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Applications of Cytogenetic and Fragile X Molecular Testing in Individuals with Mental Retardation

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ABSTRACT

Objectives: The fragile X syndrome (FXS) is the most frequent cause of inherited mental retardation (MR). It is caused by the progressive expansion of (CGG)n trinucleotide repeats located in the promoter region of the (Fragile X mental retardation 1 gene) FMR1 gene at Xq27.3. The aim of the study is to estimate the prevalance of the FXS and other chromosomal aberrations by cytogenetic and molecular analysis in patients with MR and language disorders.

Material and Method: 72 cases with MR who were sent to our laboratory for molecular and cytogenetic search in term of fragile X. The lymphocyte culture was carried out according to standard methods. DNA extraction was done using whole blood DNA extraction kit (Bangalore Genei, Bangalore). This was followed by bisulphite treatment and PCR amplification.

Results: Chromosome abnormality was found in 12 cases (16.7%). 7 cases (9.7%) were detected fragile X positive in molecular analysis. The mutations were detected such a full mutation and abnormal methylation in (4.1%) and a premutation carrier (5.5%). We concluded that chromosomal studies in mentally retarded patients help in accurate diagnosis and proper prognosis followed by genetic counseling and management rehabilitation.

Conclusion: Due to recent molecular advances, our understanding of the perplexing genetic issues surrounding fragile X syndrome has grown and diagnostic techniques have become both reliable and readily available. *©2007, Fırat University, Medical Faculty*

Key words: *Mental Retardation, Fragile X Syndrome, Full Mutation, FMR1 Gene, Chromosomal Abnormalities.*

ÖZET

Mental Retardasyonlu Bireylerde Sitogenetik ve Frajil X Moleküler Testlerin Uygulanması

Amaç: Kalıtsal mental retardasyonun (MR) en yaygın sebebi Frajil X Sendromu'dur (FXS). Xq27.3'deki (Frajil X mental retardasyon 1) FMR-1 geninin promotor bölgesinde yer alan (CGG)n üçlü nükleotid tekrar sayısının artmasıyla bu bozukluk meydana gelmektedir. Bu çalışmanın amacı, MR ve konuşma güçlüğü bozukluğu olan vakalarda, sitogenetik ve moleküler analizlerle diğer kromozomal anormallikler ve FXS'unun insidansını ortaya koymaktır.

Gereç ve Yöntem: Laboratuarımıza Frajil-X açısından sitogenetik ve moleküler araştırma için yollanan MR'lu 72 olgu çalışıldı. Lenfosit kültürü standart metodlara göre yapıldı. DNA ekstraksiyonu, tüm kandan DNA ekstraksiyon kit (Bangalore Genei, Bangalore) kullanılarak yapıldı. Bunu bi sülfitle muamele ve (polimeraz zincir reaksiyonu) PZR amplifikasyonu izledi.

Bulgular: 12 olguda (%16.7) kromozomal anomali bulundu. 7 olguda (%9.7) moleküler analizde frajil X pozitif olduğu tespit edildi. %4.1'inde tam mutasyon ve anormal metilasyon ve %5.5'inde premutasyon taşıyıcısı olduğu saptandı. Mental geriliğe sahip hastalarda genetik danışma ve yönetim rehabilitasyonu tarafından yapılan kromozomal çalışmaların doğru tanı ve düzenli prognoza yardımcı olduğu sonucuna vardık.

Sonuç: Son zamanlardaki moleküler ilerlemelere bağlı olarak, frajil X sendromunu da kapsayan zihni karıştırıcı genetik konularını daha kolay anlamaktayız. Böylece diagnostik teknikler hem güvenilir hem de kolay ulaşılabilir hale gelmiştir. *©2007, Fırat Üniversitesi, Tıp Fakültesi*

Anahtar kelimeler: *Mental Gerilik, Fragile X Sendromu, Tam Mutasyon, FMR1 Gen, Kromozomal Anormallik.*

Fragile X syndrome (FXS) is the most frequent cause of familial mental retardation and it is also one of the more common genetic diseases as it constitutes about one third to a quarter of the patients with X-linked mental retardation. It is also the second most common cause of mental disability after Down's syndrome (1,2). Most of the studies show prevalence of fragile X mental retardation (FXMR) amongst the target population of mentally retarded males of unknown etiology between 0.5 to 3% (3). The incidence of Fragile X varies from 1/1200 in males to 1/2400 in females: no differences have been observed among ethnic groups and many different loci have been identified (1,4,5).

Clinical features show marked heterogeneity. Characteristic features such as long face with prominent mandible, large ears and macro orchidism are seldom noticed in pre-pubertal children (6). Mental retardation varies from mild to profound retardation with affected males being more severely affected (7,8). There are several sub-types of Fragile X. The most common is the fragile X (FRAXA) syndrome. In the normal population, the CGG repeat varies from six to 52 units. Phenotypically normal carriers of the fragile X syndrome have a repeat in the 52 to 200 range (the premutation). Affected subjects have expanded CGG repeats (>200) in the first exon of the FMR1 gene (the full mutation) (4).

The aim of the study is to estimate the prevalance of the fragile X and other chromosomal aberrations by cytogenetic and molecular analysis in 72 patients with MR and language disorders.

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MATERIAL AND METHODS

In this study, cytogenetic and molecular analysis were performed. Fragile X can be diagnosed cytogenetically by searching metaphase chromosomes in the culture of lymphocytes using basal medium without folic acid. In our study, 72 cases with MR who were sent to our Medical Biology and Genetic Department of Medical Faculty in Firat University for molecular and cytogenetic search in term of fragile X in term of 2003 to 2006, retrospectively. At all of the cases, the chromosomes from the metaphases that obtained by either standart methods or basal medium without folic acid for fragile X were evaluated. For each case at least 100 metaphases were evaluated for fragile X. All numerical or structural anomalies were recorded according to the International System for Human Cytogenetic Nomenclature 2005.

DNA extraction was done using whole blood DNA extraction kit (Bangalore Genei, Bangalore). This was followed by bisulphite treatment and PCR amplification as per the method described earlier (9). Accordingly, DNA was diluted in 50 µl of distilled water and 5.5 µl of 2M sodium hydroxide was added. To create single stranded DNA, it was incubated at 37°C for 10 min and 30 µl of 10 mM hydroquinone was added to each tube. Hydroquinone was freshly prepared by adding 55 mg of hydroquinone to 50 ml of water. To this 520 µl of freshly prepared sodium bisulphite was added. Sodium bisulphite was prepared by adding 1.88 g of sodium bisulphite to 5 ml of water and pH was adjusted to 5.0 with sodium hydroxide.

The reagents were properly mixed with DNA and a layer of mineral oil was added. An incubation period of 16 h was followed at 50°C. The single strand DNA was purified using Wizard DNA cleanup system (Promega, USA) and then desulphoned by adding sodium hydroxide to a final concentration of 0.3 M and incubated at 37°C for 15 min. The solution was neutralized by addition of ammonium acetate (pH 7.0). The DNA was precipitated with 4 volumes of ethanol, separated by centrifugation, dried and resuspended in 50 µl of double distilled water. For the PCR amplification, the following primers (Bangalore Genie, Bangalore) were used for amplification of CpG island located upstream of the repeats-FR611R: CGT CGT CGC GTT GTC GTA C and FR690F: AAC CAC GAA CCG ACG ACG. These primers complimented the modified antisense strand and were specific for the amplification of methylated C present in affected individuals and normal FMR1 gene on the inactive X chromosome. PCR amplification was performed in 50 µl volumes containing 0.2 mM dNTPs, 0.5 µM of each primer, 200 ng of bisulphite treated DNA and 1U of Taq polymerase (Bangalore Genei, Bangalore). The thermocycling programme consisted of 5 min denaturation at 94°C, followed by 32 cycles at 93°C for 30 seconds, 65°C for 30 seconds and 72°C for 30 seconds and a final extension of 10 min at 72°C in a PTC 100 Thermocycler (MJ Research Inc., USA). For amplification of fragments containing repeats, the following primers were used. FR526R: GGG AGT TTG TTT TTG AGA GGT GGG and FR754F: CAA CCT CAA TCA AAC ACT CAA CTC CA. The PCR products were separated by electrophoresis on 2 per cent agarose gel containing ethidium bromide and photographed using Gel Doc system (Hero Lab, Germany).

RESULTS

The majority of the 72 cases were obtained from Firat University Medical Center Neurology Department after clinical observation. Included in the study were 72 cases (23 girls and 49 boys). The mean age of cases was determined as 11.63 (1.5-18). The first two complications of the cases were MR and preservative speech. Seven cases were diagnosed as fragile X syndrome cytogenetically (Figure 1). The chromosomal abnormalities seen in our patients have been showed in Table 1. A chromosome abnormality was found in 12 (16.7%) cases. Chromosomal abnormalities are an important cause of mental retardation and its frequency increased with the severity of mental retardation. Fragile-X testing by PCR analysis were detected be fragile X positive in 7 cases (9.7%).

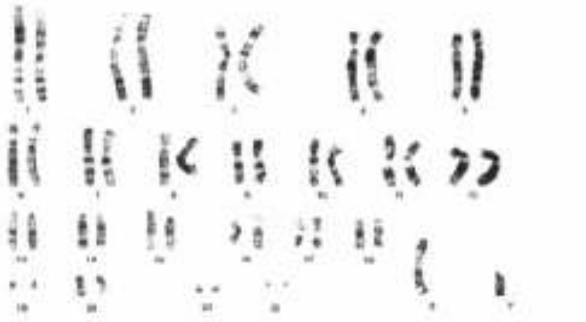

Figure 1. Fragile site at Xq27.3 as observed in the karyotype of case no: 36.

Table 1. Lists the chromosomal abnormalities seen in our patients.

Case No	Sex	Age	Karyotype
14	M	7	46,XY, 15ps(+)
35	M	7	46,XY, Fra Xq27.3 (%10)
36	M	16	46,XY, Fra Xq27.3 (%12)
37	F	18	46,XX, Fra Xq27.3 (%17)
44	M	4	46,XY, Yqh (+)
45	M	1.5	46,XY, inv9 (p13-q21)
55	M	8	46,XY, inv9 (p13-q21)
57	M	1.5	45,XY, rob (15:15)
66	M	3.5	46,XY, Xq27.3 (%2), Fra Xq22 (%2), Fra 3p14(%4)
67	F	18	46,XX, Fra Xq27 (%3.2) , Fra 3p13(%5)
68	M	8	46,XY, Fra 1q24(%3.3), Fra 5q23 (%3.3)
75	M	12	46,XY,Yqh(+), Fra Xq27 (%2)

The PCR products were assessment by electrophoresis. In 72 cases, the number of repeats ranges from 7 to 200 copies. The 31 repeat allele (90.2%) was the most common, followed by the 30 and the 28 repeat alleles. Four cases (5.5%) were found to have premutation (60 repeat) (cases no: 66, 67, 68 and 75). Cases with full mutation (4.1%) were determined as 10% in case no 35, 12% in case no 36, 17% in case no 37 ratio of fragile X positive by cytogenetic analysis (Table 1). Five male

boys and their mother who were sent to our laboratory for cytogenetic search in terms of fragile X were determined as increase in satellite of chromosome 15 and heterochromatin region of Y chromosome, chromosome 9 with a pericentric inversion, robertsonian translocation of chromosome 15 (case no:14, case no: 44, case no: 45, case no: 55 and case no: 57). The boys and their mother had no fragile X chromosome. The rest of cases were not determined fragile X chromosome. Sixty five of all cases were determined 31 repeat allele (90.2%). In our study, 90.2% as frequency of normal allels, 4.1% as frequency of allels in patients with full mutation and 5.5% as frequency of allels in premutation carriers were estimated. In conclusion, the incidence of fragile X was consistent with the result of the fragile screening studies made in the individuals with MR.

With the first set of primers (FR611R and FR690F), designed for the CpG island upstream of the trinucleotide repeats, an 80 bp fragment was amplified in affected males, where as in healthy and carrier males, no PCR amplification could be detected (Figure 2). This is because of the mismatch between bisulphite treated DNA and primers. The second set of primers (FR526R and FR754F) amplified the fragment containing non methylated trinucleotide repeats. All normal samples showed bands at 280-300 bp. A 400 bp fragment was identified in a normal carrier males (Figure 3).

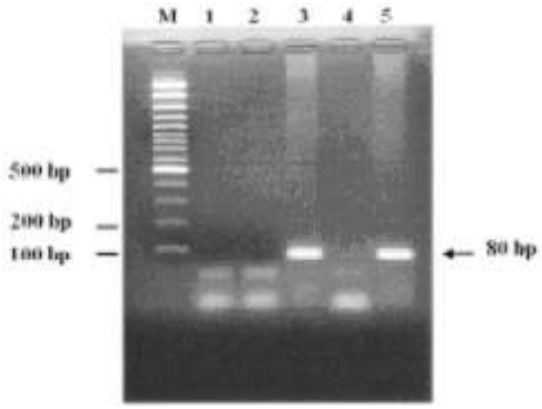

Figure 2. Result of PCR using primers FR611R and FR690F for amplifying the methylated C residues. Lane M, GeneRuler100 bp DNA ladder plus (Fermentas, USA); lane 1, negative control (PCR without DNA); lanes 2 and 4, negative samples (health males); lane 3, full mutation; lane 5, positive control.

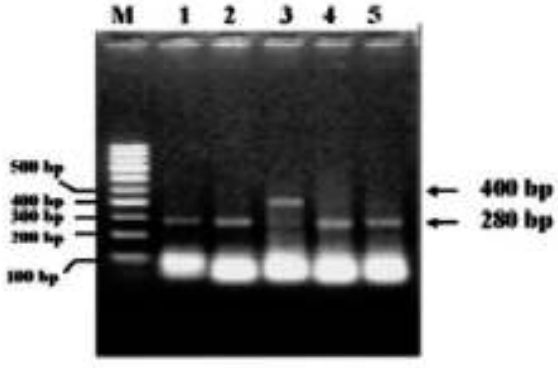

Figure 3. Result of PCR with primers FR526R and FR754F which amplifies the bisulphite treated unmethylated DNA sequences. Lane M, 100 bp ladder (Fermentas, USA); Lanes 1, 2, 4, 5 normal; Lane 3, carrier.

DISCUSSION

Of a total of 72 cases referred for fragile X testing, 7 (9.7%) were found to be positive for fragile X by either cytogenetics alone or by both cytogenetics and DNA testing, 12 (16.6%) were found to be positive for structural chromosomal abnormality, while 4 (5.5%) were found to exhibit a hetero-morphism. Positive chromosomal findings included abnormalities of the sex chromosomes and autosomes, deletions, translocations. Heteromorphism mostly involved an increase in the length of heterochromatic regions of certain chromosomes as well as a pericentric inversion of a chromosome 9, usually considered normal variants. It is concluded that chromosomal abnormalities other than fragile X are found with equal and, in some cases, higher frequency than the frequency of fragile X positivity in patients referred for a question of the Fragile X Syndrome. Our figures consistent with those reported in the literature, underscore the value of routine karyotyping in this population of patients (10).

Fragile X syndrome is inherited as an X-linked dominant inheritance (11,12). Frequencies of fargile X in previous studies have reported as 2.6 to 8.7% among moderate to severely retarded males and 2.9 to 5.4% in mildly retarded females (13). In our study population, frequencies of MR were determined as 3.75% in males and 2.5% in female. Our study consistent with previous studies.

Considering the technical aspects of cytogenetic analysis for FXS, usually a longer exposure to colchicine causes more chromosomal condensation, which turns fragile X easier to be detected by microscopic analysis. We used a shorter exposure (30 to 40 minutes instead of 60 minutes) which is less likely to interfere with the detection of other cytogenetic abnormalities. Although cytogenetic evaluation was sensitive and specific for the diagnosis of FXS, the fact that it is based in a 96-hours cell culture, which can fail, may require re-testing in several occasions. When the culture is successful, the study demands many hours of microscopic analysis, especially for the female specimens. In addition, it is well known that this method may not detect all carrier females, and would miss most male carriers, since only a small proportion of their cells will express the fragile X (14). The best advantage of this method is that it makes it possible to detect other chromosomal abnormalities, including the other fragile X sites with one single test.

Gender of carrier parent, gender of the offspring and the number of CGG repeats are important factors that influence disease expression. The complex pattern of inheritance poses an extraordinary challenge for accurate diagnosis and genetic counselling of affected families. Though a variety of clinical phenotypic characteristics has been described, none are singly or in combination helpful in definitive diagnosis. Though cytogenetic methods and PCR have been used in Turkish studies on Fragile X syndrome, methylation sensitive PCR has not been reported (15,16). In our study with 72 samples three cases with full mutation and another with premutation (carrier state) were detected. The carrier state and healthy normal samples were clearly distinguishable by the size of the amplified PCR product, as also the fragile X positive sample. The basic principle of this method is that it relies 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 primer pairs 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. The same C residues are however, unmethylated in healthy males, normal transmitting males and in the active X chromosome in females (17,18).

The disadvantage of this methylation PCR is that it cannot reliably diagnose affected females with fragile X syndrome due to the fact that the inactive X chromosome is already methylated. Recently modifications have been incorporated into methylation sensitive PCR strategies which reliably differentiate normal from carrier and full mutations, in both females and males (17,18). All our DNA samples in the present study were from male and female children with mental retardation. There is no specific treatment for fragile X syndrome. DNA tests for fragile X syndrome should be done in all mentally retarded children without an obvious cause, along with genetic counselling of the involved families. Methylation sensitive PCR strategy is one of the most comprehensive methods available at present for the accurate diagnosis of fragile X carrier and disease state (18). As a result, in our study, the frequency of normal allels, patients with full mutation and premutation were estimated in Elazig and vicinity. In this population, the frequency of fragile X syndrome that is an important problem for population health was found nearly 9.7% in this study firstly.

We believe that the recording and following of families with FXS will provide prenatal diagnosis and the usage of a possible treatment opportunity in the future. Although cytogenetic analysis in mentally retarded patients help in accurate diagnosis, our data show that all the members with/without clinical findings of FXS in the families with FXS should be screened by the PCR-based method to follow the transmission of the CGG repeats and to give correct genetic counseling to families. Our data suggest that expansion of CGG repeats in the FMR1 gene can be analyzed by Methylation sensitive PCR, an efficient and non-radioactive method that can be used to monitor the expansion of premutation to full mutation, which would eventually lead to reduce the FXS prevalence.

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