# Autism phenotype in fragile X premutation males is not associated with *FMR1* expression: a preliminary evaluation

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**ABSTRACT**

To explore the association between autism phenotype and *FMR1* protein (FMRP), *FMR1* mRNA and CGG repeat length in 31 male *FMR1* premutation carriers aged 3.0 to 27.9 years old (mean 13.0 ± SD 6.5) using the ADOS communication, social interactive and total scores. FMRP levels were determined using the sandwich Enzyme-linked Immunosorbent Assay (ELISA) method, *FMR1* mRNA expression levels were measured by qRT-PCR, and CGG repeat size was determined using Southern blot and PCR analyses. There was no significant difference in FMRP, CGG repeat length, and *FMR1* mRNA between fifteen subjects without (ASD / PDDNOS / autism and sixteen subjects with ASD / PDDNOS / autism. ADOS scores were not significantly associated with either FMRP or *FMR1* mRNA, This preliminary evaluation found that autism phenotype is not associated with the level of expression of either *FMR1* mRNA or FMRP. However, CGG was significantly negative associated with both ADOS communication score (p= 0.0173) and ADOS total score (p= 0.0358).

Key-words: Autism, CGG, *FMR1* mRNA, FMRP, Fragile-X Premutation

The expansion of the CGG repeat in the premutation range (55-200 CGG repeats) of the fragile X mental retardation 1 gene (*FMR1*) can lead to a range of clinical involvement, including psychological problems [1,2]; fragile X-associated primary ovarian insufficiency (FXPOI) [3,4]; immune-mediated disorders [5,6]; hypertension [7]; fragile X-associated tremor/ataxia syndrome (FXTAS) [8–10] and neurodevelopmental disorders, such as autism spectrum disorders (ASD) and attention deficit hyperactivity disorder (ADHD) [11,12]. Some of behaviours associated with autism such as avoidance of eye gaze, hand flapping, repetitive behaviours, and speech perseverations have been reported in more than 60% of all individuals with fragile X syndrome (FXS) [13–15].

A lack or deficiency of the *FMR1* protein (FMRP) in individuals with the full mutation (>200 CGG repeats) leads to the clinical features of FXS[16]. However, FMRP may be also mildly deficient in some individuals with the premutation, particularly those with CGG repeats in the upper premutation range as well as the premutation CGG Knock-In (CGG KI) mouse model [17–20]. In addition, elevated level of *FMR1* mRNA, which rises with increased CGG-repeat number, is the most consistent molecular abnormality observed in both human and mouse premutations [20–23]. Elevated mRNA also leads to central nervous system (CNS) toxicity and neurological disease, such as FXTAS and psychopathology in older carriers [1,2,24].

Although most individuals with the premutation are unaffected by intellectual disability, a subgroup of children experience ASD, ADHD, anxiety, seizures, and learning difficulties or intellectual disability [12,13,25–29]. The prevalence of ASD in boys with the premutation whose parents sought medical attention for their sons' behaviour problems in the clinic (probands) is

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high (73%); however, if the premutation is identified through cascade testing, brothers with the premutation (non-probands) are then found to have a lower prevalence of ASD (14%)[11]. A questionnaire study of more than a thousand families demonstrated a prevalence of autism or ASD of 13% in boys with the premutation and 1% in girls with the premutation [28].

The cause of the developmental problems in some premutation children is likely related to mild deficits of FMRP [12,17] in addition to the toxic effect of elevated mRNA [30–32]. A recent report by Chonchaiya et al. found that seizures were common in male probands with the premutation and were significantly associated with ASD and intellectual disability in these boys [29]. A study demonstrated that 11.3% had seizures of 57 premutation boys from a family survey [28]. In the CGG KI mice, the CGG premutation animal model, neurons appear to die more easily in culture than in control neurons [33], and these neurons have an enhanced frequency of electrophysiological spikes [34]; thus the premutation neurons appear to be more vulnerable to seizures or other environmental toxicity. There are case reports of carriers who have been exposed to environmental toxins, including chemotherapy [35] or industrial contaminants [36], and have suffered more severe neurological problems than typically seen in carriers.

The prevalence of the fragile X premutation in the general population is estimated at one in 130 to 259 females and one in 250 to 813 males [37,38]. Because the premutation is relatively common, some of the problems in premutation carriers may be exacerbated by other factors, in addition to low FMRP or to the RNA toxicity of elevated mRNA. The association between autism and *FMR1* expression has been studied in the full mutation range such an association has not been reported in those with premutation. In this study, we utilized enzyme-linked immunosorbent assay (ELISA) to measure FMRP levels in blood [39] and assessed the association between FMRP, *FMR1* mRNA, and CGG repeats and three ASD scores: communication score, social interactive score, and total score.

## SUBJECTS AND METHODS

### Subjects

Subjects participated in studies at the MIND Institute at the University of California, Davis, between 2006 and 2011. The subjects included 31 male premutation carriers with mean age 13.0, SD 6.5 (3.0 to 27.9 years). Parents and participants older than 8 years signed consent approved by our institutional review board. Premutation status was confirmed in all participants, as described below. Subjects underwent a full medical evaluation, which involved a medical history and a physical / neurological examination by one of the authors (RJH), an expert in neurodevelopmental disorders. The medical history involves a detailed review of systems and questions regarding medical conditions, such as history of development, and medical problems, including seizures. We classified the subjects into probands and non-probands. Probands were individuals who presented to the clinic because of their medical or developmental problems, while individuals who were identified by cascade testing named as non-probands.

During the clinical evaluation, assessment of specific measures for ASD were completed by licensed psychologists. The diagnostic algorithm of the Autism Diagnostic Observation Schedule (ADOS) module 1,2,3 and 4 were used to examine the profile of autism. [40] From the ADOS examination, the subjects were diagnosed with ASD, PDDNOS or autism. The participants were then divided into two groups, group A with no ASD/ PDDNOS/autism or were in typical range and group B with ASD/PDDNOS/autism

### Molecular Measures

FMRP was determined using the sandwich ELISA method published previously [39]. *FMR1* mRNA expression levels, measured by qRT-PCR were as described [41]. CGG repeat size was determined using Southern blot and PCR analyses, as previously described [37].

### Statistical Analysis

Descriptive statistical analysis was based on Fisher exact test for categorical variable and student's t test for continuous variables. To test association between ASD scores and molecular biomarkers (FMRP, *FMR1* mRNA, CGG repeats), the analysis of covariance (ANCOVA) was used to with covariates group effect and FMRP or *FMR1* mRNA or CGG repeat. Differential association between ADOS variables depending on whether premutation carriers in group A or B were assessed with inclusion of interaction term with group; however, the all interaction terms were not statistically significant. All statistical analysis was conducted in SAS version 9.2.

## RESULTS

### Characteristics of study subjects

The initial cohort included 31 males who carried an *FMR1* allele with CGG repeats number from 55 and 186 (Mean 101, SD 40). Relative FMRP levels were measured from blood lymphocytes with levels ranging from 0.65 to 2.27 (Mean 1.57, SD 0.5), where a value of 1.0 is the mean FMRP value derived from controls with normal alleles. The analysis cohort included 31 subjects based on ADOS overall diagnosis: 8 (25.81%) subjects had PDDNOS; 7 (22.58%) subjects had autism; 1 (3.23%) subject had ASD, and 15 (48.39%) subjects either did not have ASD/PDDNOS/autism. Among participants, three individuals (9.67%) experienced seizures and all of them had been diagnosed with ASD.

Although FMRP levels were negatively correlated with CGG repeat length (p= 0.0465). FMRP levels in group A (N 15, Mean 1.53, SD 0.52) were not significantly different (p= 0.7782) from values for the group B (N 16, Mean 1.59, SD 0.59).

Group A consisted of 9 (60%) subjects who were identified as probands and 6 (40%) as non-probands. Group B consisted of 11 (68.75%) subjects who were identified as probands and 5 (31.25%) as non-probands. Among the probands in group A, 7 (64%) had ADHD, 3 (27%) individuals had learning difficulties, and 1 (9%) individual had intellectual disability. The prevalence of probands was not significantly different between the two groups (p=0.716). As expected, ADOS total, communication and social interactive scores differed between the two groups.

Table 1 summarizes the study cohorts, including molecular variables, and ADOS outcome score

| | | **A= No ASD/PDDNOS/autism** | | | **B=ASD/PDDNOS/autism** | | | |
|---|---|---|---|---|---|---|---|---|
| **Variable** | | **N** | **Mean** | **SD** | **N** | **Mean** | **SD** | **P-value** |
| Age | | 15 | 13.50 | 7.70 | 16 | 12.56 | 5.70 | 0.7014 |
| CGG | | 15 | 92 (56-144)* | 29 | 16 | 109 (55-186)* | 48 | 0.233 |
| *FMR1* mRNA | | 15 | 3.23 | 1.29 | 16 | 3.21 | 1.84 | 0.9613 |
| FMRP | | 15 | 1.53 | 0.52 | 16 | 1.59 | 0.59 | 0.7782 |
| Seizures | | | | | | | | |
| Proband | | **N** | **%** | | **N** | **%** | | |
| | Yes | 9 | 60.00 | | 11 | 68.75 | | 0.716 |
| | No | 6 | 40.00 | | 5 | 31.25 | | |

*Association between ADOS communication, social interactive and total score with, FMR1 expression and CGG expansion*

Table 2 summarizes the primary analyses to assess the association between ADOS scores with FMRP, *FMR1* mRNA, and CGG among premutation groups (A= No ASD/PDDNOS/autism, B = ASD/PDDNOS/autism).

There was no significant association between the three ADOS scores (communication, social interactive, total score) and either FMRP or *FMR1* mRNA expression. CGG repeat length was significantly negatively associated with both ADOS communication score (p=0.0173) and ADOS total score (p=0.0358); see Table 2. For example, the total ADOS score declined by a modest 0.0394 unit for one CGG repeat increase (Table 2); thus, the model average ADOS total scores for an individual with 60, 90 or 130 CGG repeats are 5.1, 3.9, and 2.3, respectively (for premutation carriers without ASD).

Table 2 ADOS outcome score

| Variable | Parameter | Estimate | Standard Error | P-value |
|---|---|---|---|---|
| **Model #1: ADOS score= Group* + FMRP** | | | | |
| ADOS Communication Score | Intercept | 0.1465 | 0.9106 | 0.8736 |
| | Group=B | 2.5085 | 0.5920 | 0.0003 |
| | FMRP | 0.5119 | 0.5358 | 0.3498 |
| ADOS Social Interactive Score | Intercept | 0.8415 | 1.3301 | 0.5335 |
| | Group=B | 5.1733 | 0.8647 | <.0001 |
| | FMRP | 0.8998 | 0.7827 | 0.2627 |
| ADOS Total Score | Intercept | 1.0685 | 2.2496 | 0.6393 |
| | Group=B | 6.8448 | 1.4227 | <.0001 |
| | FMRP | 1.8700 | 1.3108 | 0.1671 |
| **Model #2: ADOS score= Group + *FMR1* mRNA** | | | | |
| ADOS Communication Score | Intercept | 1.8301 | 0.8217 | 0.0365 |
| | Group=B | 2.4577 | 0.5843 | 0.0004 |
| | *FMR1* mRNA | -0.2778 | 0.2111 | 0.2017 |
| ADOS Social Interactive Score | Intercept | 3.4861 | 1.2147 | 0.0089 |
| | Group=B | 5.1296 | 0.8637 | <.0001 |
| | *FMR1* mRNA | -0.3934 | 0.3120 | 0.2206 |
| ADOS Total Score | Intercept | 6.7056 | 1.9556 | 0.0023 |
| | Group=B | 6.7464 | 1.4064 | <.0001 |
| | *FMR1* mRNA | -0.8645 | 0.5170 | 0.108 |
| **Model #3: ADOS score= Group + CGG** | | | | |
| ADOS Communication Score | Intercept | 2.5550 | 0.7503 | 0.0025 |
| | Group=B | 2.7371 | 0.5271 | <.0001 |
| | CGG | -0.0177 | 0.0069 | **0.0173** |
| ADOS Social Interactive Score | Intercept | 3.4725 | 1.2191 | 0.0093 |
| | Group=B | 5.4328 | 0.8564 | <.0001 |
| | CGG | -0.0139 | 0.0112 | 0.2274 |
| ADOS Total Score | Intercept | 7.4645 | 1.8688 | 0.0006 |
| | Group=B | 7.5273 | 1.3513 | <.0001 |
| | CGG | -0.0394 | 0.0177 | **0.0358** |

*Group: A= No ASD, as reference; B = ASD/PDDNOS/Autism

## DISCUSSION

ASD is common in the general population and occurs in as many as one in 88 individual.[42] The etiologies of autism are numerous, including a number of known genetic disorders, though FXS is the most common single-gene disorder associated with autism.[43] FMRP regulates the translation of many other proteins important for synaptic plasticity and whose genes are also associated with autism when mutated; when FMRP is absent, many of these gene products are dysregulated. [44] Fragile-X carriers especially boys who have CGG repeat number in premutation range can present with ASD although their etiology may be heterogeneous similar to idiopathic autism. [45] In this study, we investigated the role played by *FMR1* molecular measures in leading to the ASD phenotype among premutation boys and found no correlation between autism phenotypes and both the level of FMRP and *FMR1* mRNA. However, we observed that CGG repeat number was significantly negatively associated with both ADOS communication score and ADOS total score; although the effect size was clinically modest. A variety of mRNA transcripts arise from a combination of alternative splicing, alternative transcriptional start sites selection and

differential usage of polyadenylation sites. In fact, these events generate the vast majority of diversity of gene expression and have been described for over 180 000 mouse transcripts. Besides alternative splicing, which can produce extraordinary protein diversity, regulation at the level of the 5′- and 3′-UTRs modulates mRNA processing, nuclear export, stability, subcellular localization and translational efficiency. Such processes are crucial for differential expression of a gene during development, tissue differentiation and under certain pathological conditions. Since our analysis cohort only had a small sample size of 31 subjects in total, the results need to be interpreted with caution. Larger studies are needed to provide more accurate estimate and to confirm the observed association reported here. Another important limitation of this preliminary evaluation is this association may be confounded by other factors that we may not have in the study, potentially genetic and environmental factors. Those with low CGG-repeat numbers are the most common group of premutation males and perhaps most likely to have autism for additional causes, confirming that the etiology of their autism may be multifactorial or similar to idiopathic autism. A publication demonstrates that approximately 20% of premutation carriers with ASD, ID or neurological problems can have a second genetic hit that may be additive to the premutation specifically a copy number variants (CNV) that may also be associated with autism or ID [46].

The study population reported here included boys who were diagnosed with autism or ASD either before or after premutation status was established. Some presented as the proband of their family, and others were identified by cascade testing once a proband was diagnosed with FXS or premutation involvement. Because of the inclusion of probands with the premutation, our study is biased towards clinical involvement, particularly ASD in this group of patients. However, this is an optimal population of carriers to see if the levels of FMRP or mRNA are related to this type of clinical involvement. We observed a significant negative correlation between FMRP and CGG repeats. However, FMRP was not significantly associated with ADOS measurements (communication score, social interactive score, and total score). The large range of FMRP values in these premutation boys is similar to what has been reported in individuals with normal CGG repeats [39]. Lowered FMRP levels have been seen in the blood and brain tissue of some premutation patients with developmental problems [12,17], and in both blood and brain of premutation mice[18]. Brain levels of FMRP may be significantly lower than blood levels in premutation carriers, particularly in areas of the brain important for social deficits such as the amygdala and insula. Therefore, low levels of FMRP may be present in the brain even when FMRP is not low in blood [47]. Fatemi et al have demonstrated low levels of FMRP in the brain in individuals with idiopathic autism without an *FMR1* mutation, and in those with neuropsychiatric disorders such as schizophrenia[48]. Both age of onset and IQ correlates with level of FMRP in the blood of those with schizophrenia who do not have an *FMR1* mutation [48]. Therefore, FMRP may be important for the clinical phenotype in a number of neuropsychiatric disorders.

Premutation neurons lose viability more rapidly than control neurons in cell culture [33], and display an increase in spike-wave discharges [34] compared to controls. Therefore, environmental factors, such as infections or toxins, associated with autism would be more likely to cause deleterious effects in premutation neurons. There is also evidence of mitochondrial dysfunction in premutation carriers [49], which has been seen in fibroblasts and brain tissues from premutation carriers with and without FXTAS [34]. Mitochondrial problems are also commonly seen in idiopathic autism [50]. Mitochondrial abnormalities could possibly be related to the autism or ASD phenotype linked to RNA toxicity, even without lowered FMRP levels. A variety of environmental factors may elevate FMRP. Jeon et al. have reported that HeLa cells exposed to cell-death inducer etoposide up-regulated FMRP levels.[34] This effect was synchronized with phosphorylation of Akt, a known cell-survival-related signaling molecule. Induction of FMRP apparently plays a protective role against the stressed status of cells and, with reduced FMRP, cell survival is compromised. The premutation is associated with higher rates of seizures in both the current premutation group and I n reports in the literature[28]. Chonchaiya et al. have shown that the presence of seizures is closely associated with the diagnosis of autism or ASD in premutation boys [29]. On a cellular level, premutation neurons demonstrate enhanced spikes in culture, so it appears that RNA toxicity can predispose to seizures in premutation carriers [34]. Seizures in turn can lead to neurochemical changes in the CNS that can further exacerbate developmental problems [51] and seizures can pull FMRP away from the dendritic spines and into the cell body thereby depleting the dendrites of the regulatory effects of FMRP [52].

We do not yet know why some individuals with the premutation have developmental problems or

ASD and others do not. The reasons are not simple, and they not seem to be related to FMRP levels or *FMR1* mRNA levels in blood. There are likely additive and multifactorial effects that involve both background genetic and environmental effects. Further studies of neurotoxicants are warranted, and more detailed genomic studies are needed to better understand the neurodevelopmental effects of the premutation.

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