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QUARTERLY

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

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The unstable DNA sequence in the *FMR1* 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 *FMR1* (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 *FMR1* 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 $(CGG)_n$ 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: FMR1, fragile X mental retardation; NTMs, normal transmitting males; PIC, polymorphism information content.

FMR1 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 *Eco*RI and methylation-sensitive enzymes *Eag*I or *Bss*HII was applied to determine both expansion range and methylation status of *FMR1* gene. DNA fragments were electrophoretically separated on 1% agarose gels and blotted onto positively charged nylon membranes (Boehringer Mannheim). The probe pP2 [13] or StB12.3 [14] (both having the same *Pst*I fragment of the *FMR1* gene) were labeled by random primer extension method with DIG-dUTP (Boehringer Mannheim) or [α-^{32}P]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 $MgCl_2$, 10% DMSO, 100 μM dATP, 100 μM dTTP, 100 μM dCTP, 25 μM dGTP, 75 μM 7-deaza-dGTP, 200 nM primer c (5'-GCT CAG CTC CGT TTC GGT TTC ACT TCC CGT-3'), 200 nM primer f (5'-AGC CCC GCA CTT CCA CCA CCA GCT CCT CCA-3'), 5% detergent W-1 (BRL), 1.5 μCi of [α-^{32}P]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 1

Results of analysis of unstable $(CGG)_n$ sequence in 85 individuals from fragile X families

Sex	Mental retardation	FRAXA expression	CGG expansion*	CpG island methylation**	Number of individuals
Male	–	–	–	–	13
Male	–	–	small	–	1
Male	+	–	large	+	1
Male	+	+	large	+	21
Male	+	+	large, small***	+, –***	4
Male	+	+	large, –***	+, –***	2
Female	–	–	–/–	–/–	11
Female	–	–	small/–	–/–	17
Female	–	–	large/–	+/–	1
Female	–	+	large/–	+/–	5
Female	+	+	large/–	+/–	8
Female	+	+	large, small/–***	(+,–)/–***	1

*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.

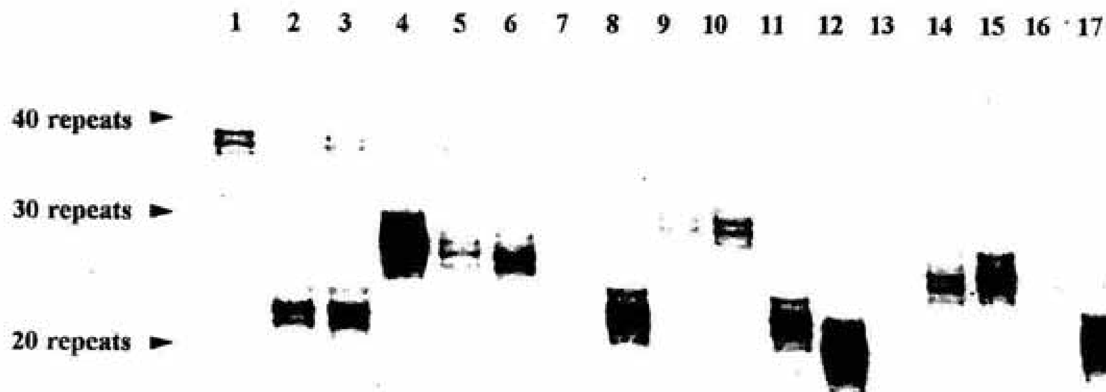

Fig. 1. PCR amplification of FMR1 gene region containing $(CGG)_n$ sequence.
Polymorphism of $(CGG)_n$ sequence in *FMR1* gene is illustrated by different length of PCR products corresponding to alleles found on normal X chromosomes. Fully mutated alleles found in affected males did not amplify in PCR reaction (lanes 7, 13 and 16). Lanes 3, 4 and 5 correspond to normal females, whereas normal males are represented by lanes 1, 2, 6, 8–12, 14, 15 and 17.

Normal alleles

Using PCR method we estimated the number of CGG repeats in 38 normal *FMR1* genes. The $(CGG)_n$ 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 $(CGG)_n$ sequence (full mutation) was found in all affected males

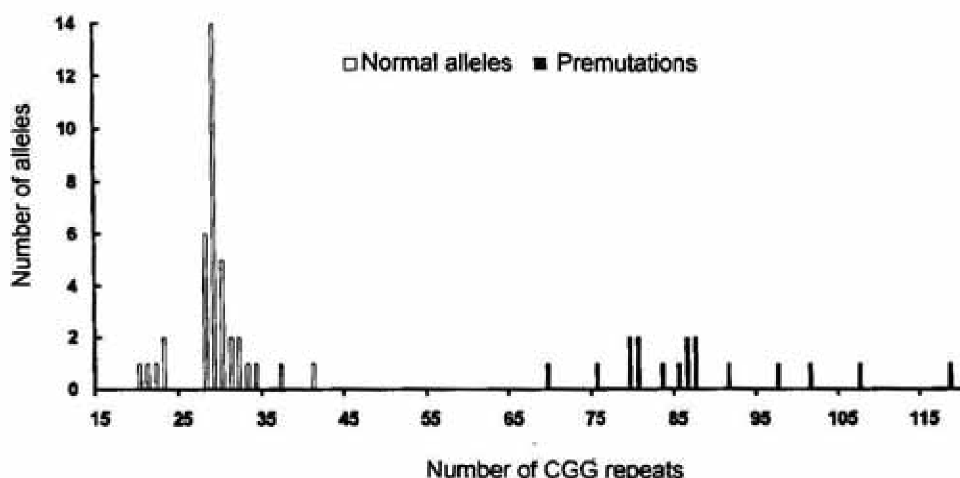

Fig. 2. The difference between length of normal and premutated FMR1 genes.
(The longest premutation containing about 200 CGG repeats which did not amplify in PCR reaction is not included).

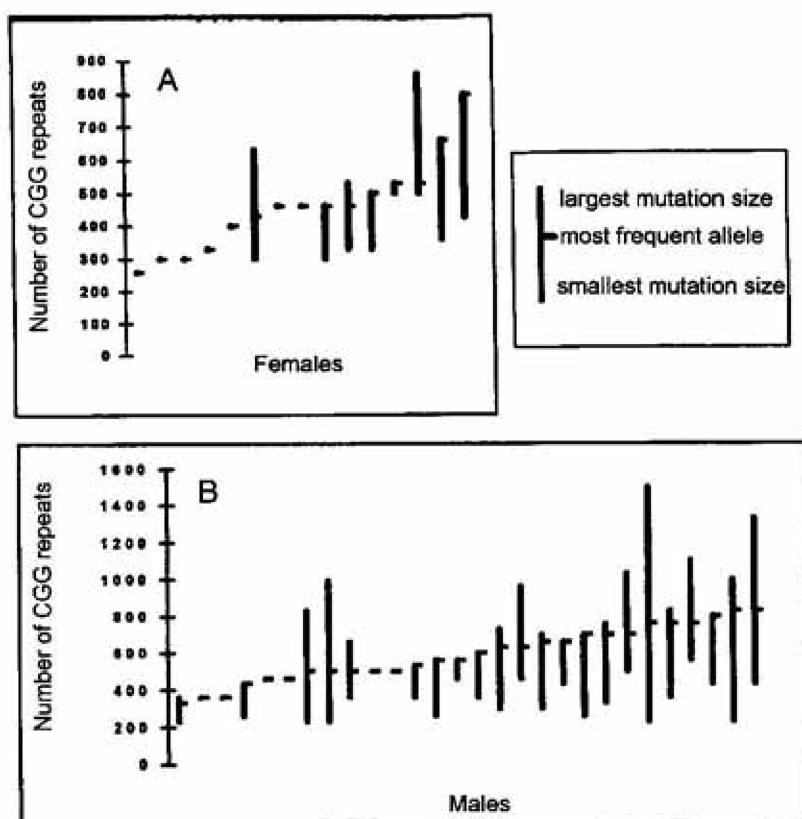

Fig. 3. The number of CGG repeats in full mutations found in 15 females (A) and 28 males (B) from fragile X families.

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.

Mutations and activation ratio

The proportion of mutated alleles located on active and inactive X chromosome was nearly

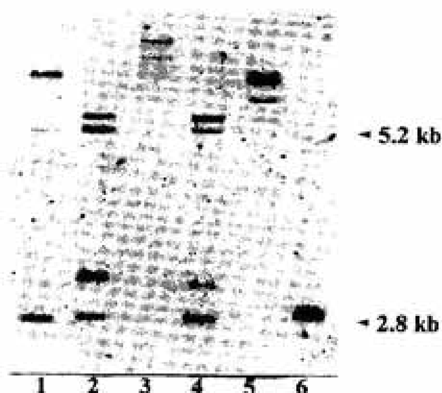

Fig. 4. Southern blot analysis of EcoRI+BssHII digested genomic DNA hybridized to DIG-labeled StB12.3 probe.

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) *FMR1* 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 *FMR1* gene. However, some of detected premutations and full mutations in females were found almost exclusively on the methylated (inactive) or, in other cases, on unmethylated (active) X chromosome (Fig. 4). The activation ratio, i.e. proportion of active normal X (normal unmethylated/normal unmethylated + 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 observation was found.

DISCUSSION

The expansion of unstable $(CGG)_n$ sequence in *FMR1* gene is the cause of majority of cases of Martin-Bell syndrome. The drastically increased 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 mutations. The distribution of normal alleles in Polish population is very similar to that found in other Caucasian [15–18], as well as in Japanese population [19]. We did not find alleles containing 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 results are consistent with previous reports indicating that such an unusual distribution of alleles is maintained across different populations. 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 transmission 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 molecular analysis of $(CGG)_n$ sequence and cytogenetic testing for fragile site expression leads to conclusion that indirect analysis of mutations in *FMR1* gene is much more reliable diagnostic 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. Presented results indicate that about 5% of individuals with full mutation do not express the fragile site. The full mutations found in cytogenetically negative individuals were larger than some full mutations observed in cytogenetically 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 appearence 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 exclude 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 usefulness in routine fragile X diagnosis is rather low.

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