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Molecular analysis of *FMR1* alleles for fragile X syndrome diagnosis and patient stratification

Daman Kumari[1,*], **Karen Usdin**[1]

[1]Laboratory of Cell and Molecular Biology, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, Maryland 20892, USA

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1. Introduction

Fragile X syndrome (FXS), a neurodevelopmental disorder, is the leading form of heritable cognitive disability and autism, affecting approximately 1 in 5,000 males and 1 in 4,000 to 8,000 females [1]. The classic clinical phenotype of FXS males includes intellectual disability, behavior problems, attention deficit hyperactivity disorder (ADHD), anxiety, sleep disorder, social deficit and autism spectrum disorder (ASD). There is no cure or effective treatment for FXS. Available options for management of FXS include pharmacological interventions focused on treating anxiety and hyperactivity, interventions for behavioral problems and speech and language therapy [1].

FXS is caused by mutations in the *Fragile X Mental Retardation-1* (*FMR1*) gene, located on the X-chromosome, that result in a deficit of the encoded protein, FMRP or impairment of its function [1]. FMRP is an RNA binding protein that regulates the transport and translation of its target mRNAs in brain and is important for synaptic plasticity [2]. Recent studies also suggest a number of other roles for FMRP including involvement in alternative splicing, RNA editing and DNA repair [3]. Decades of research into understanding FMRP functions has identified critical pathways altered in the absence of FMRP. This has paved the way for clinical trials of compounds that showed great promise in preclinical studies in animal models [1]. However, the expected therapeutic benefit has not yet been seen in FXS patients. Some of the reasons for the observed differences in the drug response between the FXS mouse model and patients may be the phenotypic and genotypic variability of FXS patients,

[*]**Corresponding author:** Daman Kumari, Laboratory of Cell and Molecular Biology, National Institute of Diabetes and Digestive and Kidney Diseases, Building 8, Rm 2A19, 8 Center Drive, Bethesda, Maryland 20892, USA, damank@niddk.nih.gov.

Author's contributions

Both contributing authors have participated in writing of the manuscript and approved the final version of the manuscript.

Declaration of interest

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lack of objective outcome measures and/or the age at intervention [4]. Currently, the average age of FXS diagnosis in males is 3 years and is even later in females [5]. An earlier age at diagnosis would not only enable early interventions and access to treatment that provide added clinical benefit but also reduce the considerable stress associated with the "diagnostic odyssey" that families endure before a diagnosis is made. It would also enable timely genetic counseling that would provide parents with the information they need to make future reproductive decisions. Therefore, there is an urgent need for earlier diagnosis of FXS. The search is also on for molecular/biochemical markers that could help in patient stratification and also serve as an endpoint for clinical trials for targeted treatments.

2. Molecular genetics of FXS

In about 99% of FXS patients, the underlying mutation is the expansion of a polymorphic CGG repeat in the 5' untranslated region of the *FMR1* gene. *FMR1* alleles are classified into four groups based on the number of CGG repeats, with 5–44 considered normal, 45–54 as intermediate/gray zone, 55–200 as premutation (PM) and >200 as full mutation (FM) [6]. The PM alleles are often hyper-expressed and associated with two well characterized clinical conditions, fragile X-associated tremor and ataxia syndrome (FXTAS) and fragile X-associated primary ovarian insufficiency (FXPOI). Other comorbid health conditions associated with the PM have also been described [7,8]. In contrast to the hyperexpression that is often seen from PM alleles, FM alleles are generally methylated and transcriptionally silenced resulting in the absence of FMRP. The remaining 1% of FXS patients who lack the CGG expansion, harbor other mutations including deletions and point mutations in the *FMR1* gene that result in a deficit of functional FMRP [9,10]. Of the FXS patients with FM alleles, approximately 41% are mosaic with respect to the CGG repeat size as well as DNA methylation [11]. This mosaicism can differ between or within tissues in the same individual [12]. Size mosaicism can be extreme in those cases where the FM allele somehow escapes methylation [13]. To make matters more complicated, PM alleles are also occasionally methylated [13,14]. Some cases may reflect a lower than normal methylation threshold, whilst others likely reflect contractions from methylated FM alleles that, for reasons that are still unclear, then fail to become demethylated [13]. The size and methylation mosaicism can modulate the expression of *FMR1* mRNA and FMRP, and the levels of residual FMRP can influence the clinical presentation in FXS patients [15]. FM females generally present with a mild and variable clinical phenotype which in part can be attributed to the presence of a normal X-chromosome and the process of X-inactivation that determines what fraction of cells carry the FM allele on the active X chromosome and thus the proportion of cells that lack FMRP [16]. In addition to the variability in the levels of FMRP among patients, expression of *FMR1* mRNA from PM and unmethylated FM alleles can have its own "toxic" effects that are thought to result from sequestering proteins that bind expanded repeats or as a result of repeat associated non-AUG (RAN) translation [17]. This can also influence the clinical presentation. There have been only a limited number of studies on molecular-clinical correlations in mosaic FXS patients [11,14,18–21]. Expanding such studies to include longitudinal analysis will further our understanding of the clinical risk associated with the presence of different *FMR1* alleles in mosaic individuals. In the following sections, we discuss various molecular methods for complete characterization of *FMR1* alleles that can

facilitate rapid and inexpensive FXS diagnosis and earlier interventions that may provide therapeutic benefit to patients.

2.1. Detection of mutations in the *FMR1* gene

The American College of Medical Genetics and Genomics recommends FXS testing for all individuals who present with developmental delay, intellectual disability and/or behavioral problems [6]. Two methods that are commonly used for estimating the CGG repeat size and methylation of the *FMR1* gene include Southern blot analysis and polymerase chain reaction (PCR) [22]. Over the years the PCR-based assays have improved, and the current methods can now provide more precise estimates of the repeat size, AGG interruptions and methylation status quickly and more cost-effectively with much smaller sample sizes (Reviewed in [23]). Small pool PCR may also be a useful approach for individuals with extensive size mosaicism [13]. In individuals who present with a clinical phenotype suggestive of FXS but who are negative for CGG expansion, additional tests like sequencing and multiplex ligation-dependent probe amplification (MLPA) can be useful for the identification of copy number variations and single nucleotide differences (Reviewed in [23]). In female carriers of PM and FM alleles, it is also necessary to determine the X-inactivation ratio which can influence the clinical phenotype [16].

2.2. Quantification of *FMR1* mRNA and FMRP protein

Depending on the CGG repeat size, the unmethylated alleles may produce some FMRP resulting in a milder FX phenotype. Furthermore, unmethylated FM alleles can make *FMR1* mRNA, potentially resulting in toxicity like that seen in some PM carriers. Therefore, assays that measure the *FMR1* transcript levels and FMRP levels can help assess the full clinical spectrum. This information may also be useful for patient stratification during clinical trial design and may also serve as an outcome measure for treatments based on restoring *FMR1* expression. Quantitative reverse transcription coupled PCR is widely used to measure *FMR1* mRNA levels in peripheral blood and other cell types derived from FXS patients and is a good indicator of the transcription status of the *FMR1* gene [24]. Highly sensitive assays for measuring FMRP in cell lysates and dried blood spots in a high throughput format are also now available [25]. The caveat with these tests being that, because of tissue differences in the extent of methylation and size mosaicism, it is not possible to be certain that the levels measured in peripheral sources such as blood and saliva accurately reflect the levels present in brain. Nonetheless, a recent study suggests that FMRP levels ≥35% of normal in fibroblasts may be sufficient for normal intellectual functioning [26]. In rare individuals who have a clinical presentation of FXS but are negative for the expansion mutation, similar studies on *FMR1* mRNA and FMRP levels may allow diagnosis and help inform appropriate tests for identifying other mutations in the *FMR1* gene.

2.3. Population screening

Newborn screening (NBS) programs would facilitate early diagnosis. Some of the recently described FX assays are less costly and/or labor-intensive than the older assays. This should help reduce the barrier to including FXS in the Recommended Uniform Screening Panel (RUSP) for NBS. However, screening for CGG repeat size also results in the identification of carriers of the much more prevalent PM allele. Because insufficient information is

currently available for risk assessment, disease prognosis prediction, or treatments for FXTAS or FXPOI, this complicates the inclusion of FX testing based on CGG-repeat size in standard NBS in the United States. Voluntary screening programs such as the Early Check Program being implemented in North Carolina [27], may go some way to addressing this problem. Additionally, incorporating NBS assays for FXS that are based on detecting abnormal methylation of the FM alleles [28,29] or reduced FMRP levels [30] would limit the detection of PM alleles.

3. Conclusions

In recent years, the development of sensitive, high-resolution assays for molecular characterization of *FMR1* alleles have made it easier to evaluate the extent of *FMR1* methylation and size mosaicism than ever before. The assays for FMRP quantitation have also improved considerably. There is evidence to suggest that in some cases the efficacy of drug treatments aimed at compensating for the absence of FMRP may vary depending on the methylation status of the *FMR1* gene [31]. It is also likely that FM individuals that express some *FMR1* mRNA may benefit from therapies designed to benefit PM carriers. Thus, the careful consideration of the extent of DNA methylation and size mosaicism, along with residual *FMR1* mRNA and FMRP levels may provide useful clinical benefits and perhaps help optimize patient stratification for clinical trials. In addition, many PM carriers also present with clinical features characteristic of FM carriers. This includes ADHD, ASD, depression and anxiety [7]. The more recent assays may be helpful in resolving the basis of this overlap.

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