Analysis of unstable DNA sequence in FRM1 gene in Polish families with fragile X syndrome

Michał Milewski\textsuperscript{a}, Marta Żygulska\textsuperscript{b}, Jerzy Bą\textsuperscript{a}, Wout H. Deelen\textsuperscript{c}, Ewa Obersztyn\textsuperscript{a}, Ewa Bocian\textsuperscript{a}, Dicky J.J. Halley\textsuperscript{c}, Jürgen Horst\textsuperscript{b} and Tadeusz Mazurczak\textsuperscript{a}

\textsuperscript{a} Department of Genetics, Institute of Mother and Child, 01-211 Warsaw, Poland
\textsuperscript{b} Institute of Human Genetics, WWU, D-4400 Münster, Germany
\textsuperscript{c} Department of Clinical Genetics, Erasmus University, 3000 DR Rotterdam, The Netherlands

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The unstable DNA sequence in the FRM1 gene was analyzed in 85 individuals from Polish families with fragile X syndrome in order to characterize mutations responsible for the disease in Poland. In all affected individuals classified on the basis of clinical features and expression of the fragile site at X(q27.3) a large expansion of the unstable sequence (full mutation) was detected. About 5% (2 of 43) of individuals with full mutation did not express the fragile site. Among normal alleles, ranging in size from 20 to 41 CGG repeats, allele with 29 repeats was the most frequent (37%). Transmission of premutated and fully mutated alleles to the offspring was always associated with size increase. No change in repeat number was found when normal alleles were transmitted.

The fragile X syndrome (Martin-Bell syndrome) is the most frequent familial form of mental retardation and affects 1 in 1250 males and 1 in 2500 females [1, 2]. The syndrome is associated with the expression of a fragile site FRAXA at X(q27.3) [3], although NTMs (normal transmitting males) and about half of the carrier females do not express the fragile site. The genetic defect responsible for the fragile X syndrome has recently been identified as the mutational expansion of an unstable CGG repeat located in the first exon of the FRM1 (fragile X mental retardation 1) gene [4–7]. Two categories of such mutations are usually distinguished. Premutations containing 60 to 230 repeats are not associated with any symptom of the disease. However, they are able to expand to full mutations consisting of more than 230 CGG copies and observed in affected individuals. The large expansion is accompanied by abnormal methylation of the 5' region of the FRM1 gene and inhibition of transcription [8]. Rarely, intragenic mutations other than expansion of the unstable CGG repeat may lead to the clinical phenotype of the syndrome [9, 10]. In order to characterize mutations responsible for fragile X syndrome we analyzed the unstable (CGG)\textsubscript{n} sequence in 18 Polish fragile X families.

MATERIALS AND METHODS

Patients. Eighty five individuals from 18 families including 28 mentally retarded males and 9 females with mental impairment were tested for the presence of mutations in the

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Abbreviations: FRM1, fragile X mental retardation; NTMs, normal transmitting males; PIC, polymorphism information content.
FMRI gene. All affected patients were classified on the basis of clinical features and expression of the fragile X(q27.3) site (FRAXA). The fragile site expression was analysed using two different induction systems [11].

**Southern blot analysis.** Genomic DNA was isolated from peripheral blood leukocytes by the method of Miller et al. [12]. Samples of 10 μg DNA were digested with 10-fold excess of appropriate restriction enzymes. The double digestion with EcoRI and methylation-sensitive enzymes Eagl or BsaHI was applied to determine both expansion range and methylation status of FMRI gene. DNA fragments were electrophoretically separated on 1% agarose gels and blotted onto positively charged nylon membranes (Boehringer Mannheim). The probe pI2 [13] or StB12.3 [14] (both having the same PstI fragment of the FMRI gene) were labeled by random primer extension method with DIG-dUTP (Boehringer Mannheim) or [α-32P]dATP and hybridized to the blots at 42°C in 50% formamide hybridization mixture or at 65°C without formamide. Color detection was carried out following manufacturer’s instruction to detect hybridization products labeled with digoxigenin. Radiolabeled hybridization products were identified by autoradiography.

**PCR analysis.** Amplification of DNA region encompassing unstable (CGG)n sequence was performed according to the method of Fu et al. [15], with minor modifications. Genomic DNA (50 ng) was amplified in a total volume of 10 μl containing BRL Taq polymerase buffer, 1 mM MgCl2, 10% DMSO, 100 μM dATP, 100 μM dTTP, 100 μM dCTP, 25 μM dGTP, 75 μM 7-deaza-dGTP, 200 nM primer c (5’TGCCTCGCTCGGGTGTTCCCTGGT-3’), 200 nM primer f (5’TAGGCCCTCCACGCCGTC-3’), 5% detergent W-1 (BRL), 1.5 μCi of [α-32P]dCTP and 0.6 U Taq DNA polymerase (BRL). After a 10 min denaturation at 94°C, samples were subjected to 30 cycles of amplification (45 s at 94°C and 7.5 min at 68°C). Radiolabeled products of amplification were separated on 6% polyacrylamide sequencing gels and identified by autoradiography.

**RESULTS**

Hybridization analysis showed that abnormal elongation of (CGG)n sequence and methylation of adjacent CpG island were the cause of disease in all families tested. The classification of detected alleles was based upon both size increase and methylation status (Table 1). In total we identified 43 full mutations and 18 premutations. In 24 individuals normal alleles only were detected.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Mental retardation</th>
<th>FRAXA expression</th>
<th>CGG expansion</th>
<th>CpG island methylation</th>
<th>Number of individuals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>-</td>
<td>-</td>
<td>small</td>
<td>-</td>
<td>13</td>
</tr>
<tr>
<td>Male</td>
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<td>-</td>
<td>large</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
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<td>+</td>
<td>-</td>
<td>large</td>
<td>+</td>
<td>1</td>
</tr>
<tr>
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<td>+</td>
<td>+</td>
<td>large</td>
<td>+</td>
<td>21</td>
</tr>
<tr>
<td>Male</td>
<td>+</td>
<td>+</td>
<td>large, small***</td>
<td>+,<strong>,</strong></td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>11</td>
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<td>-</td>
<td>small/-</td>
<td>-</td>
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<td>-</td>
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<td>large/-</td>
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<td>+</td>
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<td>8</td>
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<tr>
<td>Female</td>
<td>+</td>
<td>+</td>
<td>large, small/***</td>
<td>(+,-)/**</td>
<td>1</td>
</tr>
</tbody>
</table>

*Small expansion stands for Δ ≤ 0.5 kb, large expansion for Δ > 0.5 kb, and (-) for lack of expansion (normal allele); **(+)-methylation, (-) lack of methylation; in females only active X chromosomes were considered; ***mosaic cases.
Normal alleles

Using PCR method we estimated the number of CGG repeats in 38 normal \( \text{FRM1} \) genes. The \((\text{CGG})_n\) sequence was found to be highly polymorphic (Fig. 1). We identified 13 different alleles ranging in size from 20 to 41 CGG repeats (Fig. 2). The most frequent allele containing 29 repeats was found on 37% of normal chromosomes. Polymorphism information content (PIC) for this sequence was 0.81. In contrast to mutated alleles the normal alleles remained stable during transmission, i.e. no case of change in CGG number was found when alleles were transmitted to the offspring.

Premutations

Premutations were found in 17 fra(X) negative females and in one transmitting male. The smallest premutated allele consisted of 69 CGG copies whereas the largest one had about 200 repeats (Fig. 2). The only premutation found in male has 83 repeats. Because alleles containing more than 130 trinucleotide repeats did not amplify efficiently in PCR reaction, their repeat number was calculated from Southern blots only. Some of detected premutations were transmitted to progeny. Maternal premutations expanded to full mutations or very large premutation (about 200 repeats when maternal premutation consisted of 80 repeats) in the offspring. When paternal premutation was transmitted much smaller increase in size was observed. The premutation in daughter of the transmitting male had only 18 repeats more than original paternal premutation.

Full mutations

The large expansion of the \((\text{CGG})_n\) sequence (full mutation) was found in all affected males.

![Diagram showing normal alleles and premutations](image-url)
and in all fra(X) positive females irrespective of their mental status. The size of fully mutated alleles was ranging from 230 to about 1500 repeats (Fig. 3). Twenty one percent of full mutations in males and 47% in females were observed as single alleles, whereas the rest of them was heterogeneous (somatically unstable) (Fig. 4). In some fully mutated individuals we found also additional premutation or normal allele resulting probably from somatic instability of full mutation. Mosaicism of this kind was found in six males (21.4%) and one female (6.7%) with full mutation. All fully mutated alleles were associated with methylation of the CpG island, in contrast to premutation and normal alleles which were methylated only on inactive X chromosomes in females. When full mutations were transmitted to the next generation (only maternal transmissions of such alleles were observed) longer fully mutated alleles were always found in the offspring.

We did not detect somatic mosaicism in two "cytogenetically negative" individuals with full mutation. The male who did not express the fragile site had heterogeneous full mutation (mean mutation size = 530 repeats) whereas the "cytogenetically negative" female had single fully mutated allele containing approximately 300 repeats.

**Fig. 4. Southern blot analysis of EcoRI-BssHII digested genomic DNA hybridized to DIG-labeled StB12.3 probe.**

The samples represent males (lanes 3, 5 and 6) and females (lanes 1, 2 and 4) from fragile X families. The 2.8 kb and 5.2 kb DNA fragments correspond to normal active (unmethylated) and normal inactive (methylated) FMRI genes, respectively. The bands observed above them correspond to premutations (lanes 2 and 4) and full mutations (lanes 1, 3 and 5). Skewed X inactivation pattern observed in some females is visible in lanes 1 and 4. Fully mutated alleles in lanes 3 and 5 are heterogeneous and the single full mutation is observed in lane 1. Lane 6 corresponds to normal male (only 2.8 kb fragment corresponding to one normal X chromosome is observed).
1:1 for majority of females carrying the mutation in FRM1 gene. However, some of detected
premutations and full mutations in females were found almost exclusively on the methyl-
lated (inactive) or, in other cases, on unmethyl-
lated (active) X chromosome (Fig. 4). The
activation ratio, i.e. proportion of active normal
X (normal unmethylated/normal unmethyl-
lated + normal methylated) was very high
(>0.9) in five females with premutation and five
with full mutation, whereas in two females
with premutation and one with full mutation
the activation ratio was very low (<0.1). No
age-dependent correlation in regard to this ob-
ervation was found.

DISCUSSION

The expansion of unstable (CGG)_n sequence
in FRM1 gene is the cause of majority of cases
of Martin-Bell syndrome. The drastically in-
creased instability of this locus is observed
when the number of repeats exceeds 50-60
CGG copies. However, the high polymorphism
of normal (smaller) alleles gives evidence that
such alleles are also unstable, although to much
smaller extent than premutations or full muta-
tions. The distribution of normal alleles in Pol-
ish population is very similar to that found in
other Caucasian [15-18], as well as in Japanese
population [19]. We did not find alleles contain-
ing 24-27 repeats, while the most frequent were
alleles consisting of 28-30 CGG copies. The
presence of the secondary peak between 20 and
24 is also a characteristic feature of almost all
populations studied so far. Therefore, our re-
sults are consistent with previous reports indi-
cating that such an unusual distribution of
alleles is maintained across different popula-
tions. Jacobs et al. [17] suggested that “CGG
number even within the normal range is subject
to natural selection”.

All maternally transmitted premutations and
full mutations were elongated during the trans-
mission process and no case of contraction was
found. So the tendency to increase in size is
very strong in case of mutated alleles. This
reduce the chance to obtain a shorter allele by
children of carrier females.

The comparison of results obtained by mole-
cular analysis of (CGG)_n sequence and cyto-
genetic testing for fragile site expression leads
to conclusion that indirect analysis of muta-
tions in FRM1 gene is much more reliable di-
agnostic method, especially when carrier
diagnosis is performed. Even if only affected
individuals are considered there is a small risk
of negative result of cytogenetic analysis in
spite of the presence of full mutation. Present-
ed results indicate that about 5% of individuals
with full mutation do not express the fragile
site. The full mutations found in cytogeneti-
cally negative individuals were larger than
some full mutations observed in cytogeneti-
cally positive patients. This suggests that there
is no strict limit of repeat number determining
the fragile site expression and that other factors
may contribute to the appearance of the fragile
site.

The high incidence of mosaic cases among
individuals with full mutation should draw
our attention to extremely careful treatment of
PCR results when Southern blot analysis was
not done. In such cases the presence of PCR
product of normal length in males can not ex-
clude the possibility that such individuals are
carriers of mutation. Therefore, the PCR
method is unreliable when used as the only
diagnostic technique and it seems that its use-
fulness in routine fragile X diagnosis is rather
low.

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