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Original Research Article

Molecular studies in children with fragile X syndrome

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ABSTRACT

Background: Fragile X Syndrome (FXS) is the most common single gene cause of Learning (intellectual) Disability (LD). FMR1 gene mutation is the commonest cause for this syndrome. The present study aims to analyze the incidence of the syndrome in Kerala population.
Methods: Study was conducted among 86 children belonging to different places of Kerala. Children were selected on the basis of IQ scores and typical features of FXS. Blood samples were taken and routine karyotype was performed. PCR analyses were also conducted.
Results: Majority of the children showed typical features of FXS. Out of 86 samples, six showed chromosomal aberrations were excluded. PCR analyses in 55 samples, screened 35 samples with FMR1 mutation, in which 26 samples having pre- mutation and 9 samples with full mutation.
Conclusions: Through this genetic study, differential diagnosis of LD children with FXS, LD children with constitutional chromosome abnormalities, and LD children without any apparent genetic abnormalities could be established.

Keywords: CGG repeats, Fragile X syndrome, Fragile site, Learning disability, PCR analysis

INTRODUCTION

Fragile X Syndrome (FXS) is the most common single gene cause of Learning Disability (LD). It is an X-linked recessive disorder, which affects approximately 1 in 2500 females, and 1 in1250 males worldwide. The gene responsible for FXS is Fragile X Mental Retardation (FMR-1) gene. Affected male individuals are having large ears, long and narrow face, prominent jaw, and macro-orchidism after puberty.[1,2] The fragile site is a non-staining gap which is located in the long arm of X-chromosome (Xq27.3) and is designated as FRAXA. Lubs, demonstrated the presence of fragile site in long arm of X chromosome in children with FXS and an analysis of their fragile karyotyping revealed a slight break or fracture on X chromosome in this region.[3] The most suggestive clinical criteria for the diagnosis of FXS are LD, large or prominent ears, an enlarged face, ADHD and Autistic like behaviour. If a patient has five of these features, then no case of FXS could be missed.

There are genes coding for intellectual function located on X chromosome and many disorders associated with the X chromosome aberrations, a large number of these have neurobiological roots and behavioral manifestations. One of such disease is FXS, which is the most common inherited form of LD. The elusive mutant gene, which causes FXS is called FMR1 gene, which is transmitted stably from parent to offsprings in humans as well as in divergent organisms. It contains a variable trinucleotide repeat sequence, CGG (Cytosine- Guanine-Guanine) which can become unstable over successive generations. The most common mutation reported is the expansion of triplet CGG in the 5' untranslated region of the FMR1 gene on Xq 27.3. The mutation consists of excessive copies of the CGG repeats (triplicate), which codes for

the amino acid arginine coincident with the cytogenetic folate-sensitive fragile site (FRAXA).[4,5] This expansive repeat results in the inability of FMR1 gene to be expressed. Females with FXS are usually less severely affected than males because of the presence of two X chromosomes.

FXS is caused by an expanded CGG above 200 units in the FMR1 gene resulting in the inactivation of FMR1 promoter and absence of the FMR1 mRNA and protein. The FMR1 protein is proposed to act as a regulator of mRNA transport or translation that plays a role in the synaptic maturation and function. For the identification of a fragile X mutation, several methods have been developed for detection of the expanded CGG repeat and abnormal methylation of the FMR1gene, both of which are diagnostically important factors. These protocols utilize either Southern blotting or PCR methodology. Non-radioactive PCR method has been reported by Chowdhury et al to be rapid and cheap method for initial screening followed by Southern blots for confirmation. Molecular characterization of the FMR I triplet expansion region requires the combined use of PCR to amplify normal and pre-mutation length alleles and Southern blot analysis to detect fully expanded alleles and assess methylation.[6] Analysis by PCR is useful for defining the precise repeat lengths of very small pre-mutations or alleles in the "*g*rey zone".

There are a number of children who showed typical features of FXS attended the OP clinics of Institute of Communicative and Cognitive Neuroscience (ICCONS), Trivandrum and Shornur, for treatment, counselling and further management. Even though genetics has become an area of significant interest in this century, no scientific studies on the genetic factors involved in the etiology of LD children with FXS has been carried out so far in Kerala population. Hence there is the need to elucidate the genetic factors underlying this syndrome, as a straight forward cost-effective service for the diagnosis, prognosis and genetic counseling. Objectives of the study was to investigate the clinical features - including phenotypic features and IQ levels in FXS suspected children. To analyze the CGG trinucleotide repeat lengths in children with FXS.

METHODS

IQ measurements

The concept of 1Q was formulated by William Stern, a German psychologist, in 1912. IQ is the quotient of mental age (MA) divided by the chronological (actual) age (CA) usually expressed as a multiple of 100 to avoid fractions. IQ has a positive correlation with LD.

The formula for calculation of IQ is expressed as

$$IQ = \frac{MA}{CA} X100$$

The scores on the different verbal and non - verbal tests were added together and the mental age of each child was assessed. The IQ was calculated with the help of standard formula and from the score obtained, children were categorized based on the reference IQ scores specified by WISC (1939).

Cytogenetic studies-karyotype analysis

Karyotyping or chromosome analysis is a fairly standard test in all the genetic studies. In the present study, karyotype analysis was done to investigate the presence of any constitutional chromosomal abnormality in any of the children with FXS. Metaphase chromosomes of study subjects were prepared using short-term micro - culture of the peripheral blood lymphocytes following the standard method of Arakaki and Sparkes modified by Manjunatha.[7,8] The slides were GTG banded using Seabright's method of chromosome banding.[9] For each sample, a minimum number of 20 metaphases were analyzed.

Molecular analysis

For molecular analysis, DNA from those children showing suspected features of FXS was extracted using standard phenol-chloroform extraction protocol.

Isolation of DNA from blood

Peripheral blood samples (5ml) were collected from the suspected children in plastic tubes containing EDTA. Genomic DNA isolation was carried out by routine organic method (Phenol-Chloroform extraction method), proposed by Sambrook et al.[10]

DNA quantification

The DNA quantification was done according to the standard formula. DNA protein concentration was evaluated.

Amplification of DNA

PCR master mix was prepared and Denaturation was done at 95ºC for 10 minutes. Taq Polymerase was added, Annealing was done at 64ºC and Extension at 72ºC for 30 cycles.

Agarose gel electrophoresis

Agarose gel was prepared. Ethidium Bromide was added and cooled and poured into the gel cast. Allowed the gel to solidity, submerged gel tray in electrophoresis tank, added 5µl of loading dye buffer to the 10µl PCR and centrifuged. Samples were then loaded into the sample wells. Electric current is applied. Distance migrated by the DNA in the gel was assessed.

RESULTS

Intelligence test was conducted for the children in the age group of 7 - 15. The IQ was assessed by means of standard intelligence test. IQ score was found to be ranging from 65 - 109.

So, the children were grouped under 4 categories. Those children whose IQ score was >69were under the category of difficulty in understanding and for those the IQ score ranged from 70-79 were in borderline intelligence, 80-89 were in low average group and with 90- 109 were in average group (Figure 1 Figure 2).

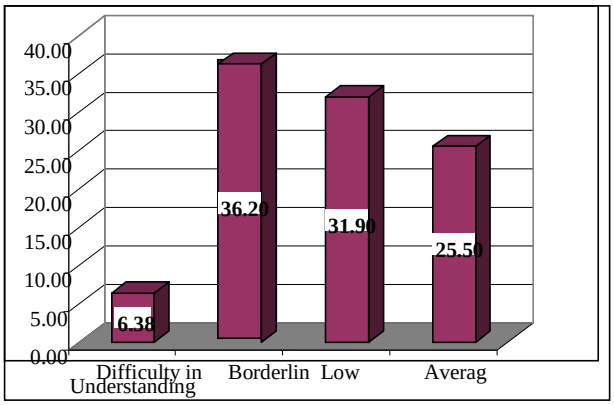

Figure 1: IQ score of male children.

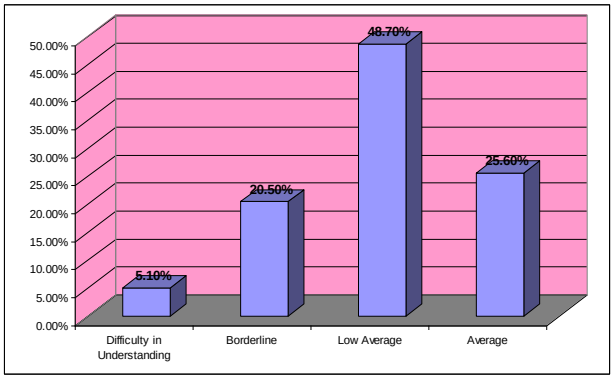

Figure 2: IQ score of female children.

Molecular analysis for FMR 1 gene mutation was carried out in 55 samples, which showed trinucleotide CGG repeats only in 35 samples.

Out of the 35 children who showed CGG repeats, 20 were males and 15 were females (Table 1 and Table 2). In the case of 20 male patients 3/20 showed more than 200 CGG repeats (sample Nos. 7, 9 and 35 respectively). Sample No 7 showed 225 trinucleotide repeats. In sample Nos. 9 and 35 the CGG repeats were 216 and 246 respectively. In the case of female patients 2/15 showed more than 200 CGG repeats (sample No.19 and 25). Sample 19 showed 231 repeats and Sample 25 showed 205 repeats.

Table 1: Number of CGG trinucleotide repeat sequence in male children having FXS.

Sample No.	Number of CGG repeats
1	62
5	70
7	225*
9	216*
11	112
13	91
14	125
17	65
20	120
22	166
26	160
30	90
32	99
34	125
35	246*
38	63
45	120
47	100
50	89
54	65

* Samples showing >200 CGG repeats.

Table 2: Number of CGG trinucleotide repeat sequence in female children having FXS.

Sample No.	Number of CGG repeats
3	110
4	91
8	99
12	90
16	180
19	231*
23	80
25	205*
27	135
28	96
31	140
33	145
41	90
49	69
51	75

* Samples showing >200 CGG repeats.

DISCUSSION

In the present investigation, intelligence test was conducted for the children in the age group of 7 – 15 and IQ score ranged from 65-109. In male LD children, IQ score >69 was shown by 6.38% and borderline IQ score was shown by 36.2%, 31.9% were of low average category and the remaining 25.5% were of average intelligence. When the female LD children are concerned 5.1% had >69 IQ and 20.5% were having borderline intelligence, 48.7% were in the low average category and the rest 25.6% were with average intelligence. The

surveys conducted by Blomquist on learning disabled populations also showed similar results and his study proved that it probably accounted for 4-8% of males with IQ less than 80.[11] In another study by de Vries et al., on a total of 105 LD samples, 21% had borderline IQ score between 70 and 85.[12]

In India, around 10% of school going children suffer from learning disorders. A national study conducted on 550 school children by the National Institute for Mentally Handicapped, Secunderabad, India in 1985, claimed the incidence of LD to be 4%. Sharma et al in his study in Indian population have shown the frequency of FXS among learning disabled males as approximately 7%.[13] Earlier, the prevalence of the FXS assessed based on cytogenetic diagnosis was originally estimated to be 1/1200 to /2600 in males and 1/1600 to 1/2400 in females. The Neurological Institute in Kerala in a survey conducted in 1997, revealed that 10% of school going children in Kerala is with one or more forms of learning disabilities. A cross - sectional, multi-staged, stratified, randomized cluster sampling study conducted among children with 8 - 11 years, from a south Indian city, reported the prevalence of LD as 15.17%.

The present study did not aim for the incidence survey of FXS among school going LD children in Kerala, as it was beyond the scope of the study objectives. But the study was mainly focused on genetic investigations on children with FXS. According to literature surveyed, LD affects about 3% of the population, yet the cause remains unknown in about 40% of the people with moderate to severe LD (IQ<50) and in 70% of people with mild developmental delay IQ>50.[14] It has been reported that 30% -50% of cases of undiagnosed LD may be genetic in origin. So it was of interest to explore and determine the contribution of genetic factors in the etiology of LD among school children in Kerala. In this study, special attention was also given to identify the spectrum of FXS among children with LD and delineate the contribution of genetic factors of FXS in the etiology of LD. The present study analyzed a selected sample population of LD children in Kerala and is the first genetic research conducted among the LD children in Kerala.

Routine cytogenetic analysis or karyotyping has successfully been used for the last 50 years in investigating the cause of patients with MR, specific organ malformation and dimorphisms, whether or not, they are part of a syndrome. Present day standard karyotyping, was carried out in dividing cells and using a 10,000 X magnification detects numerical as well as structural chromosome aberrations such as deletions, duplications, inversions and translocations, as long as they involve at least 5 to 10 million base pairs of DNA (5-10 Mb resolution). Chromosome abnormalities result from mutations which change the number of chromosomes (numerical chromosome abnormalities) or structure of chromosome (structural chromosome abnormalities) can be observed and identified through present day standard karyotyping.

Conventional cytogenetic analysis carried out in 86 children with LD, revealed constitutional chromosome abnormalities in 6 children (6.98 %). The abnormalities involved only structural chromosome abnormalities, such as chromosome deletions, chromosome translocations, ring chromosomes etc. No numerical chromosome abnormalities were observed among these patients. In this study, attention was given to exclude known syndromes such as Down Syndrome, Edward's Syndrome, Patau Syndrome, Klinefelter's Syndrome, and Turner's Syndrome, which are known chromosomal disorders associated with numerical chromosome abnormalities. This could be the reason for absent detection of numerical chromosome abnormalities among the LD children studied. Cytogenetic studies conducted by Shin on 259 children with LD, Hong, on 604 children, also proved the significance and effects of karyotyping in identifying chromosomal abnormalities.[15,16] Out of the 86 children studied cytogenetically, two showed chromosome deletions involving chromosomes 4 and chromosome 20, 2 showed translocations between chromosomes 3 and 12 and also between chromosomes 13 and 14 and another 2 showed ring chromosomes, involving chromosomes 1 0 and 15.

Molecular analysis is more sensitive test for identification of FXS. For this, trinucleotide CGG repeat lengths as a result of the FMR1 mutation, was investigated in 55 LD children doubted with FXS, employing PCR based molecular test. Molecular studies are used to test for fragile X genotype of individual with symptoms of the FXS and individuals at risk of carrying the mutation, by examining the size of the trinucleotide repeat segment and the methylation status of the FMR1 gene. These approaches are generally employed by PCR and Southern blot analysis.

According to Strelnikov et al PCR assay is a powerful non-invasive neonatal screening method capable of detecting genetic abnormalities in newborn boys.[17] The present investigation also proved that by using non-radioactive PCR, the exact number of CGG repeats could be identified in both sexes. PCR is the most suitable screening tool for detecting the FXS and is very rapid test and has high sensitivity for normal, lower pre- mutation repeat size. Wang et al developed an assay which allowed simultaneous amplification of the triplet repeat sequences at the FRAXA loci by PCR, and detection of the products on non-denaturing gels stained with Ethidium bromide.[18] In the present study, the investigator used same protocol for molecular analysis to detect the CGG rep

In the present study, out of the 55 samples analyzed by this technique, 35 showed trinucleotide CGG repeats in varying premutation and mutation ranges. In the study by Turner, the prevalence of females with a full mutation was the same but the prevalence of symptomatic females was

approximately half.[19] The present study used the molecular level of investigation as a key tool in the detection of FMR 1 gene mutation. Studies by Pieretti et al and Sutcliffe et al had shown that methylation of the CGG repeat and surrounding region correlates with transcriptional silencing of the FMR1gene and expression of the fragile X phenotype.[20,21]

In a European study of 213 FXS by Xuncla;[22], 17.6% of those were found to be FXS carriers. NBS has recently captured attention in the use of new PCR-based population screening approaches and with the introduction of targeted treatments with encouraging results.[23-25] The results of a pilot study for FXS in the United States, based on the screening of 11,217 newborns, indicated that the observed prevalence of a premutation allele is 1:188 in females and 1:480 in males, while the prevalence of gray-zone alleles is 1:70 in females and 1:107 in males.[26] Molecular screening for FMR1 gene mutation in 294 suspected cases of FXS have reported 36 cases (12.2 %) as showing FRAXA mutation. Out of this, 23 cases (7.8 %) were found to have full mutation for FRAXA and 13 females to have pre-mutation. PCR based screening has been reported to be more reliable test for screening FXS;[6]. Chetan, screened 300 patients with LD for FXS of which 8.6% showed fragile X etiology.[27] Karmasagar et al used methylation specific PCR for detection of FMR1 mutation in 25 males with LD, suspected to have FXS.[28] Out of these 25 patients, they detected one full mutation and one premutation.

Pre-mutation prevalence was found to be different in various ethnic groups; it was higher in people of mixed European descent compared with that in African American and Hispanic people and shows a higher incidence compared with that in previous studies by Fernandez-Carvajal et al.[29] Study conducted by Reiss et al, had taken the efforts to demonstrate the exact length at which the CGG repeat becomes unstable, because of the considerable overlap between repeat lengths at the high-end range found in the general population and the small range of premutation alleles detected in fragile X families.[30]

In another study by Pembrey et al on FXS patients, it has been reported that, few cases were individuals with average intelligence, although patients with FXS suffer from profound to borderline mental disability, and the presence of a methylated full mutation is routinely associated with their cognitive dysfunction.[31] The observations and findings in the present research study also proved that majority of LD children were pre-mutation carriers of CGG repeat lengths, had average intelligence.

No specific treatment is available for FXS. Supportive therapy for children and adults with this syndrome currently consists of the following.[32,33] Treatment of manifestations of FXS include early developmental intervention, special education (individual attention, small class size, and avoiding sudden change and excessive stimulation), and vocational training; individualized pharmacologic management of behavioral issues that significantly affect social interaction and routine treatment of medical problems.

CONCLUSION

Learning disabilities are developmental disorders that arise in childhood and typically affects on academic performance, affecting written expression, deficits in handwriting speed, spelling, vocabulary complexity and verbosity, deficits in visuo-spatial skills, abstract and conceptual thinking, planning and problem solving, motor, mathematical and interpersonal skills. The study subjects were selected from ICCONS, Trivandrum and Shornur, Kerala State. A large number of children with LD from all over Kerala attend ICCONS for consultation and therapies. The present study on 86 children with LD showed constitutional chromosome abnormalities (accounting for 6.98 %) and CGG trinucleotide repeats as a result of FMR1 full mutation and pre-mutation (accounting for 43.75 %) contributed as genetic factors in the etiology of LD children. Through genetic studies, differential diagnosis of LD children with FXS, LD children with constitutional chromosome abnormalities, and LD children without any apparent genetic abnormalities can be established. This kind of diagnostic accuracy has implications for prognosis and planning appropriate intervention programmes.

LD is a disorder, which not only affects those who suffer from it but also the people around them. It is an important disorder in which proper and earlier genetic investigation is necessary to modify the lifestyles of people who are affected. It is clear from the present investigation that, various parameters like IQ, cytogenetic and molecular diagnosis are very useful for differential diagnosis of LD with and without FXS.

Among this, molecular diagnosis is more reliable for confirmation of FXS. This study suggests that simple PCR combined with cytogenetic analysis for fragile site could be a reliable inexpensive test that is feasible for a large-scale screening of subjects with LD to identify the FXS cases.

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