

Review Article

Molecular diagnosis and genetic counseling for fragile X mental retardation

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The fragile X syndrome is the most frequent cause of inherited mental retardation. It is caused by a dynamic mutation: the progressive expansion of polymorphic (CGG)_n trinucleotide repeats located in the promoter region of the FMR1 gene at Xq27.3. The cloning of the FMR1 gene and the elucidation of the molecular basis of the fragile X syndrome is of great importance for the diagnosis and understanding of this unusual type of mutation. Although extensively studied, the mechanism behind the transition from stable normal (CGG)_n alleles to the carrier state (an unstable premutation) and from premutation to mutation is partially understood. The clinical diagnosis of fragile X mental retardation (FXMR) is not possible as dysmorphic features are subtle. Molecular diagnosis by Southern Blot is the confirmatory test that makes carrier detection and prenatal diagnosis possible. As the risk of recurrence of FXMR is high in the family and carrier relatives, an identification of fragile X positive children, and offering carrier detection and prenatal diagnosis to the families is very important. It is possible by screening mentally retarded children and adults even if there is no family history of mental retardation or typical behavioral or physical features associated with the fragile X phenotype. In this review we have discussed the method for the diagnosis and counseling of the families. The complexities due to premutation and the variable severity of manifestations in carrier females need to be understood while counseling fragile X families.

Key Words: Fragile X mental retardation, Genetic Counseling, Mutation, Premutation.

Introduction

Fragile X mental retardation (FXMR) is the commonest cause of inherited mental retardation. It is caused by progressive expansion of (CGG)_n repeats, in the promoter region

of the FMR-1 gene at Xq27.3.¹ FXMR primarily affects males but approximately one-third of the carrier females are also found to be affected, though the severity of mental retardation in females is less than in the males.² The fragile X syndrome was the first triplet repeat disorder identified and served as a prototype for several diseases caused by triplet repeat expansions in the human genome.

In 1943, Martin and Bell³ described a family of sex-linked mental retardation without dysmorphic features. Although this was the first description of sex-linked familial mental retardation, an association between sex and mental retardation had been known since long, as institutionalized mentally retarded patients showed an excess of males among the severely retarded patients.⁴ Several reports about many other families with idiopathic mental retardation segregating in sex-linked fashion followed the Martin-Bell paper.⁵⁻⁷ In 1969, Lubs⁸ observed a marker X chromosome in a family with four males affected with mental retardation. All affected males as well as two females (one being the mother of two affected males) expressed a constriction at the end of the long arm of the X chromosome (Figure 1). It was an important and landmark observation, which led to the development of a diagnostic method for the fragile X syndrome. In 1977, Sutherland et al⁹ showed the importance of folic acid or thymidine deficient

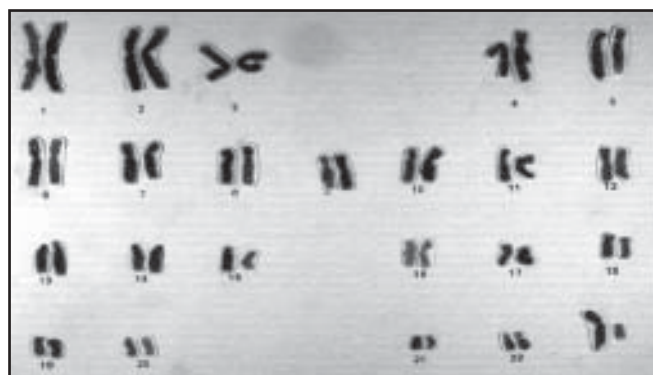


Figure 1: Karyotype of FXMR showing fragile site at Xq27.3 region

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cell culture medium for the expression of a fragile site on X chromosome patients. Therefore, folic acid deficient medium remains an important condition for expressing fragile sites for the cytogenetic diagnosis of FXMR.

During the re-examination of sex-linked mental retardation families by Turner and Turner¹⁰ fragile X expression was seen in six out of sixteen families. The presence of macroorchidism in post-pubertal affected males was also recognized as a feature of this syndrome. These findings were confirmed by several investigators¹¹⁻¹⁴ when several other families including the original Martin-Bell family were re-examined. In due course of time other clinical abnormalities were also defined. However, there is a marked heterogeneity in the clinical presentations of FXMR.

Clinical features

Fragile X syndrome is a very subtle dysmorphic syndrome and it is difficult to diagnose clinically (Table 1). Long face with prominent mandible, large and mildly dysmorphic ears and macroorchidism are the characteristic features of fragile X syndrome. The phenotype is subtle in young children¹⁵ and the features become prominent as the child grows. Hyperextensibility of the finger joints, pectus excavatum, mitral valve prolapse, strabismus and epilepsy are other commonly seen features.

Mental retardation (MR) in fragile X males varies from mild to profound with most affected males being moderately to severely retarded.¹⁶⁻¹⁸ Females are usually less severely affected than males.¹⁸⁻²⁰ A number of behavioral characteristics associated with FXMR have been described. They include hyperactivity, short attention span, stereotypic behavior (hand -flapping, -rubbing, or -biting, perservative speech, echolalia), poor eye contact, tactile defensiveness and anxiety related to social

contact.²¹⁻²⁶ Many of these features suggest the possibility of autism. A combination of 11 clinical studies on fragile X males performed between 1983 and 1990, indicated that 20% (ranging from 5 to 53%) FXMR patients had autistic features.²⁷

Due to lack of clinical diagnostic criteria, simple scoring systems have been developed to select individuals for fragile X diagnosis²⁸ but these scoring systems are mostly useful for population-based studies.

Genetics of fragile X syndrome

FXMR is an X-linked semi-dominantly inherited condition. The complex segregation pattern of the syndrome is unusual for a Mendelian trait. The occurrence of affected males and females in fragile X families suggests a dominant pattern of inheritance. However, the presence of unaffected males²⁹ who transmit the marker X chromosome to their daughters (also known as normal transmitting males, NTM) point to a mode of inheritance more complicated than a simple X-linked dominant mode.^{3,5,30} Sherman et al³¹⁻³² performed large-scale segregation analysis on fragile X syndrome pedigrees and observed a significant number of asymptomatic males and affected females and put forward a model of X-linked dominant inheritance with reduced penetrance (79% for males and 35% for females). It was proposed that an asymptomatic carrier male is more likely to have grandsons with the disorder than to have brothers with FXMR. Therefore, the penetrance of the disease increases in succeeding generations of a pedigree—an observation now known as the Sherman paradox. The mechanism responsible for the Sherman paradox became clear in 1991 with the cloning of the defective gene in the fragile X syndrome.

FMR-1 gene

In order to clone the gene responsible for the fragile X syndrome, a great deal of both genetic and physical mapping was done. Although the fragile site cosegregated with the syndrome phenotype, it was not known whether the syndrome was caused by the fragile site itself or a closely linked causal mutation. Pedigree analysis localized both the causal locus and the fragile site to a 22 cM region on the X chromosome between the factor IX gene and marker St14. Further studies revealed a number of linked markers that reduced the interval to 1–2 Mb and strengthened the localization of the causative locus to the fragile site.³³

In 1991, Fu et al¹ mapped the gene by the positional cloning method and it was designated as FMR-1 (Fragile X Mental Retardation gene 1). This gene consists of 17 exons, spanning 38 kb of Xq27.3.²⁹ The FMR-1 mRNA is 4.0 kb long, of which 1.9 kb is coding sequence, predicting a protein product of 631 amino acids.³⁴ In the non-coding part of the gene at the 5' end, there is a section of DNA composed mainly of tandem repetitive triplets of CCG, which corresponds to the fragile

Table 1: Phenotypic features of FXMR

Features	Frequency (%)
Long face	74
Macroorchidism (Testicular volume more than 30 ml)	74
Long ears	66
Flat feet	65
Hyperextensible joints	64
Prominent ears	63
High arched palate	48
Hand calluses	45
Pectus excavatum	43
Double jointed thumbs	41
Single palmer crease	35
Strabismus	33
Prominent jaw	28
Scoliosis	20
Perservative speech	91
Poor eye contact	87
Hand flapping	74
Tactile defensiveness	74
Hyperactivity	66
Hand biting	56

site on the X chromosome.

Molecular basis of the fragile X syndrome

Almost all cases of FXMR are caused by the expansion of CGG repeats in the 5' untranslated region of the FMR-1 gene²⁹ (Figure 2). Normal individuals have 6 to 50 CGG repeats, which are stably transmitted from generation to generation. In fragile X families the CGG repeat number exceeds the normal range in NTM and affected individuals. According to the size and methylation status of the expansion, fragile X mutations have been classified into premutations with small expansions of 50-200 copies of the CGG repeat (unmethylated), and full mutations ranging in size above 200 repeats, with concomitant hypermethylation of the nearby CpG island and the expanded CGG repeat itself. Full mutations are detected in individuals affected by the fragile X syndrome and also in a proportion of unaffected carrier females. As yet there is no universal agreement as to the number of repeats that should define the lower limit for the premutation range although most screening studies have used 55 repeats.

In contrast to the normal FMR-1 gene, premutations are unstable. Their instability is seen in virtually every transmission when passing to the offspring, size increases being much more common than occasional contractions of the repeat. One important factor that influences the rate and size of an expansion is the gender of the parent transmitting the unstable CGG repeat. A premutation has been documented to expand

to a full mutation in offspring with concomitant abnormal methylation only when it is transmitted by a female.¹⁹ The size of the CGG repeat expansion is another factor having a significant effect on instability; the larger the premutation, the more unstable it is upon transmission. Moreover, the risk of transition of a premutation to a full mutation, and consequently the risk of having an affected child has been shown to depend strongly on the size of the maternal premutation.^{1,36} It remains to be explained how an expansion of the (CGG)_n repeats leads to hypermethylation of the CpG islands.

Recent studies have completely changed the preexisting ideas about the premutation alleles of FMR-1 that other than serving as a source for full-mutation alleles in matrilineal transmissions of fragile X syndrome, such alleles do not give rise to clinical involvement. It is now clear that premutation alleles also contribute directly to clinical involvement. Although premutation females were reported to have normal cognitive abilities³⁵ premature ovarian failure has been observed in 21% of such carriers.¹⁹ Mild emotional problems have also been reported in 20% of the carrier females that are correlated with the number of CGG repeats.³⁷ Recent long-term follow-up studies show neurological signs involving intention tremor, ataxia, and cognitive decline, particularly among older male carriers of premutation alleles of the FMR-1 gene.³⁸ Premutation males may occasionally have cognitive involvement in childhood,³⁹ and a subgroup is at risk for late-onset neurological problems including executive function deficits, tremor, ataxia, and brain atrophy.³⁸ It is yet to be determined why certain subgroups of males and females with premutation are more vulnerable to CNS problems.

A small group of patients with fragile X phenotype do not show expansion of CGG repeats.⁴⁰ Intragenic deletion or point mutations in the FMR-1 gene have been observed in these patients.⁴¹⁻⁴⁶

Stability of CGG repeats

In the normal size range the CGG trinucleotide repeat alleles behave like other microsatellite markers, and they are stable upon transmission.¹ DNA sequencing of normal and premutation FMR-1 alleles has revealed that the number and position of AGG interruptions within the CGG repeat sequence may significantly influence the stability of the repeat, and in particular, the length of an uninterrupted CGG repeat sequence appears to be an important determinant of instability. Most normal alleles contain one or more regularly spaced AGG units and the uninterrupted CGG tracts do not exceed 30 repeats. In contrast, premutation alleles typically have none or at the most one interspersing AGG triplet and a CGG copy number exceeding 30 repeats at the 3' end of the repeat array. The AGG interspersions appear to confer stability and their absence gives rise to longer perfect CGG arrays with increased instability and predisposition to expansion.⁴⁷ Nolin et al⁴⁸ have further suggested that in addition to the repeat length and

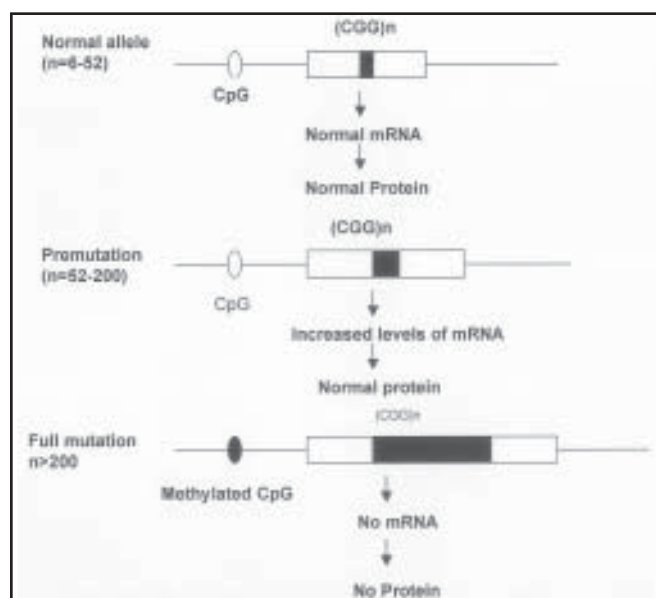


Figure 2: The molecular mechanism behind the fragile X mutation. In the normal population, the polymorphic (CGG)_n repeats of FMR-1 gene range from 6-52 CCG in size. Expansion of the repeat results in a highly unstable premutation (50-200 CGG). While passing on the premutation will expand further to full mutation (>200 CGG), which leads to hypermethylation of a CpG island located 250 bp 5' to the (CGG)_n region. Methylation of the CpG island results in the suppression of FMR1 mRNA transcription

the AGG content, as yet unidentified familial factors might influence the stability of CGG repeats.

The CGG repeat alleles that expand to pre- and full mutations have been shown to be in linkage disequilibrium with microsatellite⁴⁹ within the *FMR-1* gene or close to its 5' end. A significant linkage disequilibrium between the fragile X syndrome and certain microsatellite haplotypes suggests that fragile X mutations arose several thousands of years back and most of the present fragile X chromosomes have ancient origins.⁴⁹ This hypothesis is supported by the studies done in a genetically isolated Finnish population where 80% fragile X chromosomes and 8% of the normal chromosomes were found to be associated with certain haplotypes.⁵⁰ A similar kind of linkage disequilibrium was also observed in other Caucasian and African populations.⁴⁹ However, the disequilibrium has not been established in Asian populations including India.

Fragile X mental retardation protein (FMRP)

FMRP is a RNA-binding protein of 68-70 KD, in which extensive alternative splicing occurs. It is widely expressed with particularly high levels in the brain and testis.⁵¹ Post-mortem examination of the brains of fragile X patients and FMRP-deficient mice showed very long and thin dendritic spines in the neocortex. A unifying model for FMRP function is that it shuttles specific mRNAs from the nucleus to postsynaptic sites where mRNAs are held in a transitionally inactive form until synaptic input changes FMRP activity to allow mRNA translation. Recently, it has been observed that FMRP is associated with large numbers of mRNA whose genes are involved in important neuronal functions such as vesicle transport, signal transduction, etc. In the absence of FMRP these mRNAs become misregulated which may in turn lead to the mental retardation.⁵²

Prevalence of the fragile X syndrome

FXMR has been detected in all populations and ethnic groups studied with different frequencies. Most of the studies show prevalence of FXMR amongst the target population of mentally retarded males of unknown etiology between 0.5 to 3%.⁵³ However, higher prevalence up to 11% has also been reported (Table 2). It may vary from population to population but also depends on the selection of cases for study. Indian studies from New Delhi and Kolkata show a prevalence of 7 and 7.5% respectively amongst the mentally retarded population.⁵⁴⁻⁵⁵ Our experience of the molecular screening of 146 mentally retarded males without obvious etiology showed 2.5% prevalence of FXMR.⁵⁶ Cytogenetic analysis of individuals with mental retardation showed the prevalence of fragile X as 1/1200-1/2600 in males and 1/1600-1/4200 in females.⁵⁸⁻⁶⁰ This was probably an overestimation. Molecular techniques estimate the prevalence of FXMR as ranging from 1/3717-1/8918 in the Caucasian male population.⁶¹ For females, recent large stud-

ies have established the high prevalence of premutation carriers with a range from 1/246-1/468 in the general population.⁶¹

Diagnosis of fragile X mental retardation

Cytogenetic diagnosis of fragile X became possible when Sutherland showed that folic acid deficient cell culture medium could induce a chromosomal fragile site at Xg27.3, which was found to be linked to the mental retardation. After the mapping of the *FMR-1* gene, cytogenetic study is no longer considered a diagnostic method because of its false positive and false negative results. Major progress in molecular diagnosis was made soon after the cloning of the *FMR-1* gene and a direct molecular test became available which is confirmatory for fragile X diagnosis. In addition to direct mutation analysis, it is now possible to demonstrate the expression of *FMR-1* at the protein level by using monoclonal antibodies directed against FMRP.⁷⁰

Southern analysis

Southern blot analysis is considered as the gold standard for fragile X diagnosis. It can clearly distinguish between mutation and premutation alleles and can also provide information regarding methylation status. Digestion of genomic DNA from the patients with restriction enzymes spanning the *FMRI* (CGG)_n region followed by Southern hybridization with a radioactive probe is now the preferred method for the diagnosis of fragile X syndrome⁷¹⁻⁷² (Figure 3). A double digest using *EcoRI* and the methylation sensitive enzyme *EagI* or *BssHII* performs methylation studies of fragile X chromosomes. Since *FMR-1* is almost always methylated when the CGG expansion is beyond 230 repeats in males with full mutation, there is an increase in the size of the band corresponding to the *FMR-1* gene⁷² (Figure 3). One of the two X chromosomes in a normal female is inactivated and the *FMR-1* gene is methylated as a result of the process of lyonisation. An inactive X chromosome with a normal *FMR-1* gene shows a 5.2 kb band while an active X with a normal *FMR-1* gene in

Table 2: Prevalence of FXMR amongst cases without obvious etiology

Country	Cases	Method of analysis	Prevalence (%)
Australia ⁶²	8671	Cytogenetic	2.5
USA ⁶⁵	274	Cytogenetic	1.8
USA ⁶³	534	Molecular	0.5
Taiwan ⁶⁴	341	Cytogenetic	3.8
UK ⁶⁴	180	Molecular	2.2
Italy ⁶⁵	453	Molecular	11.0
Netherlands ⁶⁶	236	Molecular	4.2
Japan ⁶⁷	425	Molecular	2.7
Japan ⁶⁸	256	Molecular	0.8
Hong Kong ⁶⁹	324	Molecular	0.6
India ⁵⁴	130	Molecular	7.7
India ⁵⁵	98	Molecular	7.0
India ⁵⁶	146	Molecular	2.5
India ⁵⁷	360	Cyto/Mole	5.2

females and in males shows a 2.8 kb band. In the presence of premutation, the 2.8 kb PCR product increases in size. A female carrier with full mutation shows three bands corresponding to the normal unmethylated female pattern (active state - 2.8 Kb), methylated (inactive state - 5.2 kb) and an abnormal band of size greater than 5.2 kb reflecting hypermethylation and expansion of the FMR-1 mutation. Full mutations are highly unstable and give rise to smearing of the band. Mosaics for full mutations and premutation could be detected by the Southern hybridization method. Southern blot hybridization is time-consuming, costly and labor intensive. The limitation of Southern blot is that it cannot give the exact number of repeats which is especially necessary for premutation carriers and alleles in the gray zone, i.e. between 45 to 55 repeats.

Polymerase chain reaction (PCR)

Due to high guanine cytosine (GC) content, amplification of CGG repeat-containing regions becomes difficult by PCR. However, now the expansion of CGG repeat can be detected by using modified PCR methods. The advantages of PCR are faster diagnosis, requirement of only a small amount of DNA (<100 ng) and accurate sizing of the trinucleotide repeat in the FMR-1 gene. PCR-based diagnosis is feasible and reliable in the premutation (60-100 CGG repeats) carriers also. A lot of modifications have been made in the PCR protocol to increase the probability of amplifying across longer alleles. The use of nucleotide analog 7--deaza guanosine triphosphate makes amplification of long GC-rich repeats possible.

The disadvantages of PCR are that it is difficult to detect

full mutation alleles because of technical difficulties in performing PCR across hundreds of tandem repeated CGG triplets where the high content of GC and strong secondary structure make the amplification difficult. PCR also cannot detect mosaicism between premutation and normal alleles due to differential amplification. For many PCR protocols, the DNA fragment with the expanded repeats does not amplify. This is especially problematic for females and persons with repeat size mosaicism who could be misdiagnosed as normal. PCR based methods can be used for screening for Fragile X syndrome. The samples which fail to amplify by PCR and any female who appears to be homozygous should be tested by Southern blot analysis.

Antibody test

The antibody-based diagnostic method for detecting the presence or absence of FMRP is also possible in lymphocytes.⁷⁰ Cells of fragile X males with methylated full mutation produce no FMRP, while in individuals with normal FMR-1 or in

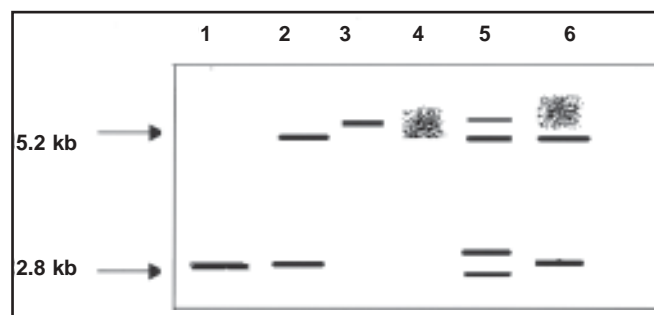


Figure 3b: Schematic representation of Southern blot hybridization pattern of FMR1 gene. Lanes 1, 2 represent normal male and female respectively; Lanes 3 and 4 show full mutation in male; Lanes 5 and 6 depict female carrier of premutation and female carrier of full mutation respectively

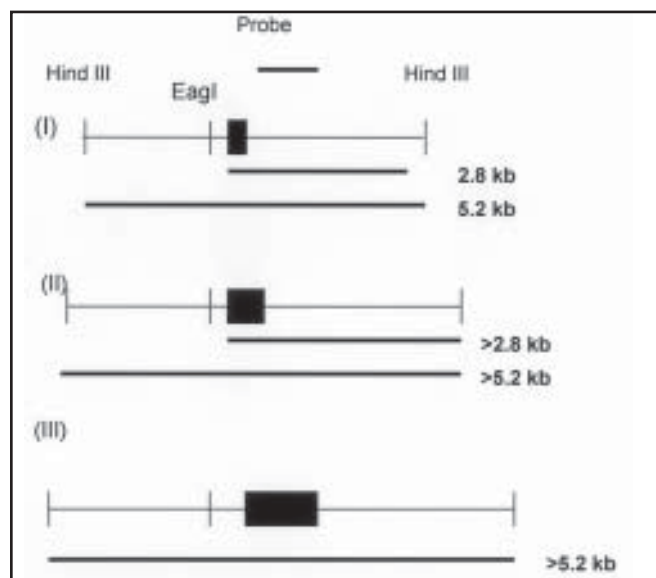


Figure 3a: Diagrammatic representation of the FMR-1 gene (normal, premutation and full mutation) along with restriction sites.

- (I) Normal FMR-1 gene
- (II) FMR-1 gene with premutation
- (III) Full mutation: The gene will be methylated and Eag I being methylation sensitive will not cut the gene giving rise to a band larger than 5.2 kb on Southern blot

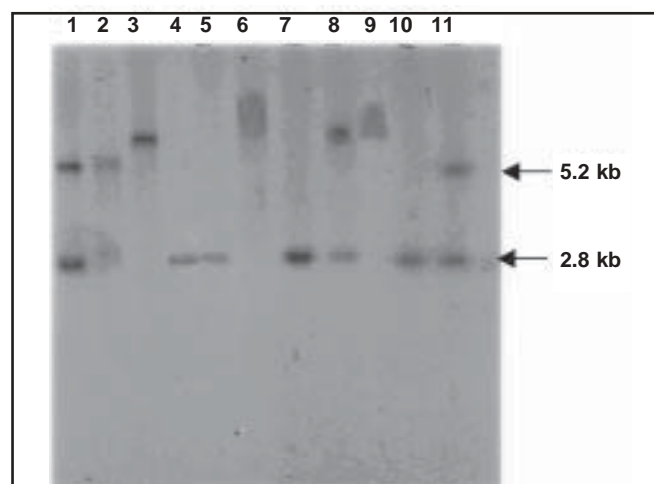


Figure 3c: Southern blot hybridization of fragile X samples identified in our study. The normal unmethylated band of 2.8 kb is indicated by the lower arrow, while the normal methylated band of 5.2 kb is indicated by the upper arrow. Lanes 1 and 2 correspond to normal and premutation females respectively; Lanes 3, 6 and 9 correspond to full mutation males. Lane 8 is a full mutation carrier female; Lanes 4, 5, 7 and 10 are normal males while lane 11 is a normal female

premutation carriers, FMRP can be detected in the cytoplasm of lymphocytes. The test can be used to diagnose affected males but cannot be used to identify female carriers of full mutation, as FMRP is still produced by the normal X chromosome.⁷⁰ In addition, the antibody test is unable to differentiate between normal and premutation alleles. Thus, at present its reliable use is limited to population screening for the diagnosis of affected males.

Genetic counseling

Associated mental impairment and high risk of recurrence makes genetic counseling essential for families with fragile X syndrome. With the availability of molecular tests, carrier detection and prenatal diagnosis is now possible. The most important step is the diagnosis of the affected child. As clinical diagnosis is not possible, especially in young children, it is necessary to test all mentally retarded children without any obvious etiology. Like any X-linked disorder the chances that the son or daughter will inherit the mutated chromosome from the mother are 50%. However, whether the child will be affected or unaffected will depend on whether the FMR-1 gene harbors full mutation or premutation. If the mother is the carrier of full mutation, her 50% sons and 50% daughters will inherit the mutation. Those sons will be clinically affected, but prediction of the clinical phenotype and severity is not possible in females.

If the mother is a carrier of premutation, the chance that the premutation will be converted to full mutation varies accordingly as the number of CGG repeats generally increase in the mother. If the number of CGG repeats in the mother is 60 to 80 or 80 to 100, then the chances that premutation will get converted to full mutation are 14% to 55% and 80% to 90% respectively. If the number of repeats in the mother is 100 to 200 the chance of it getting expanded to full mutation is almost 100%.⁷³

Molecular diagnosis can provide a reliable prenatal diagnosis. But as mentioned above, the clinical phenotype of the female carriers of full mutation can vary from normal to significant mental retardation and cannot be predicted prenatally.

As this condition is X-linked, screening of female relatives of the mother for identification of carriers is useful. An attempt should be made to educate the family, to discuss with their relatives the necessity of carrier detection and genetic counseling to prevent recurrence of similarly affected children in the family.⁷⁴

FXMR being one of the leading causes of mental retardation, screening of the general population for identification of carrier females and offering them prenatal diagnosis for prevention of the birth of a mentally handicapped child, has also been tried and found feasible in the developed countries.⁷⁵ Though this approach is not presently feasible in India, increased awareness amongst the clinicians about fragile X syn-

drome as a cause of mental retardation, is necessary. DNA tests for fragile X syndrome of all mentally retarded children without an obvious cause, and genetic counseling of the families will greatly help in reducing the burden on the families.

At present, there is no cure for the fragile X syndrome. A wide variety of therapeutic measures are used to take care of the special educational needs of the individuals with FXMR and to make them as independent as possible. Pharmacological agents like antidepressants, anxiety medications and anticonvulsants can be used as per indications. The aim of management is to help the children and adults with FXMR learn to function in the household environment and be employed in constructive occupations in sheltered atmospheres. Speech therapy may be needed. In addition, support from the family members, community and non-governmental organizations is needed for better management of these unfortunate victims of inherited mental retardation.

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