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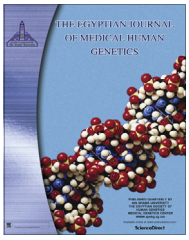

REVIEW

Fragile X syndrome: Current insight

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KEYWORDS

Fragile X syndrome (FXS);
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Abstract Fragile X syndrome (FXS) is a multigenerational disorder having massive adverse effect not only on the individuals but also on their families. It is the most common type of intellectual disability after Down's syndrome. Over two decades have passed since the discovery of FMR1, the causal gene for FXS, but still little is known about the pathophysiology of this disease. This lack of knowledge presents the major barrier encountered by the scientific community for early diagnosis and effective treatment. Since early diagnosis has important implication in determining the disease status among members of the family tree so the genetic counseling and supportive therapy get hampered in larger perspective. The present review emphasizes on the recent findings in FXS pathophysiology, therapeutics and technical challenges in molecular diagnosis.

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Abbreviations: ID, intellectual disability; FXS, fragile X syndrome; FMR1, Fragile X Mental Retardation 1; FMRP, Fragile X Mental Retardation Protein; UTR, untranslated region; PM, premutation; FM, full mutation; DNA, deoxyribo nucleic acid; FXTAS, Fragile X-associated Tremor/Ataxia Syndrome; FXPOI, Fragile X associated Premature Ovarian Faliure; mRNA, messenger ribonucleic acid; FSH, follicle stimulating hormone; AMH, anti-mullerian hormone; NLS, nuclear localization signal; NES, nuclear export signal

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1. Introduction

Intellectual disability (ID) is defined as a failure to develop a sufficient cognitive and adaptive level, caused by both genetic and environmental factors and is normally reflected in maturation, learning, or social adjustment (American Psychiatric Association 1987). It affects approximately 2% of the general population [1]. The genetic cause of ID frequently involves X chromosome, elucidating to a certain extent the lower prevalence of ID in females in comparison to males [2]. About 20% of all the X linked ID cases is because of fragile X syndrome (FXS) [3], which is by far the second most widespread inherited cause of ID after Down syndrome [4]. This cognitive disorder has an incidence of 1 in 4000 males and 1 in 8000 females [5]. It is characterized by mild to severe mental disability, often accompanied by autistic like behavior, developmental delay, augmented vulnerability to seizures, and macroorchidism in males [6] (Table 1).

2. Genetics insight of FXS

FXS (OMIM #300624) is an X linked dominant disorder caused by mutation in a single gene Fragile X Mental Retardation 1 (*FMR1*). An affected female will have 50% affected children but an affected male will have all daughters affected but all sons normal. The molecular basis of this syndrome is the expansion of a CGG repeat sequence located at the 5′ UTR of a highly conserved *FMR1* gene that consists of 17 exons and spans about 38 kb, positioned at Xq27.3, there by leading to hypermethylation of the repeat sequences and of the neighboring promoter region leading to silencing of this gene [7]. Due to X linked inheritance of *FMR1*, FXS females show variability in symptoms and are mildly affected than males because of random X inactivation. Severity of disease symptoms in FXS females is inversely related to the activation ratio for the normal *FMR1* allele and its product, FMRP (Fragile X Mental Retardation Protein) level. The FM (full mutation) males or methylation mosaic FM males too show variability in severity of cognitive impairment depending upon the amount of unmethylated DNA and FMRP level [8].

On the basis of CGG repeat length the FMR1 gene is classified into 4 allelic forms normal allele (5–44 repeats), intermediate allele (also referred as gray zone, inconclusive, or borderline) (45–54 repeats), premutation (PM) allele (55–200 repeats) and full mutation (FM) allele (>200 repeats). The most common repeat length in normal allele is 29 or 30 CGG repeats. Normal alleles are transmitted to next generation stably without any expansion and found to have AGG interruption after every 9 or 10 CGG repeats. AGG repeats anchor the repeat region during replication by preventing strand slippage. This is supported by the finding that most of the PM alleles have a single or no AGG repeat and are unstably transmitted to the offspring by mother parent [9]. The presence of AGG interruptions in all PM mothers having repeat lengths below ~100 CGG reduces the risk of the expansion to FM upon transmission [10]. The possibility of expansion to a full mutation is positively associated with the length of the premutation in the transmitting female [11]. The maternal PM repeat size as small as 56 repeats have been reported to expand to a FM in a single generation [12]. But PM or FM male can transmit only PM allele to their daughters due to selection against full mutation in sperm during spermatogenesis (Fig. 1). The presence of an FM allele causes FXS, but the carriers of PM alleles does not exhibit any of the characteristic phenotypic features associated with FXS. Unlike FXS, neuropathological changes in PM are a result of RNA toxicity related to over expression of mRNA containing the CGG repeat expansion [13]. PM is more frequent in population as compared to FXS and occurs in 1 in 113–259 females and 1 in 260–810 males [14]. PM males and, to a lesser extent, PM females are at an augmented risk of an adult-onset neurodegenerative Fragile X-associated Tremor/Ataxia Syndrome (FXTAS). 40% of PM males and 8% of PM females develop FXTAS over 50 years of age [15] which is characterized by progressive intention tremor, gait ataxia and dementia [16] and recently in 80% of FXTAS cases olfactory dysfunction was also reported. Also it was found that there is ~20% risk for PM female to develop a form of ovarian dysfunction known as Fragile X associated Premature Ovarian Insufficiency (FXPOI) [17]. FXPOI presents with a range of problems like heavy bleeding, irregular periods or increased rates of twinning, infertility and menopause before the age of 40 with reduced anti-mullerian hormone (AMH) indicative of a reduced follicle pool, and increased follicle stimulating hormone (FSH) [18].

Table 1 Characteristic features of FXS.

Features	Description
Intellectual deficiency	Mild to severe in males (IQ between 20 and 60), borderline IQ in females accompanied by learning difficulties and problems in doing mathematics [63–65]
Phenotypic features	Mild facial dysmorphia characterized by elongated face, prominent forehead, prominent ears, prominent jaw, velvety skin [66]
Connective tissue anomalies	Pes planus [67], low muscle tone [68], strabismus [69], hyper extensible joints, double jointed thumb [70,71], recurrent sinusitis and otitis media (childhood), mitral valve prolapse, macro-orchidism (post puberty) [72,73]
Behavioral abnormalities	Social withdrawal, hyperactivity, anxiety, preseverative speech, hyperarousal to sensory stimuli, tactile defensiveness, stereotypic movements (hand flapping, hand biting or rocking), autistic like features (shyness, poor eye contact, problem in face encoding) [74,75]

3. FMRP and its role in cognitive development

Extensive repeat expansion and consequential hypermethylation of the FMR1 gene in FM individuals lead to transcriptional silencing of FMRP. Insufficient FMRP in full mutation individuals leads to cognitive impairment in FXS. FMRP is a multifunctional mRNA-binding protein having three RNA interacting domains namely, two hnRNP K homology domains and a cluster of RGG (arginine–glycine–glycine) box. It has a nuclear localization signal (NLS) and a nuclear export signal (NES) for functioning as a

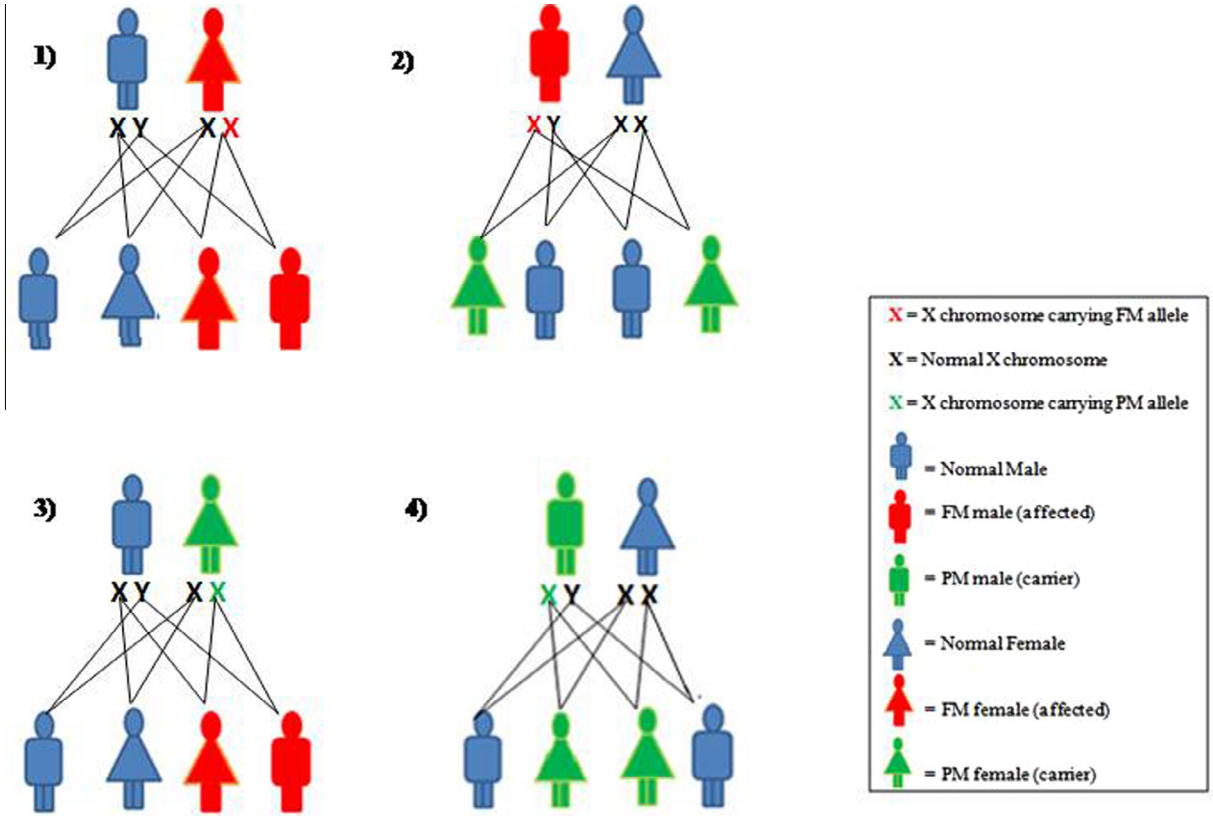

Figure 1 Inheritance pattern of fragile X syndrome. (1) Affected (FM) female will have 50% chance of having affected children [53]. (2) Affected (FM) male rarely reproduce due to cognitive/behavioral limitations, reduced fertility due to sperm malformations and oligospermia however if they reproduce they can transmit PM allele to their daughters [54]. (3) Carrier female (PM) can transmit expanded FM allele to 50% of her children depending upon the size of CGG repeat [55]. (4) Carrier male (PM) can transmit only PM allele to his daughters and all his sons will be normal [56].

nucleocytoplasmic shuttling protein, which can associate with up to 4% of all mRNAs present in the brain [19]. It is involved in activity-dependent mRNA metabolism in neurons, such as mRNA transport [20], stability [21] and regulation of dendritic kkmRNA translation. FMRP thus plays pivotal role in establishing correct synaptic connectivity [22], synaptic plasticity [23] and dendritic morphology [24]. This was consistent with the finding that the mouse models of FXS depicts extensive defects in synaptic plasticity [25], and that the *FMR1* KO (Knock Out) mice and FXS patients have augmented numbers of elongated and thin dendritic spines comparative to the mushroom-shaped spines typical of stronger and mature synapses [26,27].

The creation and elimination of dendritic protrusions are essential for establishing and maintaining normal synaptic communication and hence governs the process of learning and memory [28]. Long term synaptic plasticity requires new protein synthesis which is regulated through Group 1 metabotropic glutamate receptors (mGluRs) by several pathways [28]. Synaptic studies in Fmr1-deficient mouse depict excessive protein synthesis leading to exaggerated mGluR LTD (long term depression) [29]. These findings, along with already known translational repression potential of FMRP suggest that both FMRP and mGluR work in concert to fine-tune activity-dependent local protein synthesis.

4. Disease pathogenesis

FMRP regulates the translation of proteins important for proper synaptic function. The precise mechanism of translational regulation by FMRP is unknown. FMRP is thought to form a dimer in the cytoplasm and enters the nucleus of neurons where it interacts with target mRNA. FMRP-mRNA complex thus formed is shuttled out into cytoplasm again and is transported down to the dendritic spines, where they wait in a translationally silent state for synaptic stimulation signal like mGluR activation [60].

How FMRP mediates this translation repression is supported by two theories. According to First theory, FMRP can repress the translation of certain cargo mRNAs via specific microRNAs. On binding to its specific mRNA ligands, FMRP may recruit RISC (RNA-induced silencing complex) complex along with miRNAs allowing recognition between miRNAs and their target mRNA. The association of mammalian FMRP with RISC complex suggests its role in micro RNA-mediated translational control [30]. But there is yet another theory which states that FMRP bounds with its target mRNA by interacting with ribosome directly in RNA independent manner [31] and can interfere with normal translation process without needing the miRNA by interfering with the binding of essential translation factors to the ribosome. In agreement to

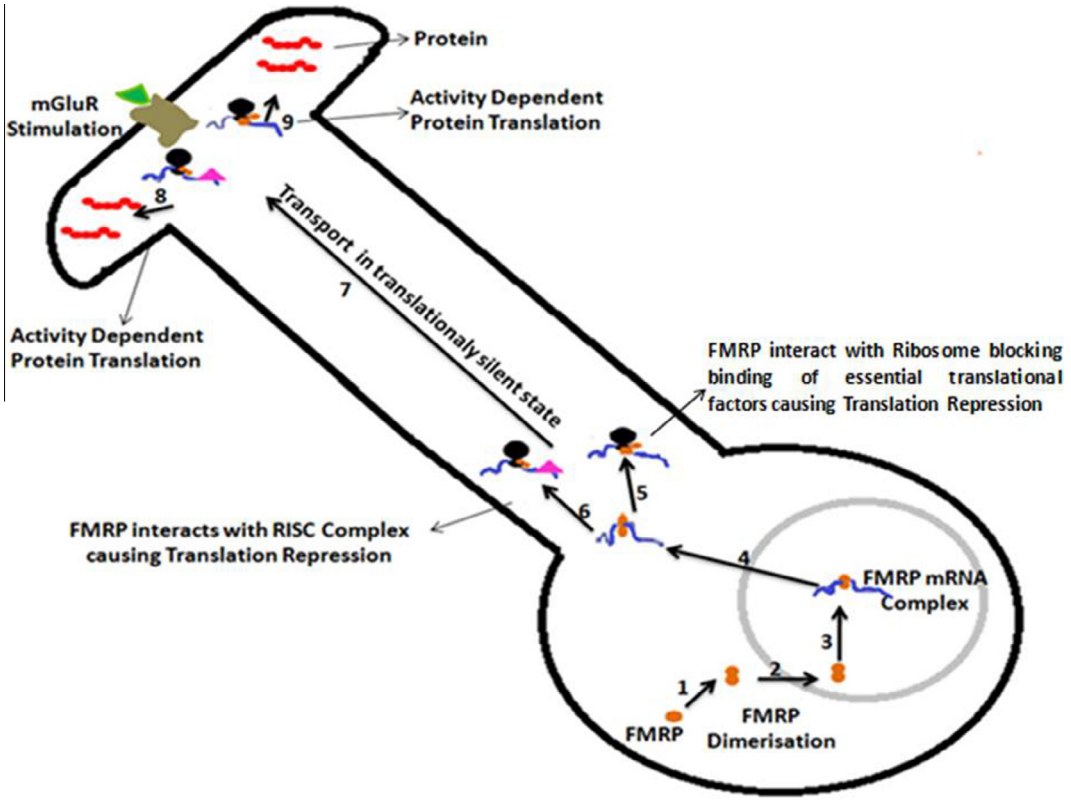

Figure 2 Translation repression by FMRP. (1) Monomeric FMRP dimerises in cytoplasm of neural cell. (2) Dimeric FMRP enters nucleus using NLS [57,58]. (3) FMRP dimer binds target mRNA through its RGG domain which interacts with G quadruplex sequences [59]. (4) FMRP-mRNA complex reenters cytoplasm using NES domain [58]. (5) FMRP-mRNA complex binds to inter subunit space within ribosome in a RNA independent manner thereby interfering with binding of translation factors thus repressing translation [31,32]. (6) Alternatively, FMRP-mRNA complex interacts with RISC complex via specific miRNA causing translation repression [30]. (7) Specific mRNAs are thus transported in translationally silent state at the dendritic spines and waits for signal [60]. (8 and 9) translation commence upon stimulation of metabotropic glutamate receptor by glutamate [61,62].

this theory, recently it was found that FMRP binds within the intersubunit space of the ribosome preventing the binding of eEF1A.GTP.aminoacyl-tRNA ternary complex and eEF2 to the 80S ribosome hence blocking translation [32] (Fig. 2).

5. Treatment

Presently there is no cure for FXS but supportive management therapies like special education and vocational training benefits FXS patients. A lot of studies have been conducted in order to understand the molecular pathogenesis of FXS to find possible treatment. The finding of exaggerated mGluR LTD in FXS has been exploited in opening many potential therapeutic interventions. The majority of therapeutic interventions being developed today for FXS focus on drugs whose action reduces the activity of Group 1 mGluRs and its downstream signal transduction pathways [33]. Different animal models of FXS like fruit fly, zebra fish, and mouse depicted rescued behavioral and cognitive deficits upon the administration of mGluR5 antagonist 2-methyl-6-(phenylethynyl)-pyridine (MPEP) [34]. MPEP has limitation like toxicity and a short half life thus it is not feasible for use in clinical trials for FXS patients. Fenobam, an mGluR NAM was administered to a cohort of 12 adult males and females with FXS. No significant adverse reactions to fenobam were detected and it is found to be safe [35]. However more high quality and placebo controlled trials on a larger group of subjects become necessary to provide a strong indication of benefit in treating FXS patients.

Preclinical trials of mavoglurant also known as AFQ056, a mGluR antagonist had shown promising results in FMR1 KO mice [36,37] and also in a double-blinded trial of mavoglurant conducted on 30 FXS adult subjects stratified according to the methylation status of FMR1 promoter, it was found that individuals with a fully methylated promoter showed significant improvements as compared to the control group [38]. However when large international clinical trial was conducted in adults, negative results are obtained which compelled to discontinue any development program for this drug. Such underwhelming results had dwindled the likelihood of using mGluR5 modulators as a single pharmacologic treatment in FXS. However it is still possible that the studies done till date were not long enough to show benefits, or that the drug may work in younger children. Currently another mGluR5 negative allosteric modulator, basimglurant is clinically found to be potent, selective, and safe with good oral bioavailability and long half-life, good brain penetration, and high in vivo potency [39]. It is now in Phase II trials for which results are still not released.

Also drugs targeting impaired GABA receptors in FXS are also under clinical trials. Ganaxolone, a $GABA_A$ receptor agonist that has anticonvulsant, anxiolytic, and sedative effects

is found to be orally active and does not have hormonal effects. It is under development for the treatment of seizure disorders and posttraumatic stress disorder. A randomized, Phase II, double-blind, placebo-controlled crossover trial to investigate its efficacy for the treatment of anxiety and attention deficits in children with FXS aged 6–17 years [40] is currently under way. If efficacy in FXS is demonstrated and there is FDA approval for an FXS indication, then it will be studied in ASD and related disorders. Arbaclofen, a $GABA_B$ agonist too has demonstrated efficacy for children with FXS and social deficits or ASD in a controlled trial [41].

Both mGlu R pathway and GABA pathway have shown to be critical in FXS pathogenesis, still other pathways may also be involved as FMRP is found to regulate many proteins which are important for the brain development. Recently a possibility of involvement of dysregulated nitric oxide signaling in the pathopsychology of FXS and other neuropsychatric disorders have been reviewed opening new avenues in the development of more drugs for FXS treatment [42]. In the present scenario it has now become important to concentrate more on the functioning of FMRP which is missing in FXS. Understanding the pathway adapted by FMRP for translation repression of its target mRNA can help in designing potential drugs which can replicate the function of FMRP and rescue FXS phenotype. With the growing concern and information about FXS, we feel optimistic to find an effective treatment for FXS very soon.

6. FMR 1 diagnosis

More than 99% of the FXS cases are the result of CGG-repeat-expansion at 5′ UTR [43]. Hence the molecular diagnosis of FXS relies on the tests that determine the number of the triplet repeat elements in the FMR1 gene. Before cloning of FMR1 gene, FXS diagnosis was done by Cytogenetic identification of fragile site at Xq27.3, induced by culturing cells in folic acid deficient medium. This method is no longer used because it is less sensitive and more costly than molecular genetic testing [76].

Cloning of FMR1 gene in 1991 has revolutionized molecular diagnosis of FXS. It requires an amalgamation of PCR and methylation-informative Southern Blot (SB) analysis [44]. Briefly, the technical simplicity and rapidity of PCR made it a preferred method for molecular diagnostics in general. But the presence of high GC rich expansions in FM, size and methylation mosaics of CGG repeats, random X-inactivation in females, and the incomplete/absent methylation in certain prenatal samples like chorionic villus sample made the molecular diagnosis of FXS complex and technically challenging [45]. Therefore long PM and FM alleles cannot be successfully amplified using conventional PCR amplification [46].

Southern blot can clearly distinguish between FM and PM alleles. SB provides information regarding methylation status and can identify female homozygous alleles that often confound interpretations of PCR data. Thus, SB supplements the result of PCR and is traditionally used for the diagnosis of FMR1 [47]. But Southern blot analysis have limitations of being labor intensive, time consuming, requires large quantities of high-quality DNA for analysis and has low resolution and sensitivity compared to PCR-based methods [48,49]. Therefore SB is not feasible for speedy diagnosis as is required in prenatal testing and carrier screening demand in clinical setup. Thus considerable efforts are put in for developing PCR technology to increase its ability to identify fragile X full mutations.

A PCR method named Triplet primed PCR (TP-PCR), has emerged as a reliable non-radioactive method that replaced Southern blot [50,51]. Triplet – primed PCR assays is showing high promise in the field of FMR1 diagnostics due to their cost effectiveness, and high sensitivity for large expansions. Different variations of this approach have been proposed [51]. Further information about the methylation pattern of FM FMR1 alleles can be supplemented using Methylation-specific PCR [52]. Thus excluding the need of SB for FMR 1 diagnosis, probably, in the near future more advanced TP PCR will be the only technology preferred for FMR 1 analysis.

7. Conclusion

It has been more than two decades since the discovery of fragile x syndrome but the disease continues to hold surprises in spite of extensive research. Among the primary goal of the researchers, it is to find effective targeted therapy for the syndrome and also to develop speedy, sensitive and cost effective diagnostic method.

The mGluR model proposed for defining the pathogenesis is accepted widely. It explains the role of FMRP in activity dependent local protein synthesis but little is known about the transport of mRNA to the dendritic spines in a translational inactive state. In the present review, two different pathways are discussed through which FMRP could repress translation, one via miRNA mediated translational inhibition and alternatively by interacting directly with ribosome. More research is required to unravel the precise pathway adapted by FMRP in upholding apt synaptic plasticity to pave way for designing and validating possible drug target.

Following advancement in therapy, early recognition of the syndrome is also a big concern. Early diagnosis of FXS is important to ensure that not only affected children and families can receive all possible benefits, including genetic counseling and intervention services but is also important for prenatal diagnosis as the risk of recurrence of Fragile X-MR is high in the family and carrier relatives. TP PCR has preferably substituted traditionally used SB technique owing to its sensitivity, selectivity, and low cost. It offers the possibility of early diagnosis in clinical suspects, prenatal testing and is also competent in mass screening for carrier status. A progress in both diagnosis and therapy would hopefully improve the quality of life lived by FXS patient in future.

Conflict of interest

The authors declared that they have no conflict of interest.

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