in the *FMR1* gene and was named "pseudodeletion" according to definition given in the two cases of similar restriction patterns that have been described.^{4,5}

The G>C transversion found in the present work was demonstrated to segregate along three generations. None of the three individuals included in the genotype assessment showed any pathological features associated with fragile X syndrome. In particular, neither signs of mental retardation nor any characteristics of fragile X syndrome were observed in the male carriers.

The frequency estimation on a normal control series led us to conclude that the variant reported in this study is a very rare polymorphism, consistent with the lack of pseudodeletions in a large sample of consecutive individuals who underwent clinical testing in our laboratory. It can be inferred that the CGG>CCG transversion in any repeat unit is a rare event, as it should be invariably observed as pseudodeletion. Other nucleotide substitutions within the repeat tract can be detected in routine Southern analysis merely if a novel restriction site is introduced. Screening the control samples we found a C>T substitution in a normal individual. This finding further supports the hypothesis that single nucleotide substitutions in the CGG repeat occur as rare mutational events that do not lead to apparent functional effects.

Taken together, the present results failed to provide any evidence in favor of a pathogenic effect of single nucleotide variants in the *FMR1* CGG tract. The sequence context of the single nucleotide substitution does not provide any obvious clue of a possible effect on *FMR1* mRNA and protein. However, the position at which the base substitution occurs might be crucial. A specific change in the higher order structures of DNA or mRNA may be functionally relevant to determine a pathological phenotype. This hypothesis could explain the discrepancy with respect to previous observation that suggested a pathogenic role for a point mutation located in the 26th CGG of a 31-repeat-long tract.⁵

In the present study no affected individual carrying the pseudodeletion was found. Nevertheless, it cannot be formally excluded that the absence of clinical manifestations in both male carriers who were examined is due to a lack of penetrance.

The laboratory report for the proband conveyed that the repeat expansion was not detected and that a polymorphism with unknown pathogenic effect was found. The risk of having children with mental retardation was not refined by the molecular analysis, thus the empirical recurrence risk was reported. However, the occurrence of a rare variant in a proband prompted us to consider the risk of erroneous interpretation of experimental data stemming from unusual findings. The molecular diagnostic procedures are far from being immune from misleading results, even though good laboratory practices are applied. Current guidelines for molecular analysis in fragile X syndrome recommend that more than one laboratory protocol should be used, because no single method can detect all types of mutations with equal accuracy and precision⁶ (see the European Molecular Genetics Quality Network, draft best practice guidelines for molecular analysis in fragile X syndrome, available online at http://www.emqn.org/emqn/BestPractice/mainColumnParagraphs/05/document/EMQN_guidelines_FRAX_2006.pdf; accessed January 4, 2008). It is recommended that Southern hybridization analysis always be conducted, even if an expanded allele is identified by PCR.⁶ The risk of misdiagnosis due to incorrect clinical testing protocols underscores the need for specific guidelines dedicated to the most widely used genetic testing procedures.

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