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ORIGINAL ARTICLE

Screening For Fragile X Syndrome Among Neurobehavioural Patients From Kolkata, Eastern India.

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ABSTRACT

Background: Fragile X syndrome (FXS), associated with abnormal functioning of the *FMR1* gene, is a major cause for inherited mental retardation (MR). The symptoms which are commonly associated with FXS are also observed in patients suffering from various neuropsychiatric disorders like autism, epilepsy, seizure disorder etc. Thus, the diagnosis of FXS that is solely based on a patient's physical and behavioural characteristics is very difficult. To avoid a false positive diagnosis which is crucial for better management of the disorder, screening for FXS with easy diagnostic tools becomes extremely important.

Aims: In this study, screening for FXS was carried out among 157 various neurobehavioural patients attending the out patients department of Manovikas Kendra, Kolkata.

Methods and Material: To screen the level of functioning of the *FMR1* gene, the percentage of leukocytes expressing the fragile X mental retardation protein (FMRP) was measured by an immunocytochemical method. CGG repeat size was analyzed by PCR amplification and *FMR1* promoter methylation status was checked by methylation sensitive-PCR.

Results: Out of 157 patients recruited for this study, only four were confirmed as FXS (3.18% prevalence among neurobehavioural outpatients). 30 distinct alleles with 12-49 CGG repeats were detected, with the 27 and 28 repeats being most common. Premutation alleles were observed in 25 subjects. Molecular biology-based analyses confirmed 5 cases as FXS; four patients were detected with promoter methylation mosaicism and one with full methylation.

Conclusion: In the present investigation, FXS screening was performed on various neurobehavioural outpatients and four were confirmed with the disorder. The CGG repeat alleles that were most frequently observed in this study were different from those found in other studies, indicating a racial or ethnic variation.

Key Words: Fragile X syndrome, MR, FMRP, CGG repeat, MS-PCR.

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Introduction

Fragile X syndrome (MIM 309550) is one of the monogenic forms of X-linked mental retardation (MR), with a worldwide prevalence of 1 in 4000 males and 1 in 8000 females among the western population [1],[2],[3]. This is the common genetic cause for MR, second only to Down syndrome (DS). Studies carried out on Indian male MR cases revealed ~7% possibility of having FXS [4],[5],[6],[7].

The primary features of FXS are mild to severe MR, long narrow face, large ears, broad nasal bridge, prominent jaw, hyperextensible finger joints, double jointed thumbs, single palmer crease, flat feet, mitral valve prolapse, velvet-like skin and macroorchidism [8],[9],[10]. General growth abnormalities i.e. increased height throughout childhood and early onset of puberty as compared to peers is also observed in affected male individuals. Other medical problems like recurrent emesis, otitis media infections, sinusitis, strabismus, and seizures are also associated with FXS [11], [12]. Patients often display autistic features such as difficulties in social interaction with peers, impaired verbal and nonverbal communication, poor eye contact, tactile defensiveness, hand flapping, hand biting and other stereotypic movements and it is estimated that nearly 25% of children with FXS meet these criteria for autism [13]. A majority of FXS children also have problems with attention and hyperactivity; deficit in attention alongwith hyperactivity in boys with FXS has been reported to vary between 70 to 100% in various studies [14],[15],[16]. Some other associated features are hyperarousal, social avoidance, anxiety, tantrums, extreme sensitivity to sensations and sometimes, aggression. Epilepsy is also reported to occur in 10 to 20% of individuals having FXS [17].

FXS and DS the two most prevalent forms of MR that are linked to faulty brain synaptic communications **(** http://med.stanford.edu/news_releases/2007/april/fragilex.html **).** A similar kind of study, earlier done by Dr. Daniel Madison on murine model of FXS and DS, showed that not only the post-synaptic cells, but also the pre-synaptic cells are important ones to study in these disorders [18],[19].

FXS is mostly caused by a mutation in the *FMR1* gene which is located on the X chromosome, approximately 38 kb in size and contains 17 exons [20], [21].The *FMR1* gene product, fragile X mental retardation protein (FMRP), is an RNA-binding protein that is expressed in foetal and adult tissue cytoplasm, with the most abundant expression in brain and testes [22]. It plays an important role in the regulation of specific target mRNA translation in the cell cytoplasm [23]. FMRP is also involved in the regulation of the synaptic activity and loss of this protein directly influences neuronal plasticity, resulting in cognitive defects [24]. An expansion of a trinucleotide (CGG) repeat at the highly conserved 5' UTR of the *FMR1* gene causes instability; size of the CGG repeats often increases during female meiosis in succeeding generations [25]. Generally, normal subjects have 2-50 copies of the repeat and those having the repeat number in between 50-200 are known as carriers, also known as premutation (PreM) condition [26]. Hypermethylation of both the repeat and its adjacent promoter region, concomitant with expansion of the CGG repeats (>200-220 repeats), leads to a decrease in the transcription of *FMR1*; a condition known as full mutation (FM). The number of repeats in the PreM condition is potentially unstable and may expand into the FM range. Although a vast majority of patients with FXS show this pattern of expanded repeats, a small number of cases have also been reported where partial deletions in *FMR1* resulted in a disease phenotype [27].

For detection of FXS, methods like Southern-blot analysis, polymerase chain reaction (PCR) and immunocytochemical analyses are used [28],[29],[30],[31]. The most commonly applied molecular genetic technique depends on either the detection of expanded repeats or *de novo* methylation analysis, or both. Although Southern hybridization is a very popular and probably the most reliable method for detection of FXS, it has several disadvantages for rapid screening. The main disadvantage is that it is a time consuming and laborious process and hence, is disadvantageous for the rapid screening of a large population. Moreover, it is expensive and requires large amount of DNA. Because of these limitations, for rapid screening, other diagnostic tests like repeat PCR analysis and methylation-sensitive PCR (MS-PCR) analysis for genomic DNA [29],[30] and an immunocytochemical technique for detection of FMRP [31],[32] were developed.

For the present investigation, population screening for FXS was performed among

individuals living in and around Kolkata, West Bengal, who attended the psychiatric out-patient department for various neurobehavioural disorders like autism , spectrum disorder (ASD), epilepsy, attention deficit hyperactivity disorder (ADHD), DS and MR. We have relied mainly on the immunocytochemical detection of FMRP, repeat PCR and MS-PCR analyses for screening.

Materials and Methods

Subjects

Probands (n=157) with various neurobehavioural disorders were recruited from the out-patient department of Manovikas Kendra Rehabilitation and Research Institute for the Handicapped, Kolkata, India. Diagnosis was carried out by mental health professionals according to the DSM-IV criteria [33]. Among the 157 patients, 71.34% were male and 28.66% were female, with age ranges of 1.5-24 yrs and 1-35 yrs, respectively. Study subjects comprised of ADHD (n=16), ASD (n=33), Rett syndrome (n=1), epilepsy (n=2), DS (n=35) and MR (n=70); among the MR cases, 22 exhibited symptoms of FXS.

Sample Collection

Peripheral blood was collected in an anticoagulant from probands and their parents after obtaining their informed written consent. Samples collected in heparin were used for the detection of FMRP and chromosomal abnormalities. Leukocytes cultured in regular as well as folate-deficient RPMI 1640 media [34] were processed for GTG-banding and karyotype analysis [35]. The protocol was approved by the Institutional Human Ethical committee.

Genomic DNA extracted from leucocytes using the standard protocol [36], was used for PCR-based diagnosis of genetic abnormalities (amplification of CGG repeat and MS-PCR analysis).

Detection of FMRP

Intracellular FMRP was detected by an immunocytochemical technique [31]. In summary, peripheral blood smears on glass slides were collected and stored at -20°C till the assay was done (a maximum of 2 wks). Before staining, the smear was fixed with 3% paraformaldehyde (Sigma, USA) in phosphate buffer (pH 7.3) and permeabilized with methanol (SRL, India). Incubations with the 1st antibody (goat FMRP-specific polyclonal IgG, Santa-Cruz Biotechnology, USA) and 2nd antibody (rabbit anti-goat antibody coupled with FITC, Santa-Cruz Biotechnology, USA) were carried out for 2hrs and 1hr, respectively, at room temperature. Cells were viewed under Zeiss Axioskop 2 plus fluorescence microscope. For each sample, nearly 1000 cells were scored and the percentage of lymphocytes expressing FMRP was determined.

Determination Of CGG Repeat Number

Primers used for amplification of the CGG repeat region of *FMR1* gene were designed in the lab, using the Primer3 software; primer sequences and PCR conditions will be available on request. PCR amplicons were separated by 12% polyacrylamide gel electrophoresis, visualized by UV fluorescence following ethidium bromide staining and analyzed using Quantity One software. Using this system, repeats in the lower range (<180) could be easily detected; however, those in the higher range (~200-250 repeats) were not detectable.

Determination Of *FMR1* Gene Promoter Methylation Status

Genomic DNA (~5 µg) was deaminated using the EZ DNA methylation kit (Zymo research, USA). Methylation-sensitive PCR (MS-PCR) was carried out by following the method of Weinhäusel and Haas [29]. Multiplex PCR for simultaneous amplification of the *FMR*1 and *Xist* (X-Inactive Specific Transcript) promoter sequences was performed. Amplification was carried out in a final reaction volume of 20° mL containing 75 ng of sodium bisulfite-treated DNA, 1.0 U Taq polymerase (Bangalore Genei, India), 0.2 mM dNTP mix (Bangalore Genei, India), 1X Taq buffer B (Bangalore Genei, India) and 1.5 mM $MgCl_2$. Primer concentrations were as described by Weinhäusel et al and Haas [29]. After an initial denaturation at 95°C for 5mins, amplification was performed for: 35 cycle of denaturation at 95°C for 30 sec,

annealing at 60°C for 20 sec and extension at 72°C for 40 sec; cyclic reaction was followed by a final extension at 72°C for 7 mins. PCR amplicons were analyzed in a 2.5% agarose gel, followed by ethidium bromide staining and visualization under UV fluorescence.

Results

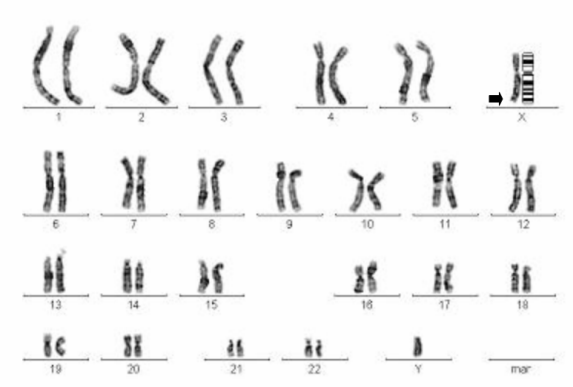
Out of the 157 cases screened, only five cases (3.18%) were confirmed as FXS, among which four were mosaic and one was homozygous for methylation of the *FMR1* promoter. Details of the results are given in [Table/Fig 1].

(Table/Fig 1) Subjects assessed during the study (N=146)

Ser. No.	Disorder	Mean Age	Subjects (n)	FMRP < 20% +ve cells	FMRP >20% +ve cells	CGG repeat number	MS-PCR for promoter methylation
1	ADHD	7.54±3.27	16	3	13	22-58	None
2	ASD	5.39±2.28	34	3	31	24-156	None
3	DS	7.88±7.28	35	3	32	18-176	None
4	MR	9.3±5.85	70	10	60	17-180	4 male subjects
5	Epilepsy	7.7±2.4	2	0	2	29	1 male subject

Chromosomal Investigation

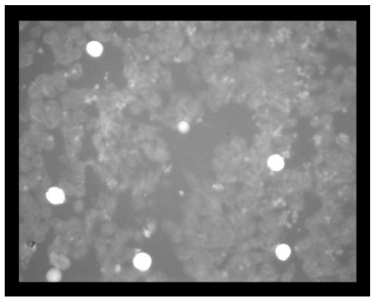
All DS cases recruited for this study showed trisomy 21. Among the MR cases, one was confirmed as Prader-Willi syndrome (deletion in 15q11-q13 region). In the FXS cases, deletion of Xq27.3 was detected in 50~54% of dividing cells [Table/Fig 2]. No other chromosomal abnormalities were observed.



(Table/Fig 2) Karyotype of a FXS patient with Xq27.3 del (inset shows deleted region at Xq27.3)

Result Of Immunocytochemical FMRP Expression Test

The percentage of FMRP positive cells [Table/Fig 3] varied between 10%-93% in different neurobehavioural patients. In ADHD cases, the average percentage of FMRP positive cells was found to be 37.26±19.64 (Table/Fig 1]. Among the ASD patients, the mean FMRP positive cell percentage was found to be 49.99±22.6. In DS subjects, the FMRP positive cell percentage was 44.56±19.64. Among the MR and epilepsy cases, the mean positive cell count was 43.27 and 48.14 respectively, of which 10 MR cases were found to have less than 20% positive cells. Among the MR cases, 2 were confirmed as FXS, one with full mutation and the other one with mosaicism [Table/Fig 4]. Other MR subjects with 35.29% and 76.47% FMRP positive cells were confirmed as FXS mosaic. One patient with epileptic seizures was also confirmed as mosaic FXS, with 42.8% FMRP positive cells.



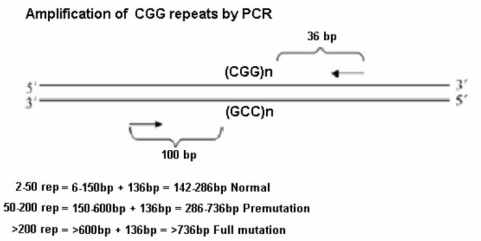
(Table/Fig 3) Representative picture of FMRP positive cells stained with anti-FMRP antibody –conjugated with FITC.

(Table/Fig 4) Cases confirmed for Fragile X syndrome.

Case ID	Sex/age	FMRP staining	Repeat PCR	MS-PCR		Remark
				fmr1 promoter	*Xist* promoter	
1 MR	Male/3.4	17.35%	No product	U/M	M	Fragile X (mosaic)
2 Epilepsy	Male/6	42.8%	No product	U/M	M	Fragile X (mosaic)
3 MR	Male/13	35.29%	69/28/22 repeats	U/M	M	Fragile X (mosaic)
4 MR	Male/9.2	13.46%	36/28 repeat	M	M	Fragile X (full methylation)
5 MR	Male/11	76.47%	65 repeat	U/M	M	Fragile X (mosaic)

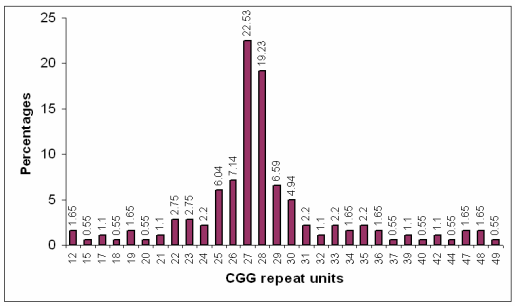
Repeat PCR Analysis

Primers used for the repeat PCR assay identified 136 bp of unique sequence along with the CGG repeat sequence [Table/Fig 5].



(Table/Fig 5) CGG repeat amplification by PCR. Excluding the repeat region the common size is 136bp (100bp starting from reverse primer and 36bp starting from forward primer). Therefore, the size of the different repeat alleles would be [136bp +(CGG)n], where n is the number of CGG repeat units.

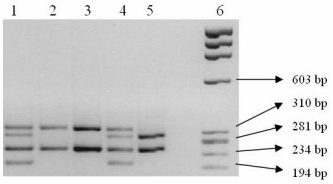
The repeat numbers varied within 12-49; the most common repeat being 27 (22.53%), followed by 28 (19.23%), 26 (7.14%) and 29 (6.59%) repeats [Table/Fig 6]. Samples from men normally showed only one allele, excepting a few cases where PreM alleles were found along with the normal allele. Among the 45 women subjects, 27 females showed homozygosity for a particular type of repeat and in the remaining 18, at least two different alleles were observed. Among ADHD patients, one patient had a PreM allele (58) along with the other normal allele. In ASD patients, 9 cases were found to have PreM alleles, with repeat sizes ranging from 53-156. In case of DS, 6 cases had PreM alleles ranging from 53-172 repeats. Out of the 70 MR cases, 9 cases had PreM alleles containing 51-180 repeats. No PreM allele was detected in the two epilepsy cases [Table/Fig 1].



(Table/Fig 6) Different CGG repeat alleles (12-49) detected during the study.

MS-PCR analysis

Only those cases which had a low FMRP level (< 20%) or had repeat alleles in the PreM range, or failed to amplify during repeat analysis, were considered for promoter methylation analyses by MS-PCR. Out of the total 80 cases evaluated, five cases were confirmed as FXS, all of which were male; four of them were mosaic, having both methylated (PM) and unmethylated (PU) promoters in the *FMR1,* as well as in *Xist*. In the fifth sample, only one band for the PM was obtained [Table/Fig 7],[Table/Fig 4].



(Table/Fig 7) Representative gel picture for MS-PCR showing PCR amplicons for PU (318bp), PM (280 bp), XistM (241 bp), XistUM (198 bp). lane1: normal female; lane 2 and 3: normal male; lane 4: male with methylation mosaicism; lane 5: male with full methylation (FRAXA) and lane 6: molecular weight marker (ϕX 174 DNA/ Hae III Digest).

Discussion

In 1943, Martin and Bell first reported a large family with 11 mentally retarded males and a few mildly affected females that showed MR segregating as an X-linked trait [37].

The cytogenetic marker, delXq27.3, is observed in ~60% cells [38] (which were cultured in folic acid or thymidine deficient culture media)[39] of affected male individuals and as well as female carriers. Until 1991, cytogenetic testing of the fragile X site was the only means for diagnosis of FXS. In the present investigation, by karyoptype analysis, Xq27.3 deletion was observed in only 50-54% of cells of FXS subjects, confirming the previous reports.

Because of the complexity and uncertainty of the cell culture method, further development in the detection of FXS was necessitated, which led to the development of methods like Southern blotting and MS-PCR analyses [28],[29],[30]. For population screening, the immunocytochemical detection of FMRP was devised [31]. The drawback of the

immunocytochemical method lies in the fact that, even in individuals with a full mutation, a base line FMRP is detectable. This is also evident from the present investigation, since in mosaic FXS cases, the FMRP positive cell percentage was ~40%. Therefore, the immunocytochemical method is not reliable for the diagnosis of FXS.

We have observed that both the repeat PCR and MS-PCR analysis are useful for the rapid screening of FXS and 157 neurobehavioural patients were screened using a combination of these two methods. We could detect only five men affected with FXS, one with full promoter methylation and four with methylation mosaicism, representing a frequency of 3.18%, which is consistent with the frequencies observed by Maino et al. [40] and Brown [41], which ranged from 2-7%. This result is also consistent with the frequency observed by Elango et al. [42] in Indian male patients with MR (2.8% prevalence of FXS). On the contrary, previous studies carried out on mentally retarded Indian males revealed ~7% possibility of having FXS [4],[5],[6],[7]; this could be due to population differences, since the Indian population is extremely heterogeneous.

One significant observation made in this study, was the low frequency of premutation alleles (6.25%) in ADHD patients who have normal intelligence [Table/Fig 1]. In the MR cases also, PreM allele frequency was comparatively lower (12.9%). No PreM allele was detected in the two epilepsy cases. On the contrary, in ASD and DS patients with neurodevelopmental deficits, PreM alleles, with repeat sizes ranging 53-172, were present in higher frequency (26.5% in ASD and 17.1% in DS). From the above observations, we conclude that premutation alleles are associated with at least moderate impairments in intellectual functioning, while it is absent in individuals with normal intelligence or mild impairment only.

Among the 45 women samples studied, seven PreM alleles were detected. Rousseau et al. [43] reported the frequency of PreM to be 1:500 X chromosomes in a large group of women samples. However, it is difficult to make such a conclusion from the present study, due to small sample size.

Among the 30 different normal CGG repeat alleles found in the present study, $(CGG)_{27}$ followed by $(CGG)_{28}$, appeared to be the most frequent alleles in this population. $(CGG)_{12}$ was the smallest and $(CGG)_{49}$ was the largest allele observed [Table/Fig 7]. A report from the southern part of the India also documented $(CGG)_{28}$ and $(CGG)_{31}$ as the most frequent alleles [5]. A study conducted on non-retarded Japanese subjects also showed $(CGG)_{28}$ to be the most frequent allele [44]. On the other hand, studies on Caucasians, Hispanics, Blacks, and Chinese reported $(CGG)_{29}$ to be the most prevalent allele [45],[46].

Methylation of the CpG island near the CGG repeat sequence, which influences expression of the *FMR1* gene, was studied by MS-PCR. [29]. We have observed full methylation in only one male MR patient and methylation mosaicism in three male MR patients and one male epilepsy patient. None of the female probands were found to be affected by FXS. However, the major drawback or limitation of the MS-PCR method was its failure to detect mosaic females [29].

Based on the data presented above, we may state that the frequency of FXS among neurobehavioural outpatients is rather low (3.18%) in the studied population. However, the present study emphasizes the importance of screening neurobehavioural patients for FXS, since psychiatric evaluation alone may not be sufficient for diagnosing FXS patients. It is also stated that for screening a large number of individuals, a combination of tests would be beneficial. PCR-based techniques could be used for short listing probands, followed by Southern blot analysis for confirmation, thus saving time while making it cost effective.

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