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Molecular diagnosis and Importance of Genetic Counseling in subjects and family members with a clinical suspicion of Fragile X Syndrome

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Abstract

Background: Fragile X Syndrome (FXS) is the most common single gene cause of learning disability, hyperactivity and mild to profound Mental retardation (MR) in male patient. Permutation status can lead to premature ovarian failure, mood swings and anxiety in a subset of females. It is most commonly caused due to an expansion of an unstable CGG trinucleotide repeat in in the fragile X gene (FMR-1), which is located on Xq27.3.

Aim: To study the frequency, pattern and importance of genetic counseling in patient with clinical suspicion of FXS and their family member by Triplet Repeat Primed PCR (TP PCR) and Fragment analysis of *FMR1* gene during July'2014 to Dec'2018 at Dept. of Medical Genetics, Metropolis Healthcare Ltd, Mumbai.

Methods: This is retrospective study done on a total of 447 patients (375 males and 72 females) aged from 5-35 years with clinical suspicion or family history indicative of FXS. The mother's and female siblings of male individuals with full mutation where also studied for the mutation status. A PCR based study was conducted for by Triplet primed - PCR (TP-PCR) followed by fragment analysis.

Results: A total of 375 male patients with history of suspicion of Fragile X were referred out of which expansion of triplet above 200 repeats was observed in 28 (7.46%) patients, 06 (1.6%) patients showed expansion of triplet between 54 to 200 and 341 (90.93%) patients showed normal range of CGG repeats (6-54 CGG). A total of 72 females (mother or siblings) studied 07(9.72%) showed permutation status, full mutation was seen in 02 (2.7%) and 63(87%) showed normal status for repeats. Since, Metropolis Healthcare Ltd is a Global Reference Laboratory; samples for Fragile X analysis are received from all parts of India. This could be the reason for variation in the percentage of abnormality detected in our study and also because of the selection bias since only the highly suspected samples were referred to our laboratory for studies.

Conclusion: The prevalence of Fragile X syndrome in individuals with clinical suspicion of Fragile X is significant and it is very important to evaluate the female siblings and offer genetic counseling to the family members as they can give birth to male child with Fragile X syndrome.

Keywords: Fragile X syndrome, Intellectual disability, CGG repeats, Fragile X mental retardation.

Introduction

Fragile X syndrome is named for the folate-sensitive fragile site, FRAXA at Xq27.3 and was detected by cytogenetically by detection fragile X sites when cells were grown in folate-deficient culture medium in late 1970s. In mid 1990s it was predominantly replaced by molecular genetic based analysis. FXS is second most genetic cause of mental deficiency after Down syndrome and the cause is usually familial (Gardner et al. 2011). The incidence is 1 in 4000-5000 males and around 1 in 7000-10000 females. The fragile site exists within the 5'-UTR of the FMR1 (fragile X mental retardation-1) locus and abnormal expansion of CGG repeats cause disease phenotype. There are other two though rare fragile sites distal to FRAXA, these are FRAXE and FRAXF. The FRAXF is reported to be harmless while FRAXE is reported to be associated with nonspecific mental retardation. FRAXD is a common fragile site is proximal to FRAXA and is reported to be harmless (Gardner et al. 2011; Hartley et al. 2011; Mundhofir et al. 2012; Jones et al. 2013). FMR1 gene encodes a protein named FMRP (fragile X mental retardation protein) which is necessary for normal development and functioning of brain (Saldarriaga et al. 2014; Majula TS. 2017).

Nearly all cases of FXS are caused by the expansion of an unstable CGG repeat in the untranslated region of the FMR1 gene. Most individuals in the general population have 54 or fewer CGG repeats, while individuals with a full mutation causing FXS have more than 200 CGG repeats. Males with FXS exhibit mild to severe intellectual disability, often associated with autism-spectrum disorders, ADHD, speech, language and developmental delay, anxiety, and other characteristic behaviours which can be in the form of frank autism, joint hypermobility, macro-orchidism in males (Turner et al. 1975; Verkerk et al. 1991; Jin et al. 200). Females with full mutations exhibit a broad spectrum of clinical presentations depending in part on the pattern of X inactivation. Approximately 50% of females with a full mutation have borderline or mild intellectual disability, and they also may exhibit shyness and anxiety. Some females with full mutations have more significant cognitive, behavioural, and physical features of FXS similar to those observed in males, while others have normal intelligence with only subtle learning disabilities. Individuals with a premutation (55-200 unmethylated CGG repeats) are carriers and may have FMR1-related disorder such as fragile X-associated primary ovarian insufficiency (FXPOI) in case of females or fragile X-associated tremor/ataxia syndrome (FXTAS) in case of males. Permutation carrier females can have premature ovarian failure in around 20% of cases, also smaller group of females may show mood and anxiety difficulties and MRI abnormalities while males with permutation carrier status may have evidence of anxiety, executive function deficit and cerebellar tremors (garber et al. 2008; Harper PS. 2010; Gardner et al. 2011; Jones et al. 2013; Majula TS. 2017)

Materials and Methods

A total of 447 patients were referred genetic studies of CGG expansion of FMR1 gene for fragile X testing at Metropolis Healthcare Ltd. Three hundred and seven five unrelated male (age 5-35 years) patients with suspicion of FXS were screened for CGG repeat expansion in in FMR1 gene. Patients were recruited from different centres all over India after evaluation by Clinical Geneticists.

Genetic Analysis

Standard protocols were used for clinical diagnosis. Peripheral blood (5 ml) was collected in EDTA and was used for genomic DNA isolation using QIAamp DNA Mini kit (Qiagen, USA) following manufacturer's

protocol. The study was approved by the Institutional Review Board. Bisulfite treatment of DNA was done using Qiagen EpiTect Bisulfite Kit following the manufacturer's instructions. Bisulfite-modified DNA was subjected to methylation-specific PCR according to a modified protocol of Zhou et al. Three sets of PCR - mTP, Met and nonMet PCR were carried out using FAM-labelled primers for all the samples as per the method described by Zhou et al. (Ref 13).

Capillary Electrophoresis (CE)

The unpurified PCR products were diluted 1:50 in nuclease free sterile water and 1 µL of diluted PCR products were mixed with 10 µL of HI-DI Formamide (ABI) and 0.2 µL of LIZ600 Size Ladder (ABI), heat denatured at 95°C for 2 minutes and transferred to 3500Dx Genetic Analyzer (Applied Biosystems Inc., ABI, Foster City, CA) for capillary electrophoresis.

Analysis

Electropherograms were analysed using GeneMapper 4.0. The Met PCR gave an amplimer of approximately 192 bp (102 bp + 3n) for 30 CGG repeats. This reaction detected and accurately sized all normal and premutation methylated alleles. The nonMet assay gave an amplimer of about 247 bp (156 bp + 3n) for 30 CGG repeats. This reaction detected and accurately sized all non-methylated alleles. The mTP assay is a TP-PCR and showed a characteristic ladder with a stutter peak of decreasing amplitude extends to the end of the Electropherograms, specifically when an allele is too large to be amplified CGG flanking PCR.

Results

A total of 375 male patients with history of suspicion of Fragile X were referred out of which expansion of triplet above 200 repeats was observed in 28 (7.46%) patients, 06 (1.6%) Patients showed expansion of triplet between 54 to 200 and 341(90.93%) patients showed between 6-54 (Figure 1). A total of 72 females, out of which 22 where mother or siblings or distant blood relative of Fragile X male with full mutation and 50 where females who presented with history of infertility or premature ovarian failure and when detailed family history was taken found to have male family member with suspicion of Fragile X syndrome .out of 22 females who presented with family history of Fragile X, 05(22.72%) showed permutation, 02 (09.09%)showed full mutation and out of 50 females presented with infertility or Premature ovarian failure 02 (04%) females showed pre mutation status. Out of 72 females referred, 63(87%)showed normal status for repeats (figure 1). The positivity observed is approximately same with other studies done on Indian patients with slight increase as Metropolis Healthcare Ltd is a Global Reference Laboratory, samples for Fragile X analysis are received from all parts of India. This could be the reason for variation in the percentage of abnormality detected in our study and also because of the selection bias since only the highly suspected samples were referred to our laboratory for studies.

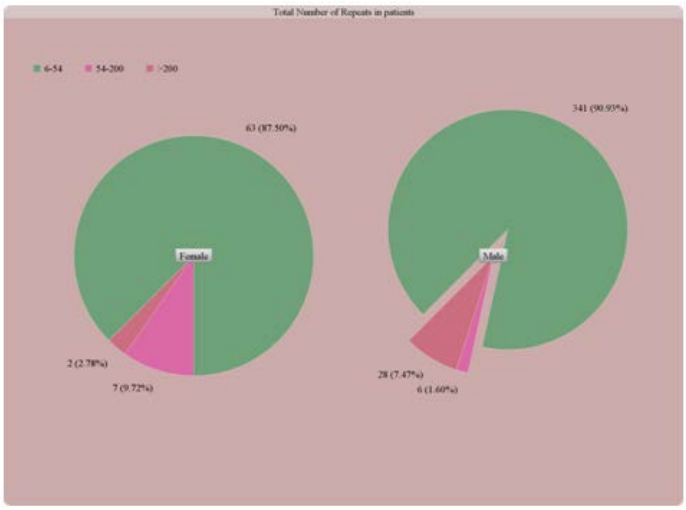

Figure 1: Showing total number of repeats in FMR1 gene amongst male and female participants.

Discussion

Fragile x syndrome is more commonly seen as a cause of inherited mental retardation Among the mentally disabled individuals fragile X positivity has been reported in around 5.9 % males and around 0.3% of females (Gardner et al. 2011; Manjula TS. 2017; Jones et al. 2013). The manifestations are more apparent in males. These individuals have normal life span, slightly increased in growth rate in early life with delayed motor milestone, increase in testicular size after puberty but may show increase before puberty, hyperkinetic behaviour, emotional instability, finger biting, cluttered speech and may have other autistic features (Hartley et al. 2011; Mundhofir 2012; Jones et al. 2013). Apart from classical manifestations some group of individuals may show abnormalities in the form of epilepsy, myopia, nystagmus, strabismus, hypertonia, hyper extensibility of fingers, flat feet, mitral valve prolapse, and aortic dilatation can be seen (Jones et al. 2013). It is also reported that the size of the expansion usually correlates with the degree of mental retardation and is often progressive from one generation to the other (Harper PS. 2010). Hence males with minimal expansion are phenotypically normal can transmit the mutation to their daughters and they will be carrier (no manifestation) but they are at high risk of having mentally retarded offspring because of significant expansion of DNA sequence (Garber et al. 2008). Majority of the males having fragile X syndrome in full mutation do not reproduce however the reproducibility is reported to be in 1% of the individuals (Harper PS. 2010). In our study 07 females were found to be permutation carriers, 06 males were found to have permutation status and 28 males had full mutation. Depending upon the findings appropriate genetic counseling was done. The patient as well as family members where counselled since genetic disorders are not related to the individual alone, but has huge implications for the family members and care takers. In patients with normal findings, other genetic tests were recommended based on clinical indication. Along with the advantages, the limitation of the test that it cannot detect abnormalities in other associated genes responsible for similar symptoms was explained.

Genetic Counseling

Appropriate genetic counseling always depends upon accurate diagnosis, which in turn helps in treatment of the individual, estimation of risk of recurrence, risk to family members and possibility of prenatal genetic testing. If a laboratory diagnosis of FXS is established, the case and the close family members should ideally be referred for genetic counseling.

Risk of recurrence depends upon the gender of index case and number of repeats. The recurrence risk of having an abnormal baby in a male with full mutation is rare as the procreation in these individuals is very rare. However, because of retrogression to a permutation in sperms the daughters will be permutation carriers and usually of normal intelligence with rare exception of full mutation. Sons of these individuals receive the Y chromosome from father so will not have any mutation. In males with permutation there is no risk to have mentally impaired child and daughters will have permutation form but never reported to have FMR Gene in full mutation form.

In females with full mutation all the offspring who have inherited the FXA locus will have full mutation and hence all male child will have FXS, while about 50-60 % of girls will have FXS but the intellectual disability in females usually will be always less severe than in males .Recently it has been suggested that the heterozygous female child may have a risk of additionally having mosaic Turner syndrome and this additional risk is around 5% (Harper PS. 2010). In females with permutation the risk of transmission to next generation is 50%, however chances

of having FXS child depends upon number of repeats. Females with low end permutations having less than 60 copies of the triplet repeats have very minimal risk of having child with FXS and females with high end permutations and having more than 90 repeats the risk ranges from 90-100% (Jin and Warren 2000; Gardner et al. 2011; Harper PS 2010; Jones et al. 2013).

If there is a risk of having FXS child, genetic counseling session should include an option of preimplantation genetic diagnosis (PGD). In couples willing to undergo prenatal diagnosis, chorionic villus sampling (CVS) is always a preferred option.

In cases where the fragile X status has shown normal pattern, depending upon the indication, recommendation for further genetic evaluation by other more relevant techniques or other disorders associated with similar symptoms should always be discussed with the referring doctor.

Conclusion

Intellectual disability, autism, behavioural disorders, premature ovarian failure especially in females is commonly seen in routine clinical practice. It is very important to know the genetics behind the disease and chromosomal karyotype for any associated abnormality as well as evaluation by fragile X studies can be very helpful in such individuals. Timely confirmation of clinical diagnosis by laboratory testing and appropriate genetic counseling can definitely help the individual and family members to improve the quality of the life of the affected individual .Our study wants to show the importance of genetic testing and genetic counseling in patients and family members with clinical suspicion of fragile x syndrome as this helps in estimation of recurrence risk, discuss treatment modalities and helps the couple to have informed choice about available reproductive options**.**

Ethical statement

Our study has been done on live subjects and written and informed consent was not necessary in our diagnostic setting. The informed consent was obtained by clinician when this material was collected for diagnosis. This is an amalgamation of our data of testing of these results. All these tests referred were part of the routine diagnostic procedure and clinical details were part of the Test Requisition Forms which were analysed anonymously and the need for ethics committee approval was ruled out by The Institutional review board.

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