

## MOLECULAR ANALYSIS OF FRAGILE X SYNDROME IN ANTALYA PROVINCE

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### ABSTRACT

**BACKGROUND:** Detection of the (CGG)<sub>n</sub> repeats in the FMR1 gene that cause the fragile X syndrome (FXS), has become a milestone for phenotype-genotype correlation in FXS. **AIMS:** To screen the FMR1 gene CGG repeats in index cases with FXS and their family members in the Antalya Province. **SETTING AND DESIGN:** This study was prospectively conducted between January 2000 and March 2005 in Department of Medical Biology and Genetics, Faculty of Medicine, Akdeniz University, Antalya. **MATERIALS AND METHODS:** A series of 132 cases from three hospitals in Antalya Province were studied. All cases were molecularly screened using non-radioactive Expand Long PCR method that was confirmed by Southern blotting. **RESULTS:** Seventeen out of 132 cases were found to have a full mutation, including three that were mosaic for premutations/full mutations. Of the 132 cases, eight were found to have the premutation size of the CGG repeats. The remaining 107 cases were identified as normal. **CONCLUSIONS:** Due to premature ovarian failure and Fragile X premutation Tremor/Ataxia Syndrome related with the premutation, the detection of the premutation will provide valuable information both for clinical follow-up and genetic counseling. In conclusion, our data suggest that expansion of CGG repeats in the FMR1 gene can be analyzed by Expand Long PCR, an efficient and non-radioactive method that can be used to monitor the expansion of premutation to full mutation, which would eventually lead to reduce the FXS prevalence.

**KEY WORDS:** Antalya, CGG repeats, expand long PCR, fragile X syndrome, FMR1 gene

The fragile X syndrome (FXS) is the most common inherited form of mental retardation. The molecular basis of the syndrome is usually an expansion of a repetitive CGG triplet sequence located in the 5'-untranslated region (5'-UTR) of the *FMR1* gene.<sup>[1]</sup> Rarely, FXS results from other molecular alterations (such as point mutations or deletions) within the *FMR1* gene.<sup>[2]</sup> The normal number of (CGG)<sub>n</sub>

repeats is polymorphic and varies from 6 to 50.<sup>[3]</sup> When the expanded repeat numbers range from 50 to 200, the allele becomes unstable in the intergenerational transmissions and is termed as premutation.<sup>[4]</sup> The expansion of the number of repeats above a threshold of approximately 200 repeats, results in hypermethylation of the FMR1 promotor region and a lack of gene expression. This allele is

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termed as full mutation. Alleles with >200 triplets are highly unstable during somatic development and inheritance. Alleles that are called 'Grey zone' (approximately 50-60 triplets) are stable in somatic development, yet their stability in transmission is unpredictable.<sup>[5]</sup> Mosaicism, methylation and size mosaicisms, is also frequent in the FXS and closely associated with clinical variability. Although the frequency of FXS has been estimated to be approximately 1 in 4000 males and 1 in 6000 females,<sup>[6,7]</sup> the frequency of premutation in the general population is ~1 in 259 females and 1 in 813 males.<sup>[8,9]</sup> The premutation in the *FMR1* gene has been reported to be associated with premature ovarian failure.<sup>[10]</sup> On the other hand, recently, Jacquemont et al<sup>[11]</sup> reported fragile X premutation tremor/ataxia syndrome in a series of 26 patients, all >50 years of age, who were carriers of the fragile X premutation, and affected by a multisystem, progressive neurological disorder. Due to increasing clinical importance and high prevalence, the identification of the premutation carriers, which can be detected only by direct molecular analysis is very important.<sup>[12]</sup> Here, we aimed to detect the mutations by using an efficient and reliable PCR protocol which enables the amplification of normal, premutated and full mutated alleles in index cases and in members of the families with FXS, and to give effective genetic counseling in the Antalya Province.

## MATERIALS AND METHODS

We prospectively analyzed DNA samples isolated from the peripheral blood of 95 males and two females who had been clinically diagnosed as having FXS according to Hagerman's Checklist (see [http://](http://www.fragilex.org/html/checklist.htm)

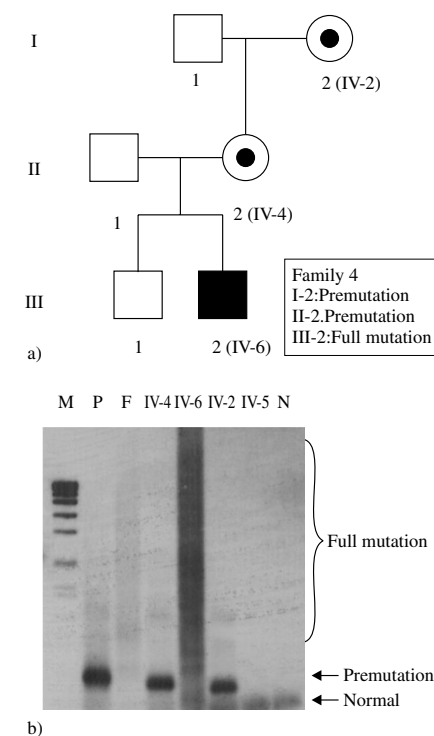
[www.fragilex.org/html/checklist.htm](http://www.fragilex.org/html/checklist.htm)) and 42 members in the seven families of those probands we detected to have FXS by molecular analysis (132 cases in total) between January 2000 and March 2005. Final examination and scoring of all cases was performed by two different professional physicians from the Departments of Pediatric Neurology and Clinical Genetics, School of Medicine, Akdeniz University. Index cases with a score of 13 or higher for the Hagerman's Checklist were included in this study. Signed written consents were taken from all subjects or their parents. This study was also approved by the ethics committee of the Faculty of Medicine, Akdeniz University. The amplification primers were Fra X-Forward: 5'-ACC TCT gCA gAA ATg ggC gTT CTg gCC CTC-3' and Fra X-Reverse: 5'-CTA gCg CCg ggA gCC CgC CCC CgA gAg gT-3'. Amplification of the CGG repeat region of normal, premutated and full mutated alleles in the *FMR1* gene was carried out by PCR reaction with a total volume of 25 µl, containing 200 ng of genomic DNA, 300 nM of each primer, 5% of DMSO, 5% glycerol, Expand Long PCR buffer 1,350 nM each of dATP, dCTP, dTTP, 7-deaza dGTP, and 1 U Expand Long PCR Enzyme Mix. Denaturation was performed at 95°C for 3 min. The samples were then subjected to 10 cycles of amplification (95°C for 30 s, 54°C for 30 s, 68°C for 8 min) in a thermocycler (Techne Genius). The reaction was stopped on ice, following the last denaturation for further addition of an enzyme (1 U), and continued for 30 more cycles (95°C for 30 s, 52°C for 30 s, 68°C for 8 min). The elongation time was extended for 5 sec. after each cycle in the last 30 cycles. The reaction was finalized by keeping the reaction at 68°C for 18 min, then

at 4°C. The PCR product was run on 1.2% agarose gel with DIG labeled molecular weight marker (MW, VII; Boehringer Mannheim) and transferred to a positively charged nylon membrane (Boehringer Mannheim) following denaturation and neutralization procedures. DNA was cross-linked to the membrane by UV light. Blot was prehybridized for 1 h then hybridized with DIG labeled 5'-(CGG)<sub>5</sub>-3' probe in DIG Hybridization Buffer at 60°C shaking in a hybridization oven for 16 h. After washing off the membrane, the bands were detected according to the procedures described in the DIG-Luminescent Detection Kit (Boehringer Mannheim). After the incubation of CSPD at 37°C for 15 min, the membrane was exposed to Hyper ECL film (Amersham) for 10-15 min.<sup>[13,14]</sup> Normal, premutation and full mutation DNA samples were ran in each study as a control and premutations and full mutations were confirmed by Southern blotting during the initial studies.<sup>[15]</sup>

## RESULTS

Ten of the 97 index cases were found to have full mutation by Expand Long PCR-based method. Two out of ten had both a premutation and full mutation band. A smear pattern was detected in the other eight patients. The remaining 87 cases were found to have normal sizes of the CGG repeats. Excluding seven index cases, 15 of 42 members in seven families were found to have an increase of the CGG repeats. Seven and eight of these were carriers of full mutations and premutations, respectively. One out of seven full mutation carriers had both premutation and full mutation sized bands. The remaining 20 members of the families had normal size of the CGG repeats.

The results of the *FMR1* (CGG)<sub>n</sub> mutation analysis in seven families are shown in Table 1. Figures 1A and 1B show the step-increasing transmission of the CGG repeats in one of the families. The premutation was transmitted with a little expansion in the premutation range from female (grandmother) (I-2) to female (mother) (II-2), then expanded to full mutation in the child (grandson) (III-2) in Family 4. Two of the mosaic patterns were transmitted from a mother with full mutation in Families 1 and 3, respectively. The remaining one, from Family 2, was observed to be transmitted by a mother with premutation (Table 1).



**Figure 1:** A family with FXS showing the inheritance of the premutation to full mutation in the generations. (A) Pedigree, (B) index case (4-6) with full mutation, his mother (4-4) with premutation, his grandmother (4-2) with premutation, and his brother (4-5) normal in Family 4. M: DIG labeled marker; N: normal control; P: premutation control; F: full mutation control

**Table 1: The results of *FMR1* gene mutation analysis in 42 members in seven families with FRXS**

Family/case	<i>FMR1</i> mutation status	Approximate (CGG) <sub>n</sub>
Family 1		
Grandfather	Normal	40
Grandmother	Premutation	85
Father	Normal	35
Mother	Full mutation	40 and 200<
Aunt	Full mutation	40 and 200<
Brother	Premutation/ full mutation	120 and 200<
Sister	Normal	35 and 40
Index case†	Full mutation	200<
Family 2		
Grandfather	Normal	30
Grandmother	Premutation	30 and 80
Father	Normal	25
Mother	Premutation	30 and 100
Sister	Normal	25 and 30
Sister	Normal	25 and 30
Index case†	Premutation/ full mutation	110 and 200<
Family 3		
Father	Normal	30
Mother	Full mutation	40 and 200<
Index case†	Premutation/ full mutation	90 and 200<
Brother	Full mutation	200<
Family 4		
Grandfather	Normal	35
Grandmother	Premutation	35 and 70
Father	Normal	25
Mother	Premutation	35 and 75
Brother	Normal	35
Index case†	Full mutation	200<
Family 5		
Father	Normal	30
Mother	Premutation	30 and 80
Aunt	Premutation	30 and 80
Aunt's husband	Normal	35
Sister	Normal	30 and 30
Index case†	Full mutation	200<
Cousin (female)	Full mutation	30 and 200<
Cousin (male)	Normal	30
Family 6		
Father	Normal	25
Mother	Full mutation	35 and 200<
Index case†	Full mutation	200<
Family 7		
Father	Normal	25
Mother	Premutation	25 and 90
Aunt	Normal	25 and 40
Aunt	Normal	25 and 40
Aunt	Normal	25 and 40
Index case†	Full mutation	200<

†All index cases are males.

## DISCUSSION

In our laboratory, the fragile X mutations had been analyzed using both cytogenetic and genomic Southern blotting (with StB12.3 probe and double digestion) until 2001.<sup>[15]</sup> We also started to analyze simultaneously the fragile X mutations using the Expand Long PCR protocol in probands and their families. In the present study, using this protocol, we found full mutation in 10 of the 97 index cases who have a score of 13 or higher according to Hagerman's Checklist. Seven full mutations and eight premutation carriers were also found among 42 members of their families. The DNA analysis of families with apparently isolated cases of FXS indicates that even distant relatives are at risk to be carriers. For this reason, it is imperative that members of these families be offered *FMR1* DNA studies. Our results suggest that this technique is useful to detect the intergenerational instability of CGG repeats in the *FMR1* gene in index cases with FXS and in their families. However, in general, the only limitation of this technique is the smear appearance of full mutations (Figure 1B). Although full mutations were detected mostly as a smear, the three patients who had both full mutation and premutation-sized fragments were also identified. These band patterns may or may not reflect the true heterogeneity of genomic DNA isolated from peripheral blood. Also, these band patterns observed in peripheral blood may or may not reflect the true heterogeneity of brain and other tissues. Two of the mosaic patients were index cases with mental retardation (Family 2-7 and Family 3-3 in Table 1). The other one was a member of Family 1 with mild mental retardation (Family 1-6 in Table 1). This clinical

difference may be due to the ratio of the premutation and full mutation in the brain neurons. Also, these findings show that all family members with or without clinical features should be screened in the families with FXS.

An issue for mothers with premutation alleles is the possibility of transmitting a full mutation to her offspring, as well as premature ovarian failure,<sup>[16]</sup> which we could not study in the women with premutation. In 2001, a new condition was reported in some adult males with premutation, it was called Fragile X Tremor ataxia syndrome (FXTAS).<sup>[11,17,18]</sup> These findings raised additional issues that should be considered by genetic counselors. In our study, no males with premutation were found, but there were three mosaic males. Due to no well-described clinical features and the high prevalence of premutation carriers, the premutation carriers must be diagnosed by an efficient molecular technique such as the PCR-based method that can identify accurately the CGG repeat size, and that we used in the present study. Two male fetuses were prenatally diagnosed with this method, one had a normal allele and the other carried a full mutation. These results were confirmed by Southern blot using *EagI/EcoRI* double digestions. We started the screening of the general population for the prevention of the birth of a child with FXS or beta-thalassemia in Antalya. The index cases that were found to be normal for the CGG repeats will be screened to detect other molecular alterations (such as point mutations and deletions) in the *FMR1* gene. We believe that the recording and following of families with FXS will provide prenatal diagnosis and the usage of a possible treatment opportunity in the future. As a result,

our data show that all the members with/without clinical findings of FXS in the families with FXS should be screened by the PCR-based method to follow the transmission of the CGG repeats and to give correct genetic counseling to families.

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