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Clinical and molecular correlates in fragile X premutation females

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ABSTRACT

The prevalence of the fragile X premutation (55–200 CGG repeats) among the general population is relatively high, but there remains a lack of clear understanding of the links between molecular biomarkers and clinical outcomes.

In this study we investigated the correlations between molecular measures (CGG repeat size, FMR1 mRNA, FMRP expression levels, and methylation status at the promoter region and in FREE2 site) and clinical phenotypes (anxiety, obsessive compulsive symptoms, depression and executive function deficits) in 36 adult premutation female carriers and compared to 24 normal control subjects. Premutation carriers reported higher levels of obsessive compulsive symptoms, depression, and anxiety, but demonstrated no significant deficits in global cognitive functions or executive function compared to the control group. Increased age in carriers was significantly associated with increased anxiety levels.

As expected, FMR1 mRNA expression was significantly correlated with CGG repeat number. However, no significant correlations were observed between molecular (including epigenetic) measures and clinical phenotypes in this sample. Our study, albeit limited by the sample size, establishes the complexity of the mechanisms that link the FMR1 locus to the clinical phenotypes commonly observed in female carriers suggesting that other factors, including environment or additional genetic changes, may have an impact on the clinical phenotypes. However, it continues to emphasize the need for assessment and treatment of psychiatric problems in female premutation carriers.

1. Introduction

Fragile X mental retardation 1 (FMR1) premutation alleles are characterized by an expansion of 55–200 CGG repeats and high levels of FMR1 mRNA [55]. In the United States, about 1 in 260–420 males and 1 in 151–209 women carry a premutation allele [48,57].

Premutation carriers are at risk of developing endocrine and neurological problems, particularly fragile X–associated premature ovarian insufficiency (FXPOI). Approximately 20% of female premutation carriers, particularly those carrying a premutation allele in the 80–100 CGG repeat range, suffer from altered ovarian function [27,39,51], compared to about 1% of women without the premutation in the general population [3,16,54]. In addition, premutation carriers are at risk for the late onset neurodegenerative disorder fragile X–associated tremor/ataxia syndrome, FXTAS [28]. FXTAS is characterized by ataxia, intention tremor, dementia, mood disorders, and global executive function deficit (reviewed in [26]). About 40–50% of males carrying a premutation allele develop FXTAS while only 8 to 16% of female carriers above 50 years old, were found to develop FXTAS [4,7,35].

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Premutation carriers can present with a variety of phenotypic characteristics including executive function deficits [13], social difficulties [18], psychological vulnerability [5], increased risk for autism spectrum disorder [5,11,17,22], hypertension, sleep apnea, migraine, and immune-mediated disorders (reviewed in [27]). Increased age, together with FMR1 mRNA gain of function toxicity may be synergistic in contributing to severity of neurodegenerative symptoms [28,55,34].

Several studies have shown an age-related deterioration in motor, cognitive, and anxiety symptoms in female carriers [1,42,52]. According to Yang et al. [61,62], female carriers have impairments in frontal lobe-mediated tasks such as working memory, performance monitoring executive functions, and processing speed. Furthermore, evidence suggests that both anxiety and depression are characterized by executive dysfunction in young women [63,64]. Interestingly, female premutation carriers demonstrated significantly higher scores of self-reported anxiety and depression compared to control subjects that were correlated with executive functions including working memory and inhibitory control [37]. These findings suggest that neuropsychological tests on executive functions might be useful in identifying markers of risk for psychiatric problems in female premutation carriers [37]. Recent studies have suggested that female carriers exhibit mild deficits in executive function [37,53], mathematical reasoning [38,49], working memory [62] and visuospatial processing [23]. They also experience a higher rate than expected in the general population of psychiatric disorders predominantly depression, anxiety, and obsessive compulsive symptoms [9,43,45,47,50].

Most prior studies examining correlation between psychiatric symptoms or cognitive profiles and molecular measures in premutation female carriers were based on CGG allele size, FMR1 mRNA expression levels and the FMR1 activation ratio (AR; indicating the percent of cells carrying the normal allele on the active X chromosome) [39]. Among these molecular measures, the correlations between CGG repeat size and psychiatric symptoms seem to be the most controversial. There have been reports of no significant associations [18,29,38], reports of inverse correlation where women with < 100 repeats have higher risk while those with > 100 repeats have lower risk for depression and/or anxiety [39,43], and positive correlations in which longer repeats have significant associations [18,29,38], reports of inverse correlation where women with < 100 repeats have higher risk while those with > 100 repeats have lower risk for depression and/or anxiety [39,43], and positive correlations in which longer repeats have higher risk of comorbidity with symptoms [36,47]. Despite the extensive correlation studies of clinical phenotypes with FMR1 premutation carriers, there is no definite biomarker to identify risk and characteristics of molecular mechanisms contributing to these clinical phenotypes. Several studies showed a curvilinear association between the size of FMR1 CGG repeat and a group of symptoms in premutation carriers, such as major depressive disorder [39,43,44], anxiety and depressive symptoms in response to stress from negative life events [48] and menopausal age [16,40]. This finding indicates that the effect of FMR1 molecular measures on the phenotype is quite complex and needs further investigation.

Recently, a novel epigenetic marker, the fragile X-related epigenetic elements 2 (FREE2), was identified and found to be hypermethylated in FXS individuals and unmethylated in controls and in the majority of premutation carriers [19]. Further studies suggested that FREE2 methylation levels can predict executive functions and/or psychiatric disorders in premutation female carriers. Specifically the methylation of several CG sites (CpG) within FREE2 (CpG 6/7 and 10–12) significantly correlated with dysexecutive functions and/or depression and anxiety [14]. It was suggested that FMR1 intron 1 methylation threshold in addition to the activation ratio (AR) could be used to classify premutation female carriers into two categories: lower and greater risk for psychiatric and dysexecutive pattern of symptoms.

In this study, we investigated if altered molecular phenotypes in the FMR1 gene, such as methylation status, CGG repeats number, FMR1 mRNA and FMRP expression levels could account for the clinical phenotypes that are observed in premutation female carriers including executive functions (working memory, response control, and attention) and psychiatric problems, including obsessive compulsive symptoms, anxiety, and depression.

2. Materials and methods

2.1. Subjects

Individuals were recruited through the MIND Institute Fragile X Research and Treatment Center, by postings in the community, through the National Fragile X Foundation or by being participants in research studies involving premutation carriers carried out at the MIND Institute. Participants provided informed consent according to protocols approved by the UC Davis Institutional Review Board.

Participants included 24 healthy females (mean age = 30, SD = 7.5) and 36 premutation female carriers (mean age = 37.5, SD = 12.8). This study is an expansion to our previously reported analysis [24], which examined acute and chronic mental health problems in 24 of the 36 premutation carriers in the current study (mean age = 30.5, SD = 8.03).

In the current study, 46.7% of the participants were married, 43.3% were never married and 10% were divorced. The overall sample included the following races/ethnicities: Caucasian-White (73.3%), Hispanic (16.6%), Asian (3.3%), not Hispanic/more than one race (3.3%), and African American (1.6%). Education level was 3.3% high school diploma, 13.3% had some college studies and 83.4% had bachelor's degree or higher.

3. Molecular measures

3.1. CGG sizing by PCR and Southern blot analysis

Genomic DNA was isolated from peripheral blood using standard procedures (Qiagen, Valencia, CA) as previously described [58]. CGG sizing and methylation status were performed using a combination of Southern blot and PCR analysis. Fully validated methylation-sensitive Southern blot, including appropriate controls, was performed on isolated genomic DNA digested with EcoRI/Nrul, transferred on a nylon membrane and hybridized with the FMR1 specified, dig-labeled, StB12.3 probe. Details of the method are as previously described [58]. CGG sizing was also assessed by AmpliDex® PCR assay, a sensitive and efficient amplification approach as described elsewhere [10]. PCR products were visualized by capillary electrophoresis (ABI 3130 XL Genetic Analyzer, Applied Biosystems) following manufacturer instructions. Analysis of fragment-sizing data for size analysis was performed using Peak scanner software (Applied Biosystems).

3.2. Methylation status

Activation ratio was measured based on the intensity of the appropriate bands on Southern blots as reported in [58]. FREE2 methylation analysis was performed by bisulfite treatment and Sanger sequencing. DNA samples were treated with bisulfite using the EZ DNA Methylation-Gold Kit (Zymo Research; Irvine, CA) as per manufacturer's instructions. Initial PCR conditions were determined and optimized to amplify the coding region of FREE2 for unconverted samples. 100 ng genomic DNA was used as the template. Reactions contained 0.5 μl each of 10 μM forward (5’-CTG AAG AGA AGA TGG AGG AGC TGG-3’) and reverse (5’-AGA GGG CCT TCC AAC AGG CCC C-3’) amplification primers (Integrated DNA Technologies; Coralville, IA). Cycling conditions were as follows: 95°C for 2 min, 35 cycles of 95°C for 1 min, 60°C for 1 min, 72°C for 1 min, followed by 72°C for 10 min, and a final 4°C hold. Each bisulfite-converted sample was analyzed in a second PCR reaction, carried out using 50 ng of converted template, 2.5 μl 10 × buffer with 15 mM MgCl2, 2.5 μl of 10 mM dNTPs, and 0.4 μl of 5 U Taq DNA Polymerase (Invitrogen Corporation; Carlsbad, CA). Amplification was conducted using 10 μM forward (5’-TTG AAG AGA AGA TGG AGG AGT TGG-3’; Eurofins Genomics;
Louisville, KY) and reverse (5′-TCC AAA CCT TCC CCA ACA ACA-3′; Integrated DNA Technologies; Coralville, IA) primers. The reaction mix was pre-activated for 15 min at 95 °C, followed by 45 cycles of 94 °C for 20 s, 61 °C for 30 s, 72 °C for 1 min, followed by 72 °C for 3 min and a final 4 °C hold. Confirmation of the correctly-sized band was determined by agarose gel electrophoresis (2%, 130 V, 30 min).

A standard protocol to chemically transform One Shot® TOP10 competent E. coli was undertaken using 2 μl of the final TOP10 TA Cloning reaction containing the PCR product (Thermo Fisher Scientific, Inc.; Waltham, MA). For control and premutation female samples, 25 single colonies were confirmed to contain the FREE2 region-of-interest by colony PCR analysis. Complete ligation and successful transformation were confirmed by agarose gel electrophoresis (2%, 130 V, 30 min) and correctly-sized amplicons were selected for sequencing analysis.

A control experiment, randomly testing eight samples, included four control subjects and four premutation carriers (the latter with activation ratios of 0.1, 0.7, 0.51, and 0.52 respectively). Samples were analyzed by bisulfit ethylation treatment to check for concordance between activation ratio, as ascertained by methylation at the Nrrl site by Southern blot analysis, and the percent methylation as measured by bisulfit ethylation at the Nrrl site. The forward and reverse primers within the promoter region for converted samples were: 1F7A (5′-AAT TTT AGA GAG GTY GAA TTG GGA TAA-3′), F3A (5′-CCC TCT TTC TTT AAA TAA CTC TAA A-3′), and 1F6A (5′-ACC CTC CAC CYA AAA TAA AAC-3′) (Eurofins Genomics; Louisville, KY). Cycling conditions were as follows: 95 °C for 2 min, 35 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, followed by 72 °C for 5 min.

3.3. RNA isolation and FMR1 mRNA expression levels

Total RNA was isolated prepared from 3 ml of blood collected in Tempus tubes (Applied Biosystems, Foster City, California, USA) or from 1 × 10⁶ cells using Trizol (Life Technologies, Carlsbad, California, USA). The measurement of FMR1 mRNA expression levels was carried out by quantitative Real Time RT-PCR on total RNA using custom designed Taqman gene expression assays (Applied Biosystems) as previously described [55,56].

3.4. FMRP expression levels

We used Cisbio Human FMRP assay (63ADK038PEC0 for detection of fragile X mental retardation protein (FMRP)). The assay used HTRF technology. It involves a sandwich format with two specific monoclonal antibodies conjugated to fluorescent dyes. The donor labeled with Eu2 + -Cryptate and donor d2. Lymphocyte and fibroblast samples were lysed with lysis buffer for 3 h, using Roche Complete Ultra Protease Inhibitor Tablets (05 892 988 001). Total protein concentration performed by Thermo Fisher MicroBCA Assay (23235). 6 μg/μl and 3 μg/μl total protein was used in triplicates in a 384-well format following the instructions on assay protocol. Samples were incubated overnight with both antibodies and read on the PerkinElmer VictorX5.

3.5. Cognitive and psychological measures

The executive function assessments included the working memory index from Wechsler Adult Intelligence Scale, 3rd Edition (WAIS-III) [60], and the Response Control Quotient and Attention Quotient from the Integrated Visual and Auditory Continuous Performance Test (IVA) [46]. Self-reported psychological symptoms, notably the obsessive compulsive symptoms T-Score, Anxiety T-score and Depression T-Score were used from the Symptom Checklist 90 revised (SCL-90-R) [15]. Life time presence of anxiety disorder was identified by the clinician-administered Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-I) [41].

3.6. Statistical analysis

Subject age was compared between groups using a two sample t-test, and distributions of categorical subject demographic characteristics were compared between groups using Fisher’s Exact Test. The association of subject age and of activation ratio with continuous clinical measures was analyzed using linear regression and the association of subject age and activation ratio with ordinal clinical measures was analyzed using proportional odds logistic regression. Continuous clinical measures were compared between groups using linear models, and ordinal clinical measures were compared between groups using proportional odds logistic regression models, with age included as a covariate in both cases. Likewise, the associations between clinical data and FMR1, FMRP and CGG repeat length were analyzed using linear models in the case of continuous clinical measures and proportional odds logistic regression in the case of ordinal clinical measures; all models allowed for separate effects in control and premutation subjects and included age as a covariate. Linear regression was used to test for associations between molecular measures. P-values were adjusted for multiple testing within each table using the Benjamini-Yekutieli false discovery rate controlling method [6]. Analyses were conducted using R, version 3.3.1 [59].

Post-hoc power calculations are not included, as these contribute no additional information beyond that provided by the confidence intervals and P-values already shown, as detailed in [32].

4. Results

4.1. Demographics and clinical features

The mean age of premutation subjects was significantly greater than that of control subjects (t = 2.83, 57.3 df, P = 0.006). The mean number of CGG repeats was 32 in the control group compared to 86 in premutation carriers (Table 1). Among the female premutation carriers, five were mothers of children with FXS, two had children with a premutation and one had a typical child. In addition, 11.1% had a history of FXPOI and none had FXTAS. We found that among the female premutation carriers, 50% had significantly elevated current depression, 33.3% anxiety and 61.1% obsessive compulsive symptoms compared to 8.3%, 4.2% and 12.5% respectively, in the control group (Fig. 1, Table 2; adjusted P = 0.017 for depression, P = 0.049 for anxiety and P = 0.019 for obsessive compulsive symptoms).

When using SCL-90R, the linear models and proportional odds logistic regression models comparing clinical measures between groups showed that mean scores for anxiety (adjusted P = 0.049), obsessive compulsive symptoms (adjusted P = 0.019), and depression (adjusted P = 0.017), were all significantly higher in the premutation group than in the control group after adjusting for age. When observing the effect of age on lifetime history of DSM-IV disorders, increased age was associated with greater likelihood of anxiety disorder (adjusted P = 0.008). None of the other clinical measures (SCID-Depression and SCID-obssessive compulsive symptoms) was significantly correlated with subject age after adjustment for multiple testing (Table 2). On the other hand, when comparing groups on lifetime history of DSM-IV disorders, there was no association of any of the SCID measures with premutation carriers compared to female controls although a marginally significant increased rate of lifetime anxiety disorder was observed.

4.2. Correlation among molecular measures

Results from linear regression models for pairs of molecular measures showed that, as expected, FMR1 mRNA expression was significantly correlated with CGG repeat number (adjusted P = 0.001). No significant correlations were observed among the other molecular measures.
4.3. Correlation between clinical outcomes and molecular measures

No clinical measures were found to be associated with FMRP, CGG repeat number, FMR1 mRNA levels, FMRP levels, activation ratio or FREE2 methylation sites following correction for multiple testing and adjusting for age (Figs. 2 and 3).

5. Discussion

This study examined correlations between molecular measures at the FMR1 locus and key clinical outcomes in female premutation carriers. Our findings show that the premutation group had significantly higher current symptoms than control group for obsessive compulsive symptoms, depression, and anxiety, and a marginally significant increased rate of lifetime anxiety disorder after multiple comparison and age adjustment.

These results are consistent with previous studies that premutation females tend to report more psychological difficulties compared to control groups [29,39,47,55,56]. Notably, in our premutation group 11% (4 out of 36 subjects) were affected by FXPOI, 13.8% (5 out of 36 subjects) have children by FXS, 5.5% (2 out of 36 subjects) and had children with a premutation. Therefore, it is unlikely that these psychiatric symptoms may be caused by distress of raising children affected by FXS, observations consistent with previous reports [24,43,44]. Roberts et al. [43] reported that nearly half of the premutation participants (48%) in their study had depression before having affected children with FXS. Another study by the same group [44] revealed that the rates of depression and anxiety disorders did not differ between females over age 40 with and without FXPOI despite the high prevalence of FXPOI in this cohort (41%). Therefore, the presence of absence of FXPOI or children with FXS was not proven to be of significant influence on the psychological symptoms in female premutation carriers.

Our previous study showed that premutation carriers were at a significantly higher risk of having psychiatric problems such as anxiety, depression and OC symptoms compared to normal females [24]. Interestingly, we found a significant positive correlation between age and anxiety confirming a finding that has been previously reported in this population.

Previous studies demonstrated that premutation carriers were prone to meet criteria for major depression, panic disorder, and specific phobias at a later onset compared to a control group. The late onset might be influenced by biological and psychosocial factors. This comes in line with the findings in our study in which 53.3% of female carriers with a mean age of 37.5 years were shown to have clinically significant anxiety symptoms compared to 8.3% of the control group. Furthermore, approximately 66.6% of premutation group had clinically elevated depressive symptoms and 23.3% reported OC symptoms. The FMR1 mRNA toxicity derived from the observed elevated FMR1 mRNA expression levels, may perturb certain cortical areas as well as the limbic system [30]. This in turn may contribute to brain changes [1] and a higher risk of having neurodegenerative disorders and difficulties dealing with multiple distresses throughout their lives [50]. However, in this study we did not observe a correlation between FMR1 mRNA expression levels and psychiatric problems, due perhaps to the small sample size, potentially to the weaker effect observed in females [29] or to the difference in mRNA expression levels between peripheral blood and different areas of the brain. Thus, this hypothesis awaits further longitudinal study.

Working memory and attention did not differ between the premutation and the control groups. These findings suggest that the premutation carriers in our study have intact cognitive abilities, despite experiencing higher levels of depression, obsessive compulsive symptoms and anxiety. The results obtained from the selected executive functions assessments (Working memory index, response quotient, and attention) indicate that the premutation carriers in this study have similar global cognitive functions to that of the control group. This was also reported to be the case in previous studies of both adult male premutation carriers [2,25,31] and of a larger female sample size [33,39].

Similar to our results, two previously published studies showed no differences in neuropsychological abilities among premutation carriers without FXTAS and non-carriers [2,33]. Allen et al. [2] investigated the effect of CGG repeat numbers on cognitive performance of 84 female premutation carriers compared with 74 control subjects using the Wechsler Adult Intelligence Scales-Third Edition (WAIS-III) and found no significant differences. However, the premutation group showed marginally decreased Verbal IQ. Hunter and colleagues [33] further expanded the study to cover 293 women with premutation alleles and 117 normal participants examining six factors of neuropsychological function including visual processing and memory, verbal comprehen-

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Table 1
Subject characteristics by group.

|                        | Control (n = 24) | Premutation (n = 36) | All subjects (n = 60) | P-value |
|------------------------|-----------------|----------------------|-----------------------|---------|
| CGG repeats            |                 |                      |                       |         |
| N                      | 24              | 36                   | 60                    | n/a     |
| Mean (SD)              | 31.8 (4.1)      | 86.1 (19.6)          | 64.4 (30.9)           |         |
| Median (range)         | 30 (28-47)      | 85 (56-152)          | 69 (28-152)           |         |
| Molecular category     |                 |                      |                       |         |
| (n, %)                 |                 |                      |                       |         |
| 0                      | 24 (100%)       | 0                    | 24 (40%)              |         |
| 1                      | 0               | 36 (100%)            | 36 (60%)              | 0.006   |
| Age                    |                 |                      |                       |         |
| Race (n, %)            |                 |                      |                       | 0.275   |
| African American       | 0               | 1                    | 1 (1.7%)              |         |
| Asian                  | 2               | 3                    | 2 (3.3%)              |         |
| Hispanic               | 3               | 2                    | 5 (8.3%)              |         |
| More than one race     | 2               | 2                    | 4 (6.7%)              |         |
| White                  | 17              | 31                   | 48 (80%)              |         |
| Marital status         |                 |                      |                       | 0.801   |
| Divorced               | 3               | 3                    | 6 (10%)               |         |
| Married                | 10              | 18                   | 28 (46.7%)            |         |
| Never married          | 11              | 15                   | 26 (43.3%)            |         |
| Education level (n, %)|                 |                      |                       | 0.064   |
| H.S diploma/GED        | 0               | 2                    | 2 (3.3%)              |         |
| Associates degree      | 1               | 2                    | 2 (3.3%)              |         |
| Some college studies   | 7               | 2                    | 8 (13.3%)             |         |
| Bachelors              | 9               | 17                   | 26 (43.3%)            |         |
| Some grad/ professional| 3               | 6                    | 9 (15%)               |         |
| school                 | Graduate/professional degree | 4 | 9 (25%) | 13 (21.7%) |

Fig. 1. Bar chart showing comparison of clinical phenotypes between the control and premutation groups using SCL-90R.

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sion and memory, processing speed, self-report of inattention and impulsivity, sustained attention, and response fluency. They found no significant correlation between these clinical measures and CGG repeat length within the female premutation group. However, they reported a moderate and significant increase in self-reported inattention and impulsivity in premutation carriers relative to controls respectively. They suggested that females with the premutation might be at higher risk of having some symptoms of ADHD.

As expected, we found that CGG repeat number, adjusted for the AR, was significantly correlated with \( FMR1 \) mRNA expression. CGG repeat size was also significantly correlated with FMRP but this significance was lost after multiple comparison adjustment. On the other hand, linear regression models showed no correlations between clinical and molecular measures including CGG repeat numbers, \( FMR1 \) mRNA, FMRP, activation ratio, and \( FREE2 \) methylation in premutation carriers compared to the control group. These findings are consistent with previous studies [8,12,18,20,21,24,29,38].

In our previous report [24], no correlation was shown between CGG repeat size or \( FMR1 \) mRNA with scores of psychiatric symptoms as measured by SCL-90R in the premutation group. In this study, we further investigated the potential correlations of psychiatric measures, using both SCL-90R and SCID, in a slightly larger sample size and confirmed the absence of correlation with molecular measures using linear regression model.

In addition, we explored the effect of molecular measures on executive functions in the premutation group but found no correlation with either CGG repeat size, or the level of \( FMR1 \) transcript, or of FMRP. The molecular correlates also included methylation at the \( FREE2 \) site earlier identified by Godler et al. [19] and shown to be hyper-methylated in some PM females. Another study [14] of executive functions and self-reported symptoms of psychiatric problems in 35 females carrying premutation proposed that methylation at the \( FREE2 \)

### Table 2
Comparison of clinical measures between groups.

| Clinical measure               | Mean (SD) in control | Mean (SD) in premutation | Age-adjusted difference in means (95% CI) | P-value Benjamini-Yekutieli adjusted P-value |
|--------------------------------|----------------------|--------------------------|------------------------------------------|---------------------------------------------|
| SCL-R90 anxiety                | 46.8 (9.2)           | 55.7 (10.7)              | 8.3 (2.6, 13.9)                          | 0.005                                       |
| SCL-R90 obsessive compulsive   | 51.8 (8.5)           | 59.9 (9)                 | 8.4 (3.4, 13.3)                          | 0.001                                       |
| symptoms                       |                      |                          |                                          | 0.019                                       |
| SCL-R90 depression             | 48.7 (10.9)          | 58 (8.3)                 | 9.6 (4.3, 14.9)                          | 0.001                                       |
| Working memory index           | 106.7 (13.4)         | 107.9 (12.1)             | 0.1 (−7.1, 7.2)                          | 0.985                                       |
| Working memory actual index    | 110.7 (12)           | 107.2 (10.7)             | −4 (−10.4, 2.5)                          | 0.226                                       |
| score                          |                      |                          |                                          | 0.999                                       |
| Response control quotient      | 90.3 (23.5)          | 99.8 (15.6)              | 6.2 (−4.2, 16.6)                         | 0.236                                       |
| Attention quotient             | 91.9 (28)            | 96 (18.1)                | 1.2 (−11.3, 13.6)                        | 0.851                                       |
| SCID lifetime depression       | 1: 10 (41.7%)        | 1: 13 (36.1%)            | 1.15 (0.40, 3.29)                        | 0.790                                       |
| SCID lifetime anxiety          | 1: 19 (79.2%)        | 1: 21 (58.3%)            | 1.71 (0.48, 6.52)                        | 0.410                                       |
| SCID lifetime OCD              | 1: 22 (91.7%)        | 1: 27 (75%)              | 3.58 (0.76, 25.97)                       | 0.110                                       |

Fig. 2. Scatter plots show scores of executive function including (a) working memory, (b) response control, (c) attention and clinical phenotypes (d) obsessive compulsive symptoms, (e) depression, and (f) anxiety as a function of CGG repeat length in PBMCs between premutation group (open circle) and control group (black circle).
region, and specifically in CpG 10–12 and 6/7, could be of benefit in classifying premutation females into two categories of higher and lower risk for impairments in executive functions and psychiatric symptoms. In our current study, we used an independent sample of PM females of comparable size. However, we did not find any correlations between this marker and either cognitive or psychiatric status after adjusting for age and multiple testing.

There are some plausible reasons why the FREE2 clinical correlation results may differ in our study compared to that of Cornish and colleagues [14]. First, the neuropsychological tests vary across the investigations; one might have higher sensitivity and specific-to-specific domain of executive functions more than others resulting in different outcomes. However, if a true association exists, we expect it to be relatively robust across executive function parameters. Second, they did not apply adjustment for multiple testing to control the family-wise type I error rate possibly resulting in significant correlation and/or differences that could occur by chance. Finally, outcomes might differ with the variation in the inclusion criteria, and method of recruitment of the subjects.

There are some limitations in our study. First, the sample size is relatively small hence its data may not be sufficiently large to detect associations that may exist and the mean age between in the 2 groups was significantly different (Table 1). Second, the full repeat length distribution was not well represented. Our study had only seven carriers with > 100 CGG repeat whereas the majority had mid-range repeats, which could contribute to different results from [14]. Lastly, although the examiners were blind to the genotype of the participants, the subjects typically knew their status and this might have affected how they responded to the testing.

In conclusion, our results showed that female carriers in this study had no apparent executive function deficits; however psychiatric problems including obsessive compulsive symptoms, depression, and anxiety were relatively common. The lack of simple correlations between FMR1 molecular measures and these clinical outcomes suggest the need to study more complex models in larger samples that can account for additional genetic changes and/or environmental influences.

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