Fragile X syndrome: diagnosis by molecular characterization of *FMR1* gene and clinical correlation

Hoda M. Abd El-Ghany[a], Eman A. Ehssan[b], Menatalla K. El-Deen[a], Rasha A. Al-Gamal[a], Rania M. Samy[a], Amany S. El-Deen[a]

Departments of [a]Clinical and Chemical Pathology, [b]Pediatric, Faculty of Medicine, Cairo University, Cairo, Egypt

Correspondence to Rania M. Samy, MD, Department of Clinical and Chemical Pathology, Faculty of Medicine, New Children Hospital, Abu El Rish, Cairo University Hospitals, Ali Basha Ebrahim, Postal Box 11562, Cairo, Egypt
Tel: +20 100 500 4325;
e-mail: rania_sami1976@yahoo.com

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Background
One of the most common forms of inherited intellectual disability (ID) is fragile X syndrome (FXS), which is caused by expansion of cytosine–guanine–guanine trinucleotide repeat at the 5′ untranslated region of the fragile X mental retardation gene (*FMR1*) at Xq27. The cytosine–guanine–guanine repeat expansion leads to hypermethylation and inactive transcription of the gene. The present study aimed to detect expected *FMR1* gene alleles by methylation-sensitive PCR and its clinical correlation for rapid screening of FXS among male patients with ID.

Patients and methods
The study included 50 male patients with ID and clinical features suggestive of FXS, who were compared with 50 healthy age-matched volunteers. All patients were subjected to full history taking, thorough clinical examination using a 15-item checklist [physical (big ears, joint hyperextensibility, Simian crease, wide forehead, macroorchidism, and elongated face) and neurological features (mental retardation, family history of mental retardation, poor eye contact, hand biting, hyperactivity, perservative speech, tactile defensiveness, hand flapping, and short attention span)], and karyotyping using GTG banding. Methylation-sensitive PCR technique after bisulfite treatment of DNA was applied for the detection of expanded alleles of the *FMR1* gene.

Results
Clinical score in patients with abnormal alleles was significantly higher compared with patients with normal alleles. GTG-banding technique showed normal 46XY male karyotype for studied patients. Frequency of normal *FMR1* gene alleles was detected in the control group (100%) and 44 (88%) patients. Abnormal alleles were detected in six (12%) patients: three (6%) patients with full mutation (FM) and three (6%) patients with premutation carrier.

Conclusion
Our study revealed that PCR-positive results of fragile X correlate with the checklist clinical score rather than a single clinical entity.

Keywords:
fragile X mental retardation 1 gene, fragile X syndrome, intellectual disability, methylation-sensitive PCR

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Introduction

Fragile X syndrome (FXS) is an inherited X-linked disease. It was described for the first time in 1943 by Martin and Bell. It is regarded the most common inherited cause of intellectual disability (ID) after Down syndrome. FXS is permanently inherited with many members in the family tree, either affected or a carrier (Ciaccio *et al.*, 2017).

It is inherited as an X-linked dominant trait with a fragile site at Xq27.3 locus named fragile X mental retardation gene (*FMR1*) (Niu *et al.*, 2017). The *FMR1* gene consists of 17 exons spanning 38 kb. The polymorphic cytosine–guanine–guanine (CGG) trinucleotide repeat is located in its 5′ untranslated region of exon 1 within the 4.4 kb of *FMR1* transcript. The number of the trinucleotide repeats ranges between 5 and 44 repeats in normal individuals, 45–54 triplets in intermediate expansion (gray zone), 55–200 repeats in premutation carriers, and beyond 200 repeats in full mutation (FM) carriers (Ciaccio *et al.*, 2017).

FXS results from an expansion mutation of a CGG repeat in the first exon of the *FMR1* gene leading to transcriptional gene silencing and absence or remarkable reduction of its product, *FMR1* protein. This protein is important for proper neuronal morphology, cognitive development, and synaptic plasticity, and its absence leads to changing levels of ID. Boys are more seriously

affected than girls because *FMR1* gene is located on the X chromosome, and there could be an unaffected second X chromosome present in girls (Kidd *et al.*, 2014).

In addition to ID, there are notable phenotypic characteristics of FXS that may be present (Rajaratnam *et al.*, 2017). Phenotypic manifestations common to several clinical checklists include large forehead, large ears, elongated face, simian crease, high arched palate, large testicles, hyperextensibility of joints, tactile defensiveness, hyperactivity, poor eye contact, short attention span, hand biting, hand flapping, perseverative speech, and a positive family history of mental retardation (MR). To increase the effectiveness of the screening programs, preselection of patients based on clinical features is required (Guruju *et al.*, 2009).

Rapid screening of large numbers of individuals suspected to have FXS could be facilitated by methods based on the PCR. It accurately detects the number of CGG repeats (Panagopoulos *et al.*, 1999). PCR techniques for amplification of CGG repeats are, however, inefficient and unreliable because of their 100% C+G composition and require subsequent Southern blot analysis and autoradiography (Kanwal *et al.*, 2015).

Methylation-sensitive PCR is rapid and relatively inexpensive technique used for diagnosis of FXS. The primary principle of that method is that it depends on the ability of bisulphite to deaminate C residues in a single-strand DNA. A characteristic of the bisulphite-treated DNA is that after modification, the sense and antisense strand are no longer complimentary. Thus, the modified strands can be amplified separately by designing primers specific for each of them. The C residues of all CpG dinucleotides flanking the CGG repeats as well as those of the CGG repeats are methylated in affected males and in the inactive X chromosome in females (Karunasagar *et al.*, 2005). The same C residues are, however, unmethylated in healthy males, normal transmitting males, and in the active X chromosome in females. However, the disadvantage of methylation PCR is that it cannot reliably diagnose affected females with FXS owing to the fact that the inactive X chromosome is already methylated. During screening, all positive female samples will have to be subjected to Southern blot analysis for confirmation of diagnosis (Karunasagar *et al.*, 2005).

The aim of the present work was to detect the *FMR1* gene expected alleles by methylation-sensitive PCR method. Clinical correlation to molecular characterization was also evaluated.

Patients and methods

The present study was conducted on 50 male patients with MR and clinical features suggestive of FXS. The patients were referred to the Genetic's Clinic, Abo El-Reech Hospital Kasr El Ainy Medical School. Their ages ranged from 3 to 21 years. Moreover, 50 healthy age-matched volunteers were also recruited as a control group. Their age ranged from 3 to 17 years. Informed consents were obtained from adult patients and parents of studied children. The study design was approved by the Scientific Research Committee of the Clinical Pathology Department, Faculty of Medicine, Cairo University.

All patients were subjected to full history taking including family history. Thorough clinical examination with careful assessment of clinical and neurological features using a 15-item checklist (Guruju *et al.*, 2009), which includes physical (big ears, joint hyperextensibility, Simian crease, wide forehead, macroorchidism, and elongated face) and neurological features (MR, family history of MR, poor eye contact, hand biting, hyperactivity, perservative speech, tactile defensiveness, hand flapping, and short attention span).

Cytogenetic studies were performed as follows: peripheral blood samples were collected for chromosomal analysis using GTG-banding technique (Verma and Babu, 1995). Metaphases with good banding quality were karyotyped using image analysis system (Applied Imaging, Shirley, New York, USA). Individual chromosomes were identified and arranged according to the International System for human cytogenetics and nomenclature (ISCN, 2016).

Genotyping method for the detection of expanded alleles of the *FMR1* gene by methylation-sensitive PCR was as follows: 3 ml of blood was withdrawn aseptically from every patient and control, and then collected in a sterile EDTA vacutainer tube. DNA was extracted from the whole blood using DNA extraction kit (GeneJET Genomic DNA Purification Kit, catalogue number: #K0721; Fermentas Life Science, Vilnius, Lithuania). Based on the method described by Chaudhary *et al.* (2014) extracted DNA was used in the bisulfite reactions. PCR amplification of the methylated CpG island located upstream of the repeats (The primers were designed for the modified antisense strand and are specific for PCR amplification of the methylated C residues present in affected individuals and on the inactive X chromosome in normal females) was done using the following primers: forward 5′-AAC GAC GAA CCG ACG ACG-3′ and reverse primer 5′-CGT CGC GTT GTC GTAC-3′.

All reactions were performed in a total volume of 25 μl. After an initial denaturation step (5 min at 94˚C), the samples were subjected to 32 cycles of 93˚C for 30 s, 65˚C for 30 s, and 72˚C for 30 s, with a final extension of 10 min at 72˚C.

PCR amplification of fragments containing the unmethylated CGG repeats (The primers were designed for the modified antisense strand after bisulfite treatment of DNA for amplifying the unmethylated sequence in normal males) was done using the following primers: forward primer: 5′-CAA CCT CAA TCA AAC ACT CAA CTC CA-3′ and reverse primer: 5′-GGG AGT TTG TTT TTG AGA GGT GGG -3′ (Chaudhary *et al.*, 2014).

All reactions were performed in a total volume of 25 μl. After a denaturation step (5 min at 94˚C), the samples were exposed to 32 cycles of 93˚C for 60 s, 58˚C for 60 s, and 74˚C for 120 s, then a final extension of 10 min at 74˚C. PCR products were analyzed on a 2% agarose gel.

Normal males showed no bands using methylated primers and a band at 280 bp representing the normal range of 19–40 CGG repeats by unmethylated primers. The absence of signal for methylated primers was not owing to any failure of PCR amplification.

FXS-positive males showed band at 80 bp using methylated primers and nothing by unmethylated primers. Premutation carrier males showed no bands using methylated primers and band at 400 bp by unmethylated primers.

Ethical statement

The study design was approved by the Scientific Research Committee of the Clinical Pathology Department, Faculty of Medicine, Cairo University. Data confidentiality was observed according to the Revised Helsinki Declaration of Bioethics.

Results

The present study was conducted on 50 patients with features suggestive of FXS. The patients were referred to Clinical Genetic Department, Abo El-Reech Hospital Kasr El Ainy Medical School. Their ages ranged from 3 to 21 years, with a mean value of 7.4 years. Family history revealed 14 (28%) patients with positive parental consanguinity and 35 (70%) patients with history of MR.

The individual clinical data of patients included both physical features and neurological features.

Physical features

Overall, 39 (78%) patients had large ears, 22 (44%) had hyperextensibility of joints, three (6%) had simian crease, 26 (52%) had elongated face, 30 (60%) had large forehead, and 10 (20%) had macroorchidism.

Neurological features

All studied patients had MR (100%), 35 (70%) with family history of MR, 46 (92%) were hyperactive, 45 (90%) with poor eye contact, 18 (36%) with hand biting, 12 (24%) with hand flapping, 34 (68%) with no preservation of speech, 29 (58%) had tactile defensiveness, and 34 (68%) had short attention span.

Guruju *et al.* (2009) found that a threshold clinical score of 5 of 15 was a good index for screening the MR population and did not miss a single Fragile X-positive patient. Using 5 as the threshold clinical score, any patient with a score of 5 or less was excluded from this study.

According to 15-item checklist, we found that the mean clinical score in patients with abnormal alleles (12.5 ± 1.64) was significantly higher compared with patients with normal alleles (8.14 ± 1.42) ($P < 0.001$). On comparing the mean clinical score in patients with abnormal alleles, patients with FM ($n = 3$) was higher than permutation (PM) ($n = 3$), 13.67 versus 11.33, but did not reach a statistical value.

Clinical presentations of the patients are shown in Tables 1 and 2 and Fig. 1.

Laboratory data of controls and studied patients

According to conventional karyotyping using GTG-banding technique, all controls and patients had a normal 46XY male karyotype.

Table 1 Collective clinical data of the studied patients (*n*=50)

Features	*n*/*N* (%)
Physical abnormalities	
Large ears	39/50 (78)
Large forehead	30/50 (60)
Long face	26/50 (72)
Hyperextensibility of joints	22/50 (44)
Macroorchidism	10/50 (20)
Simian crease	3/50 (6)
Neurological abnormalities	
Mental retardation	50/50 (100)
Hyperactivity	46/50 (92)
Poor eye contact	45/50 (90)
Family history of mental retardation	35/50 (70)
Short attention span	34/50 (68)
No preservation of speech	34/50 (68)
Tactile defensiveness	29/50 (58)
Hand biting	18/50 (36)
Hand flapping	12/50 (24)

Table 2 Descriptive data of studied fragile X syndrome patients: clinical score (*n*=6)

Case no.	Large ears	Large forehead	Long face	Hyperextensibility of joints	Macroorchidism	Simian crease	Mental retardation	Hyperactivity	Poor eye contact	Family history of mental retardation	Short attention span	Preservation of speech	Tactile defensiveness	Hand biting	Hand flapping	Clinical score
7	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	14
12	1	0	0	1	0	1	1	1	1	1	1	1	1	1	1	12
25	1	1	1	1	0	0	1	1	1	1	1	1	0	1	1	12
28	1	1	1	1	1	0	1	1	1	1	1	1	0	0	0	11
37	1	1	1	1	0	0	1	1	1	1	1	1	0	1	0	11
48	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	15

A score of '1' is given for the presence of the feature and '0' for its absence. Clinical score is done according to 15-item checklist.

Figure 1

Case no. 7 showing clinical features of fragile X syndrome (elongated face, large forehead, and big ears).

Detection of expanded alleles of the *FMR1* gene by methylation-sensitive PCR after bisulfite treatment of DNA

A total of 44 (88%) patients showed normal PCR results by using the unmethylated primers. Amplified products were detected at 280 bp, whereas these samples showed unamplified products using the methylated primers.

Six (12%) patients, which represent cases 7, 12, 25, 28, 37, and 48, showed positive PCR results. Three patients (cases 7, 48, and 25) showed FM. Cases 7 and 48 showed amplified products (80 bp) using methylated primers and unamplified products using the unmethylated primers. Case 25 showed amplified products using both methylated primers (80 bp) and unmethylated primers (280 bp), indicating the presence of both methylated and unmethylated alleles, thus indicating mosaicism for FXS carrying cells.

Descriptive data of the studied patients with FXS (n = 6) regarding conventional karyotyping and molecular analysis are presented in Table 3.

Three patients, which represents cases 12, 37, and 28 showed PM (carrier state). Using the unmethylated primers, amplified products were detected at 400 bp, representing PM with a range of 55–200 CGG repeats, whereas these patients showed unamplified products using the methylated primers (Fig. 2).

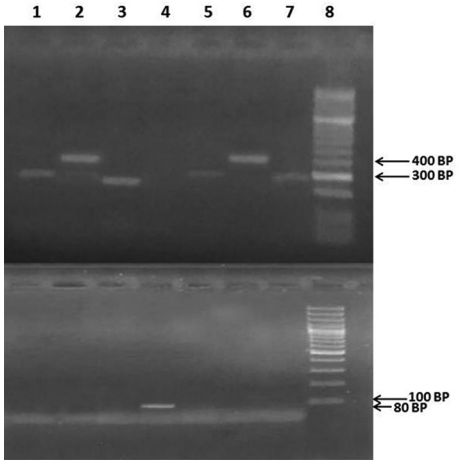
Clinical features of the two patient groups showing normal (n = 44) and abnormal alleles (n = 6) were compared regarding physical and neurological abnormalities, but insignificant clinical correlations were observed.

Table 3 Descriptive data of studied fragile X syndrome patients (n=6)

Case no.	Investigations			
	Karyotype	Methylation-sensitive PCR		
		Unmethylated primer	Methylated primer	PCR results
7	46XY	-	>200 CGG repeats (80 bp)	Full mutation
12	46XY	55-200 CGG repeats (400 bp)	-	Premutation (carrier state)
25	46XY	19-40 CGG repeats (280 bp)	>200 CGG repeats (80 bp)	Full mutation (mosicism)
28	46XY	55-200 CGG repeats (400 bp)	-	Premutation (carrier state)
37	46XY	55-200 CGG repeats (400 bp)	-	Premutation (carrier state)
48	46XY	-	>200 CGG repeats (80 bp)	Full mutation

Conventional karyotyping and molecular analysis. CGG, cytosine-guanine-guanine.

Figure 2



PCR results. Above: results using unmethylated primers. Below: results using methylated primers. Lanes 1, 3, 5, and 7 (case no. 4, 10, 27, and 31): normal alleles: amplified band at 280 bp with unmethylated primers and unamplified band at 80 bp with methylated primers. Lanes 2 and 6 (case no. 28 and 37): abnormal alleles (premutation carrier):amplified band at 400 bp with unmethylated primers and unamplified band at 80 bp with methylated primers. Lane 4 (case no. 48): abnormal alleles (full mutation): unamplified band at 280 bp with umethylated primers and amplified band at 80 bp with methylated primers.

Discussion

FXS stands for about one-third of all cases of X-linked MR (Chaudhary *et al.*, 2014). It is inherited as an X-linked dominant trait with a fragile site at Xq27.3 locus named *FMR1* (Niu *et al.*, 2017).

In the FM, CGG repeats expanding beyond 200 repeats cause hypermethylation of the *FMR1* promoter region, chromatin condensation, silencing of the gene, and insufficient synthesis of *FMR1* protein (Chaudhary *et al.*, 2014).

The typical adult male with the FM is characterized clinically by prominent ears, elongated face, prominent ears, and macroorchidism (Guruju *et al.*, 2009).

Conventional karyotyping and cytogenetic analysis for fragile X detection identify the fragile site at Xq27.3 as well as other cytogenetic abnormalities (El Sobky *et al.*, 2008). Methylation PCR is used for its significant sensitivity and specificity (Kanwal *et al.*, 2015).

Developing a screening procedure for early diagnosis in newborns (or within the first year) is essentially needed because of difficult clinical diagnosis of FXS in early childhood and can supply early behavioral intervention for the child and information for parents before a second at-risk child is born (Chaudhary *et al.*, 2014).

The present study aimed to detect expected alleles for *FMR1* gene by methylation-sensitive PCR-based method with clinical correlation for rapid screening of FXS susceptible patients.

The study was conducted on 50 male patients with MR and clinical features suggestive of FXS, who were compared with 50 healthy age-matched volunteers. All patients were subjected to full history taking and proper clinical examination with careful assessment of clinical and neurological features. Because many clinical features of FXS are seen in MR cases of unknown etiology, a cumulative score of the checklist characteristics was useful in evaluating clinical severity (Guruju *et al.*, 2009).

Conventional karyotyping was done in this study for the detection of any concomitant numerical or structural chromosomal abnormalities and showed a normal 46, XY male karyotype.

Methylation-sensitive PCR after bisulfite treatment of DNA was applied for the diagnosis of FXS based on the methylation-sensitive conversion of C residues to U by bisulfite treatment of DNA (Ozbey *et al.*, 2007). Male children with MR represented all DNA samples in the present study.

Normal alleles were found among 44 (88%) of studied patients. The mutation was detected as FM in three (6%) cases and PM carrier in three (6%) cases. Our data agreed with previous studies (Karunasagar *et al.*, 2005; Ozbey *et al.*, 2007). We noticed the presence of methylated and unmethylated alleles in 33% (1/3)

of patients with FM indicating mosaicism for FXS carrying cells. The same result was found in the study by Chaudhary *et al.* (2014) which revealed mosaicism in 33% (3/9) of patients with FM. Mosaicism for unmethylated and fully methylated *FMR1* alleles in affected males has been reported by other investigators with highly variable frequencies ranging from 12 (Rousseau *et al.*, 1991) to 41% (Nolin *et al.*, 1994), which probably reflect the differences in assay resolution.

An Egyptian study by Meguid *et al.* (2005) was done aimed at identifying the prevalence rate of fragile X males in Egypt. A total of 20 500 males were screened. The original work involved inhabitants of the three different large governorates: Cairo (Shobra and Rod El-Farag districts), Giza (Dokki district), and Suez (Suez district). The participants included students of primary and high schools. According to school records, parent reports, and clinical examinations, 130 males were selected and subjected to molecular analysis. The prevalence of FXS mutation among Egyptian males was 0.9 per 1000. Moreover, it was 6.4% among mentally subnormal males.

In another study by Omar *et al.* (2016) on 64 males with idiopathic MR, ranging in age from 4.2 to 19 years, amplification of *FMRI* gene by PCR of tested patients revealed that eight (12.5%) cases have FM and six (9.4%) cases have PM.

Among patients with abnormal alleles, FM and PM carrier (n = 6), family history of MR was present in all patients, which is in agreement with the study by Guruju *et al.* (2009) where 92% of cases had a family history of MR. Family history of MR was observed by other investigators in 69.4% (Lachiewicz *et al.*, 2000).

Parental consanguinity was present in 16.6% of patients with fragile X-positive in contrast to a study done by Fahad Al-Hur and Alsuhaibani (2017). They reported a higher prevalence (76.47%), which may be attributed to higher consanguinity prevalence in their country. On the contrary, there was low parental consanguinity in a study done by Pouya *et al.* (2009). This difference may be owing to different consanguineous marriage rates among different countries and different sample size.

In our study, the percentage of patients with hyperextensibility of joints, tactile defensiveness, large ears, elongated face, macroorchidism, poor eye contact, and family history of MR were higher in patients with abnormal alleles than other patients with normal alleles but did not reach a significant variation. These results were previously supported by Guruju *et al.* (2009).

Among male patients with abnormal alleles, 50% (3/6) of patients had macroorchidism and 17% (1/6) were postpupertal. Macroorchidism can be a useful clinical sign for screening postpubertal males. However, 9.9% of non-fragile X males also had macroorchidism. This would lead to a 10% rate of false-positive cases if macroorchidism alone was considered (Guruju *et al.*, 2009).

The mean clinical score of patients with abnormal alleles was higher than patients with normal alleles. A previous study revealed significant difference between the mean clinical score of fragile X and non-fragile X patients (Guruju *et al.*, 2009). This indicates that PCR-positive results of fragile X correlates with the clinical score of checklist rather than a single clinical entity. The clinical checklist used in the present study is useful to clinicians as an initial screening tool of a large MR population for individuals affected.

Regarding the degree of MR in this work, PM carrier was found to have mild to moderate MR (IQ level between 35 and 70), whereas patients with FM showed severe MR (IQ level between 20 and 34), indicating less degree of affection of PM carrier than those with FM. We also observed that patients showing mosaic patterns are less severely affected than those with complete methylation. Case no. 25 showing mosicism had an IQ level between 35 and 70 (mild to moderate), whereas cases no. 7 and 48 with FM had an IQ level between 20 and 34 (severe), which was in accordance with Chaudhary *et al.* (2014).

Methylation-sensitive PCR technique is one of the most valuable methods available at present for the proper diagnosis of fragile X carrier and disease state (Weinhausel and Haas, 2001). However, the pattern of methylation in our study needs further investigation using larger number of cases and different analysis methods.

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Conflicts of interest
There are no conflicts of interest.

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