FMR1 Gene Mutation Analysis and CGG Repeat Number Distribution from a Single Center

Tek Bir Merkezden FMR1 Gen Mutasyon Analizi ve CGG Tekrar Sayısı Dağılımı

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ABSTRACT

Background: Mutation occurring in fragile X mental retardation 1 (*FMR1*) gene is acknowledged as the most common cause for X chromosome linked intellectual disability/mental retardation (XLID/XLMR). This gene harbors unstable CGG triplet repeats within its 5'UTR (untranslated region). Loss of function of the *FMR1*, which is mostly due to the hypermethylation of the CpG islands on its promoter region, causes fragile X syndrome (FXS). Displaying different frequencies, the FXS is a common phenomenon all over the world, the studies focus mostly on the Caucasian population.
Purpose: We aimed to reveal the CGG repeat number distribution and the mutation profile of the *FMR1* gene in clinically pre-diagnosed FXS patients and in family members of the patients who were diagnosed as full mutation.
Methods: We evaluated the copy number of the CGG triplets in 767 FXS patients and their family members in Antalya province by employing fragment analysis molecular technique. We also assessed, by segregation analysis, whether there is unusual genetic transmission pattern of CGGs.
Results: The molecular analysis shows the most common copy numbers of CGGs are thirty, twenty-nine and thirty-one. Present study is the first report concerning Antalya city of Turkey about the frequencies of the normal CGG repeats number, grey-zone, pre-mutation and full mutations, we updated our molecular test results with two unusual transmittance patterns of the CGG repeats.
Conclusion: Since the potential of CGG repeat properties may cause differential intergenerational transmission patterns, its' population specific evaluation can contribute to provide a better genetic diagnosis and genetic counseling services for the related clinical entities.

Key words: *FMR1*, CGG triplets, pre-mutation, UTR, Fragile X Syndrome.

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ÖZET

Amaç: Frajil X mental retardasyon 1 (*FMR1*) genindeki mutasyonlar, X kromozumuna bağlı entellektüel yetersizlik/zeka geriliği (XLID/XLMR)'nin en yaygın sebebi olarak bilinmektedir. Bu genin 5' kodlanmayan bölgesinde, kararsız CGG üçlü nükleotid tekrarları bulunmaktadır. Büyük oranda, CpG adacıklarının hipermetilasyonu sebebiyle ortaya çıkan *FMR1*'in fonksiyon kaybı, Frajil X sendromuna (FXS) neden olmaktadır. Literatürde daha çok Kafkas populasyonuna odaklanılmış olsa da farklı FXS frekansları bildirilmiştir. Çalışmamızda klinik olarak FXS ön tanısı almış bireylerin ve ailelerinin *FMR1* geni mutasyon profilleri ile CGG tekrar sayılarının dağılımlarını ortaya çıkarmayı amaçladık.
Yöntem: Antalya'daki, aile üyeleriyle birlikte toplam767 bireyin CGG tekrar sayısını fragment analizi yöntemiyle ortaya çıkardık. Ayrıca CGG tekrarlarının bir sonraki nesile aktarılmasında beklenmeyen bir patern olup olmadığını da inceledik.
Bulgular: Moleküler analiz sonucu en sık görülen CGG sayılarını otuz, yitmi dokuz ve otuz bir olarak hesapladık. Bu çalışmada CGG tekrarları normalin dışında, sınırda, premutasyonlu ve/veya full mutasyonlu bireylerin ilk defa bildirirken, beklenmeyen kalıtsal patern gösteren 2 ailede segregasyon çalışmalarıyla verilerimizi güncelledik.
Sonuç: Kuşaklar arası farklı kalıtım modellerine neden olabilme potansiyeli nedeniyle; CGG tekrarlarının, populasyona özgü değerlendirilmesi, ilgili klinik durumlar için daha iyi bir genetik tanı ve danışmanlık hizmeti sağlamada katkıda bulunabilir.

Anahtar sözcükler*: FMR1*, CGG tekrarları, premutasyon, UTR, Frajil X Sendromu.

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INTRODUCTION

The gene *FMR1*, which is responsible for the fragile X syndrome (OMIM, #300624), is located on FRAXA locus, Xq27.3, and spans 17 exons (1). 5′ UTR of *FMR1* has repetitive and polymorphic CGG triplet sequences varying from 5 to 200 tandem copies, and more than 200 in reported cases for FXS. There are four levels of the CGG expansion in promoter region, $(CGG)_{5-44}$, $(CGG)_{45-54}$, $(CGG)_{55-200}$, and $(CGG)_{>200}$, which are considered as normal, grey-zone/intermediate, pre-mutation, and full mutation alleles, respectively (2-3). The penetrance and severity index for this syndrome is different between males and females because of hemizygosity, being higher in males due to genetic anticipation and X chromosome inactivation (4-7). According to the Hagerman checklist, a long face, large protruding ears, hyper-extensible finger joints, hypertonia, and intellectual disability constitute some of the most prominent characteristic features of the FXS (8).

Highly expanded CGG repeats on promoter region of *FMR1* tend to be modified by the hypermethylation mechanism. Methylation or hypermethylation of the CpG island within the increased CGG triplets represents not only parts of the normal X inactivation process in females but also can be seen in affected males and is implicated in female meiosis (9). The stability of the CGG repeats is highly variable as well as affected by the AGG interruptions. The presence of one or more AGG breaks within the CGG repeats, as stated above, ameliorates this hazardous parental inheritance to offspring (10-12).

In our laboratories, since 2000, the molecular analyses of the FXS was evaluated by different techniques. In our previous study, concerning the period between 2000-2005 and also focused on Antalya city, we aimed to screen the CGG repeats in 132 cases with family members with non-radioactive expand long PCR technique and we found the incidence of full mutation state to be of 12.8 percent. 87 out of 97 index cases had been calculated as normal CGG copies in *FMR1*'s 5′ UTR (13). After having discovered new genes in cases with mental retardation, such as *aristaless* homeobox gene, *ARX* (OMIM, #300419) in the last decade, we also identified, for the first time in Turkey, the most common *ARX* mutation (0.2%) in a family with borderline non-syndromic intellectual disability (14).

Lastly, several studies on different types of human population concerning normal CGG triplet distribution have informed us that there are different profiles in quantities of both AGG interruption and CGG copy numbers (15-20). In the present study, given the intercontinental geographic location of our country between Asia and Europe, we aimed to identify the CGG distribution of the Turkish population and update the recent outcomes pertaining to FXS.

METHODS

Since 2000, in our laboratory, the diagnosis of the FXS was performed both by cytogenetically and southern blotting methods.

With regard to the gross development in molecular genetic methods, we have attempted to investigate and diagnose the FXS by using the fragment analysis only, which is dated from 2008. Between July-2008, and December-2014, we have included in our research a number of 767 people, who were pre-diagnosed with FXS by different doctors from the Department of Paediatric Neurology and the Department of Clinical Genetics belonging to the School of Medicine at Akdeniz University. After the gDNA isolation (PureLink™ Genomic DNA Kit, Invitrogen) from peripheral blood samples, we have performed the PCR conditions following the instructions of the Abbott Fragile X Kit (Abbott, Illinois, USA): thirteen µl High GC Buffer, 0.8 µl *FMR1* primers, 0.6 µl gender primers, 1.2 µl Enzyme Mix, 1.4 ul ddH_2O and 20 ng gDNA in 20 µl total reaction volume with 10 seconds at 98.5 °C, 60 seconds at 58°C, six minutes at 75°C (15 cycles), 10 seconds at 98.5 °C, 60 seconds at 56°C, six minutes at 75°C (15 cycles). Two microliters of PCR product were added to 3 µl of Cleanup Enzyme Mix (Abbott, Illinois, USA) and consequently incubated for ten minutes at 75 °C. 10 µl formamide and 3 µl of ROX™-1000 Size Standart (Abbott, Illinois, USA) were added to 5 µl purified PCR product and then denaturated for sixty seconds at 93 °C.

By using the fragment analysis (ABI 3730 Genetic Analyzer), we have calculated the copy number of CGG triplets in 5′ UTR of *FMR1*. In order to measure the CGG repeat correctly, we used a common calculation formula in Abbot FRX assay protocol. The local ethics committee approved our study with *2017-KAEK-189_2021.05.26_01* protocol code. Furthermore, our study was conducted in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. We performed basic statistical methods by using PASW-Statistics 18 and Microsoft Office Excel 2010 programs for the evaluation of powerful cumulative data of the participants.

RESULTS

Concerning the origin of the index cases, Antalya, the city in which the present study has been conducted, amounts to 56%, with Muğla and Konya provinces placed in second position with 12% each.845 alleles displayed CGG_{5-44} repeats and were grouped in normal CGG distribution, whereas 31 index cases and parents were evaluated as pre-mutation carriers. Also, 12 individuals were placed into the grey zone group. We also found 34 index patients with full mutation, which means they have more than 200 copies of CGG repeat in *FMR1* gene.

Among the 845 normal CGG allele carriers, we have identified the most common alleles to be in the order of $(CGG)_{30}$, $(CGG)_{29}$, and $(CGG)_{31}$ (Figure 1). All types of allele sizes were obtained with different times from 11 to 44 repeats in normal range. We detected a shift on predominant allele size from 29 to 30 for the last two years without an obvious reason, as it can be seen in table 1. In our pre-mutation group, the highest CGG copy number was 141 repeats and it was found in an index female. Both full mutation and normal copy of the CGG repeats were detected for 5 times, whereas both pre-mutation and normal allele was of 19 times in females because of heterozygosity. The shortest CGG repeat number, which is 11, was detected in 2008 in a four-year old male child.

Table 1: Descriptive statistics of CGG repeat number in index cases and their family members (M:Male, F:Female)

	2008 (*n=34*) M:30/F:4	**2009 (*n=93*) M:80/F:13**	**2010 (*n=82*) M:71/F:11**	**2011 (*n=108*) M:99/F:9**	**2012 (*n=106*) M:82/F:24**	**2013 (*n=164*) M:126/F:38**	**2014 (*n=180*) M:141/F:39**
Mean	31	29	29	30	33	32	31
Median	30	29	29	29	29	30	30
Mode	29	29	29	29	29	30	30
Min	11	13	15	19	20	12	16
Max	70	107	89	108	139	141	95

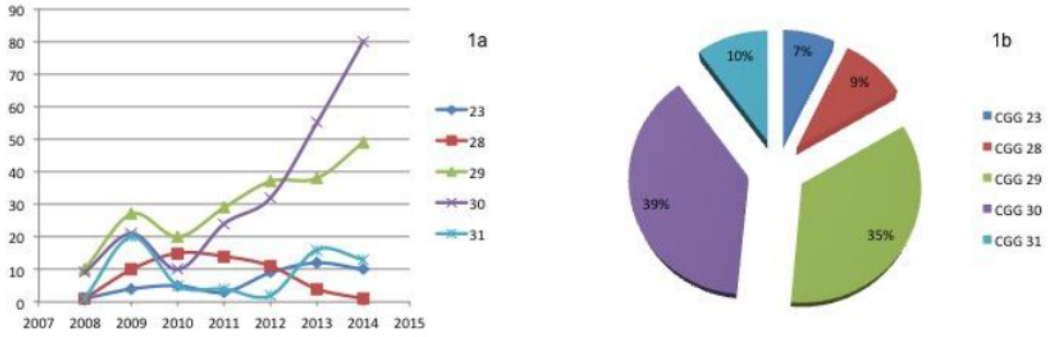

Figure 1: Distribution of the most common repeat numbers of CGG tri-nucleotides.
From 2008 to 2014 (1a) the number of $(CGG)_{30}$ and $(CGG)_{29}$ were seen as the most common alleles, respectively. 1b:Frequencies of the most common alleles in normal range.

According to the present study, the prevalence of full mutation in FXS is of 4.1% in index cases and of 5.1% along with family members. The pre-mutation frequency in both cases was calculated as 1.7% and 4%, respectively. The medical histories of the index cases and their families informed us that apart from the intellectual disability, there were some other clinical manifestations such as epilepsy, autism, tremor/ataxia, and ovarian insufficiency occurring separately or together. All of the cases were evaluated as FXS firstly and sent Medical Biology and Genetic Department in order to find if there is CGG repeat number expansion. Having found any expansion warranted, segregation analysis and detailed anamnesis showed us whether fragile X-associated tremor/ataxia syndrome (FXTAS, OMIM #300623), The premature ovarian insufficiency (POI, OMIM #311360) and etc. were accompanied or not.

The family relations based on the anamneses reports revealed that there were four consanguineous marriages out of 34 index cases (11.7%). The families of 24 index cases were informed about the inheritance of the expanded CGG three-nucleotide repeats. Unfortunately, no data could have been obtained about the rest of the families so far (Table 2). We also detected an interesting family inheritance, with 61 CGG repeat pre-mutation allele did not expand in a male offspring. Furthermore, only in one case (Index 27, Table 2), dated from 2012, a different mosaic expansion pattern was detected for a male foetus, whose mother was in a 17-week gestational pregnancy period when she underwent invasive prenatal diagnosis known as amniocentesis. In the cells of both the mother and male foetus, we observed similar 51 CGG repeats, and the mother also carried another 36 CGG repeats heterozygously (Figure 2). The pregnancy was terminated following the mother's declaration.

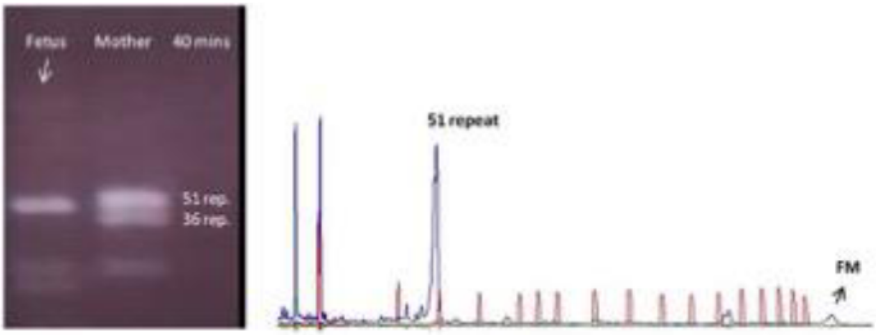

Figure 2: CGG repeat analyses of index 27 and mother: On the left panel we see FM (full mutation) and 51 repeats of CGG in index 27 and his mother. After electrophoresis, 51/36 heterozygous band pattern was observed exactly in mother's line and FM band showed in a pale shape in foetus's line. On the right panel it is very clear that index case has 51/FM allele at the same time in a mosaic status.

Table 2:CGG repeat number analysis in the families of index cases with full mutation.

Case/Family	CGG repeat status	Case/Family	CGG repeat status
index 1	FM	**index 18**	FM
Mother	70/31	**index 19**	FM
index 2	FM	Mother	84/38
Twin brother	FM	**index 20**	FM
Mother	76/26	**index 21 (foetus)**	FM/30
Father	29	Mother	108/28
Foetus	FM	Father	30
index 3	FM	**index 22**	FM
index 4	FM	**index 23**	FM/28
Mother	67/23	Mother	139/30
Sister	106/33	Brother	30
index 5	FM	**index 24**	FM
Mother	107/40	Brother	**FM**
Brother	107	Mother	FM/30
index 6	FM	**index 25**	FM
Sister	39/30	Brother	FM
index 7	FM	**index 26**	FM
index 8	FM	**index 27 (foetus)**	FM/51
index 9	FM	Mother	51/36
Mother	FM/29	Father	*
index 10	FM	**index 28**	FM
index 11	FM	Mother	101/30
Mother	89/29	**index 29**	FM
index 12	FM	Mother	72/23
Mother	77/22	**index 30**	FM
Brother	FM	Mother	86/30
index 13 (foetus)	FM	**index 31**	FM
Mother	79/29	Mother	95/30
index 14	FM	**index 32**	FM
Mother	83/30	**index 33**	FM/24
index 15	FM	Mother	FM/30
index 16	FM	**index 34**	FM
index 17	FM	Mother	FM/30
Mother	FM/102	Father	30

* indicates that there is no informative data about father of index 27. As far as we know parents are not in a marriage relationship after they got divorced.

The frequencies of epilepsy, autism, tremor, and ovarian insufficiency were calculated to be of 5.2%, 2.5%, 1.6%, and 0.5%, respectively (Table 3). It shows that among our patients with intellectual disabilities, epilepsy is the most common phenotype.

Table 3: Descriptive statistics of CGG repeat number in different phenotypes.

	Epilepsy (*n=40*)	Autism (*n=20*)	FXTAS*/Tremor (*n=11*)	POI# (*n=4*)
Mean	33	28	29	36
Median	30	29	30	30
Mode	30	29	30	29
Min	22	19	20	21
Max	58	>200	37	83

*FXTAS, Fragile X-associated tremor/ataxia syndrome #POI, Premature ovarian insufficiency

Regarding the epilepsy group, only 3 cases were found to display pre-mutation allele size of less than 60 repeats; and 1 case, evaluated in grey-zone group, showed 53 repeats. Only one female carried grey-zone allele with 48 and 29 repeats heterozygously in autism group. In total, 17 patients were grouped in grey-zone.

POI is defined as the beginning of menopause before the age of 40; it has been related clinically as significant when CGG number exceeds 79 repeats (21). In our small numbered POI group, we found an 83/29 CGG heterozygous repeat pattern in a female. Detailed clinical re-examination and genetic counselling in this case can be achieved in terms of further follow-up studies. None of the other 3 POI pre-diagnosed female had a normal copy number of CGG repeats. In our FXTAS group with 11 patients, neither the male nor the female participants had a pre-mutation.

DISCUSSION

In the present study, we aimed primarily to reveal the normal range of CGG repeat number on 5′ UTR in *FMR1* gene of FXS pre-diagnosed patients, which would be the first report on the genetic features of normal range sized CGG repeats in literature for Turkey.

Relying on two detailed and well-appreciated recovering studies, the dominant allele size in different populations displays some small differences, but on the whole it constitutes 30 repeats for the population of the U.S.A (of both African and European origin), Brazil, Chile, United Kingdom, Spain, Estonia, Croatia, Finland, and Ghana (22-23). The dominant allele size for the Asian population is 29 repeats (15,20-24). Only Mexicans (Mestizos origin) and Japanese display a different CGG size with 32 and 27 repeats, respectively (25-26). The present study conducted in Turkey, with its territory located between Asia and Europe, reveals that the pre-dominant allele sizes are 30 and 29 repeats with 39% and 35%, respectively (Figure 1).

We also intended to update the new incidence of the FXS for the last seven years with quantifiable numbers using advanced molecular methods rather than the southern blotting, as we employed until 2005 (13). We found the frequency of full mutation in FXS of the index cases to be of 4.1%. When considering other family members, the pre-mutation and grey-zone frequencies are of 4% and 2.2%, respectively. The decrease from 12.8% to 4.1% in full mutation frequency within 10 years may be related to both the efficient prenatal genetic consultation and the increase in number of the genetics centres around the country. As it can be seen in table 2, there are only 4 affected children in families numbered 2, 12, 24, and 25, which suggests an increase in the public awareness concerning the genetic factors.

Among the factors causing the instability of the CGG repeat, the two major ones are the pre-mutation allele size and the AGG interruption status. The AGG interspersion within CGG repeats is likely to block the harmful expansion of the *FMR1* allele. In normal size (5-44), the most common two AGG haplotypes are indicated as $(CGG)_9+(CGG)_9+(CGG)_9$ and $(CGG)_{10}+(CGG)_9+(CGG)_9$ within nine different world populations (13,26). We have not determined what is the Turkish type of the AGG interspersion in any normal or pre-mutation group and, actually, no Turkish study on this matter has been reported yet, although it is planned to be conducted in the future.

Also, we did not analyse any other deletional type or deleterious intragenic mutations causing FXS without any expansion of CGG repeats which are defined in very low frequencies (27-29).

It has been hypothesized and confirmed that if CGG repeat size exceeds 54 without any AGG interspersion, then the expansion to full mutation is seen on children (30). We detected compatible expansion pattern from pre-mutation to full mutation for all the families included in our work, except two cases; index 27, mosaic for the full mutation and normal alleles, and the other case who had 61 CGG repeats. Nine years old male case showed 61 CGG repeats. When we analysed the mother, we found that she also exhibited same fragment size and 28 CGG repeat allele, which meant there was no expansion. This can be explained by the AGG interruptions making stable the pre-mutation sized allele, which needs to be clarified for further studies. On the other hand, the interesting findings concerning index 27 emerged for the first time but it still remains unclear how full mutation has expanded from the 51-sized maternal allele.

CONCLUSION

In order to give an accurate genetic counselling to pre-mutation carriers, the exact knowledge of the AGG interspersion status has become an important aspect of the activity of the genetic centres dealing with the prenatal and postnatal genetic diagnoses. Apart from the AGG interspersion in pre-mutation carriers, the known point mutation analyses in symptomatic patients who have epilepsy, autism or tremor with FXS should be carried out in routine laboratories after performing the validation studies.

Conflict of interest

No conflict of interest was declared by the authors.

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