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RESEARCH ARTICLE

Assessment of FMR1 triplet repeats in patients affected with mental retardation, fragile X syndrome and primary ovarian insufficiency

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Abstract. The CGG repeats in the *FMR1* gene expand in patients with fragile X syndrome, fragile X-associated tremour/ataxia syndrome and fragile X-associated primary ovarian failure. In this study, the CGG repeats in the *FMR1* gene were studied in 449 males and 207 females using traditional polymerase chain reaction and triplet repeat primed PCR methods, also 18 CVS samples (six males and 12 females) were tested for prenatal diagnosis. Further, methylation sensitive multiplexed ligation dependent probe amplification was performed on some samples to confirm the results. Regarding the male patients, 1.1% and 9.7% had premutation (PM) and full mutation (FM) alleles, respectively. Also three (0.66%) male patients were mosaic for PM and FM alleles. Among females, 1.9% were GZ carriers and 5.8% were PM carriers. Prenatal diagnosis resulted in detection of two PM and one FM males as well as one FM carrier female. Our results were in concordance with the previously published results.

Keywords. fragile X syndrome; mental retardation; gray zone; full mutation; primary ovarian insufficiency.

Introduction

Fragile X mental retardation syndrome (FXS, OMIM: 300624) is the most common inherited mental retardation syndrome, affecting 1:4000 men and 1:8000 women globally (Hantash *et al.* 2010). It is an X-linked dominant disease which is caused by the deficiency or absence of fragile X mental retardation protein (FMRP). An expansion of a trinucleotide of CGG repeats in the 5′ untranslated region of *FMR1* gene (OMIM: 309550) cause disease in 99% of cases which is followed by hypermethylation and silencing of expression of *FMR1*. Also, any type of deletion or inactivating mutation in *FMR1* may be causative (Hantash *et al.* 2010; Monaghan *et al.* 2013).

According to the American College of Medical Genetics (ACMG) definition, normal, intermediate (gray zone (GZ)), permutation (PM) and full mutation (FM) alleles have 5– 44, 45–54, 55–200 and >200 CGG repeats, respectively. Men with FXS have delayed speech, mild to moderate mental retardation, and distinctive physical and behavioural traits. Females have milder symptoms such as attention deficit disorder or personality disorder (Hantash *et al.* 2010; Monaghan *et al.* 2013).

Normal alleles have no meiotic or mitotic instability and are transmitted with the same CGG repeat number. Intermediate (grey-zone) alleles do not cause FXS. However, about 14% of these alleles are unstable and may expand PM range when transmitted by the mother (Nolin *et al.* 2011). They are not known to expand to FMs; therefore, offspring are not at increased risk for FXS. However, expansion of an FMR1 grey-zone allele to a FM may occur in two generations (Fernandez-Carvajal *et al.* 2009; Nolin *et al.* 2011).

PM alleles are not associated with FXS, but they increase the risk of fragile X-associated tremour/ataxia syndrome (FXTAS) and primary ovarian insufficiency (POI) (Wheeler *et al.* 2014; Campbell *et al.* 2016). These alleles may be instable while transmitted from a women to her chid, thus the child is at risk of being affected with FXS. However, some individuals with high repeat sizes (>100 repeats) have been identified with learning difficulties, emotional problems, or even intellectual disability (Hantash *et al.* 2010; Monaghan *et al.* 2013).

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FM alleles (>200 repeats) cause hypermethylation of the FMR1 promoter. Often, patients with a FM allele show somatic variation of repeat number in their peripheral blood sample (Hawkins *et al*. 2011; Pretto *et al*. 2014).

To prevent the birth of new cases, population-based carrier screening, prenatal screening and newborn screening for FXS are important issues. FXS patients are often diagnosed at three years old, by then another sibling has already been born. Thus, newborn screening could allow family planning for future pregnancies (Nygren *et al*. 2008; Gatta e*t al.* 2013; Monaghan *et al*. 2013). According to a previous study, 6.3% of Iranian families with MR have FXS (Pouya *et al.* 2009).

Different methods such as Southern blot analysis, traditional polymerase chain reaction (tPCR), repeat-primed PCR methods or methylation PCR and methylation sensitive multiplexed ligation dependent probe amplification (MS-MLPA) are used to characterize the FMR1 alleles (Nygren *et al*. 2008; Gatta *et al*. 2013; Monaghan *et al*. 2013).

In triplet repeat primed PCR (TP-PCR) for fragile X, one primer hybridizes completely outside the CGG repeat region, whereas the other overlaps the CGG repeat and the adjacent nonrepeated sequence. A third primer (M13 reverse linker primer) which hybridizes to the linker sequence of reverse primer, over amplifies the CGG repeats and help to boost the amplification signals. Also, accurate sizing of alleles up to 200 CGG repeats may be possible using this method (Hantash *et al*. 2010; Monaghan *et al.* 2013).

Materials and methods

Patients

In total, 674 cases (449 male, 207 female and 18 chorionic villus sampling (CVS)) were recruited in this study. The reasons of referral were suspicion of FXS, intellectual disability, mental disorder, familial history of MR/FXS, developmental delay, speech problem, autism, attention deficit, hyperactivity, carrier testing for FXS, premature ovarian failure, early menopause and infertility. CVS samples were studied for prenatal diagnosis. This study was approved by the local ethics committee, and an informed written consent was obtained from all patients and their parents.

DNA extraction

Genomic DNA was extracted from peripheral whole blood and CVS samples using salting out and phenol–chloroform methods, respectively.

Traditional PCR

All samples were at first tested by traditional PCR to determine the presence or absence of normal alleles. PCR amplification was performed using 300 ng DNA, 1× PCR buffer, 1.4 mM $MgCl_2$, 0.25 mM dNTP, 0.9 pmol each primer (table 1), 1 unit of *Taq* DNA polymerase (CinnaGen, Iran) and 1× GC-rich buffer. PCR condition was as follows: an initial denaturation step for 5 min at 94°C, followed by 35 cycles of denaturation for 30 s at 94°C, annealing for 35 s at 64.5°C, extension for 4 min at 72°C, and a final extension for 10 min at 72°C. The PCR products were separated on the polyacrylamide gels.

TP-PCR

The male samples with no amplification of normal alleles as well as female samples with only one band on gel were tested further by TP-PCR to determine the PM and FM alleles. TP-PCR was performed using 100 ng DNA, 1× PCR buffer, 2 mM $MgCl_2$, %6 DMSO, 1.7x Q-solution, 0.2 mM of each 7deaza-dGTP, dATP, dCTP and dTTP, 0.6 μmol of each primer (table 1) and 1 unit of Fast Start *Taq* DNA polymerase. TP-PCR condition included an initial denaturation step for 10 min at 98°C, followed by 10 cycles of 35 s at 97°C, 2 min at 64°C and 8 min at 68°C, and then 25 cycles of 35 s at 97°C, 2 min at 64°C and 8′:20″ at 68°C, and a final extension for 10 min at 72°C and incubation at 25°C for 2 min. The TP-PCR products were separated on 3130 genetic analyzer using GeneScan 1000 Rox dye size standard (Thermo Fisher Scientific).

MS-MLPA

MS-MLPA (ME029 kit) was also performed on 34 samples to assess the conformity of the results. It is capable of detecting copy number changes in males and females as well as hypermethylation in *FMR1*/*AFF2* genes in male patients. After hybridization of the probes to their targets, the reaction is split into two tubes: in one tube the standard MLPA reaction (ligation and amplification) is performed, providing information on copy number changes. The other tube undergoes ligation and simultaneously digestion by *Hha*I endonuclease. A universal PCR primer set will amplify only probes that are ligated and undigested, revealing methylated alleles (https://www.mlpa.com) (Nygren *et al*. 2005, 2008).

Results

In this study, 449 male, 207 female and 18 CVS samples were studied using traditional PCR and TP-PCR (table 2). Thirty-four of these samples were also assessed by MLPA method to authenticate the results. PM and FM alleles were found in five and 44 male samples, respectively. Besides three male samples were mosaic for PM and FM alleles (table 2; figure 1). Concerning females, four and 12 samples were carrier of GZ and PM alleles, respectively.

Table 1. Primers used in traditional PCR and TP-PCR methods.

Traditional PCR	F: GACGGAGGCGCCGCTGCCAGG R: GTGGGCTGCGGGCGCTCGAGG
TP-PCR	FMR1-F: TGTAAAACGACGGCCAGTGCTCAGCTCCGTTTCGGTTTCACTTCCGGT FMR1-R: CAGGAAACAGCTATGACCCTCGAGGCCCAGCCGCCGCCGCC M13reverse linker primer: CAGGAAACAGCTATGACC

Table 2. Results obtained in this study.

Samples	Number	Normal	Intermediate (GZ)	PM	FM	Mosaic (PM/FM)
Male	449	397 (88.4%)	0	5 (1.1%)	44 (9.7%)	3 (0.66%)
Female	207	191 (92.2%)	4 (1.9%)	12 (5.8%)	0	0
CVS male	6	3 (50%)	0	2 (33.3%)	1 (16.6%)	0
CVS female	12	11 (91.6%)	0	0	1 (8.3%)	0

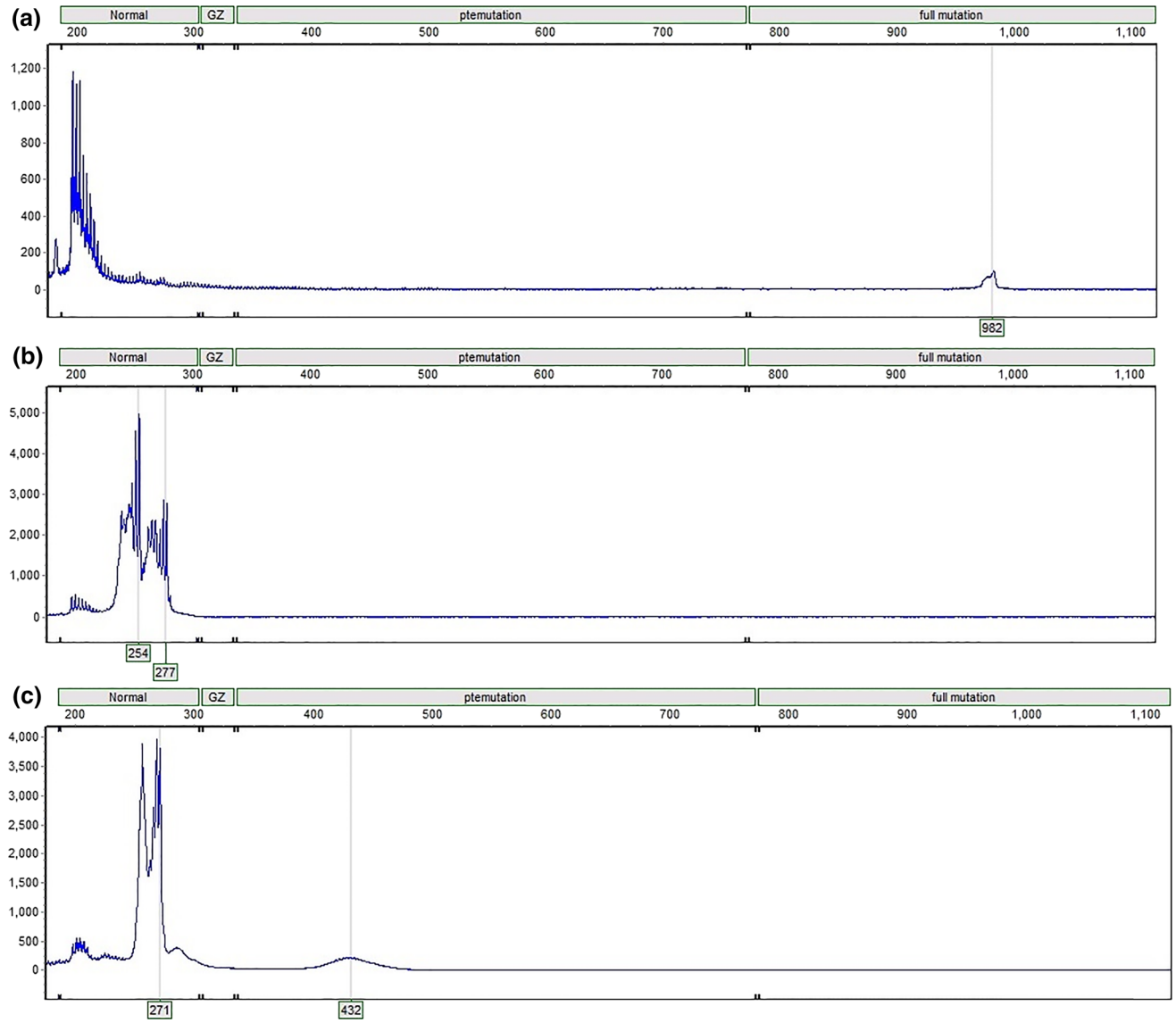

Figure 1. TP-PCR results. (a) Male with a FM allele. (b) Female heterozygous for two normal alleles. (c) Female heterozygous for normal and PM alleles.

Discussion

In the majority of the fragile X patients, the disease is due to the expansion of CGG repeats in the 5′ UTR of *FMR1* gene (Peprah 2012). Also, less frequent defects including expansion of GCC repeats (>200) in the *AFF2* (*FMR2*; *FRAXE*) gene on Xq28 cause fragile XE syndrome which is a form of nonsyndromic X-linked cognitive disability (Tzeng *et al*. 2000). Few deletions in *FMR1* and *AFF2* are known, however, their frequency might have been more, especially in the very large *AFF2* gene. Microdeletions in *AFF2* gene may cause premature ovarian failure (Murray *et al*. 1999).

Carrier detection, prenatal diagnosis and newborn screening are crucial. In this study three different methods including traditional PCR, TP-PCR and MS-MLPA were used to study the patients with intellectual disabilities or for carrier detection and prenatal diagnosis.

TP-PCR is an accurate and robust method which detects expansions and FMs including mosaics in both females and males. Being capable of distinguishing between normal homozygous females from FM carrier ones, this method reduces the number of required Southern blot analyses (Hantash *et al.* 2010; Lyon *et al*. 2010; Tural *et al.* 2015).

Male patients with PM alleles have different phenotypes from those with both PM and FM alleles (mosaic males) (Todorov *et al*. 2009). According to the ACMG recommendations, in cases with a PM allele identified by traditional PCR, Southern blot analysis should also be performed. However, newer TP-PCR or methylation PCR eliminate the need to perform Southern blot analysis on each sample (Monaghan *et al*. 2013). In this study, we used TP-PCR to detect the FM even if the PM alleles were identified.

MS-MLPA is a rapid, easy to perform, and reliable method which is capable of detecting copy number changes as well as identifying males with methylated FMR1/AFF2 alleles. The results are highly reproducible on good-quality DNA samples. The length of the repeat cannot be measured by MS-MLPA. However, it is possible to quantify the methylation at certain CpG sequences in the promoter regions of these genes by this method. It is possible to make a reliable distinction between FMR1 PM and FM alleles in male DNA samples. However, a distinction between PM alleles and normal alleles cannot be made. Also, it cannot detect point mutations unless they are near the ligation site of the right and left oligonucleotide probes (Nygren *et al.* 2005; Stuppia *et al*. 2012).

We performed traditional PCR and TP-PCR on 449 male, 207 female and 18 CVS samples. Also, MS-MLPA was performed on 34 random samples to compare the results. The results of all the three methods in this study were in concordance. We found PM and FM alleles in five (1.1%) and 44 (9.7%) male samples respectively. Also three male samples were mosaic for PM and FM alleles. Four and 12 females were carrier of intermediate and PM alleles, respectively. It was revealed that the CVS samples included six males (three normal, two PM and one FM) and 12 females (11 normal and one carrier of FM).

We could not find any mutations in 397 males (88.4%) as well as 191 females (92.2%). In these cases, the disease may be due to point mutations, deletions, or insertions of a few nucleotides in the *FMR1*/*AFF2* genes or the diagnosis may be incorrect.

Similar studies have also reported the frequency of FMR1 alleles. Although FXS is known to be the most common cause of inherited intellectual disability in males, the precise frequency of this syndrome in the general population is unknown. Also there is variability in the frequencies reported by different groups. In a research, the incidence of FXS in 5000 consecutive newborn males in Catalonia in Spain was found to be 1 in 2466. Also one in 1233 males had the PM allele (Rife *et al.* 2003).

In a study of 1275 samples submitted for fragile X analysis, 15 intermediate females, seven intermediate males, 10 premutated females, three FM females and three FM males were found. No expansion was found in 1237 samples (Hantash *et al.* 2010). TP-PCR of the *FMR1* gene revealed 1.7% GZ carriers and 0.3% PM carriers among 300 women of reproductive age and 3.6% GZ carriers and 2.14% PM carriers among 140 women with POI. The frequency of GZ and PM carriers did not significantly differ between the cohorts (Dean *et al.* 2018). In a study of 50 patients (including 12 (24%) POF and 19 (38%) fragile-X patients) using TP-PCR, all suspected fragile-X patients had normal FMR1 alleles and 6.6% of POF patients were observed to have PM (Tural *et al.* 2015). In a screening study of 200 patients with intellectual disability in the west of Iran using cytogenetic and molecular methods, the frequency of FXS revealed to be 8% (Hadi *et al.* 2018). In another study of 508 Iranian families with MR, full FMR1 mutations were found in 32 families (6.3%) (Pouya *et al.* 2009).

In this study, we did not find any deletion in *FMR1* gene in 34 samples tested with MS-MLPA. Also, no deletion or hypermethylation was detected in *AFF2* gene. However, it is suggested that the remaining samples be studied with MS-MLPA method to find deletions in FMR1 as well as deletion and hypermethylation in *AFF2* gene. In addition, there are some reports of point mutations (Suhl and Warren 2015; Rajaratnam *et al.* 2017) in *FMR1* gene in FXS patients. These mutations can be detected by cycle sequencing method.

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