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Linking the FMR1 Alleles with Small CGG Expansions with Neurodevelopmental Disorders: Preliminary Data suggest an involvement of Epigenetic Mechanisms

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To the editor

Three allelic classes of expanded CGG trinucleotide repeat in the fragile X mental retardation 1 (FMR1) gene have been recognized. The full mutation, FM (>200 repeats), which causes a Fragile X syndrome, is normally associated with epigenetic silencing of the FMR1 gene's promoter, leading to a gross deficit of a specific protein product (FMRP), and subsequent synaptic abnormalities [Irwin et al., 2000]. In contrast, the premutation (PM) alleles, ranging from ~55 to 200 repeats [Maddalena et al., 2001], are associated with the obvious elevation of FMR1 mRNA levels [Tassone et al., 2000]. Apart from the late-onset neurodegenerative disorder termed 'fragile X associated tremor/ataxia', FXTAS [Hagerman et al., 2001], this elevation may account for some neurodevelopmental changes, manifesting as learning deficits [reviewed in: Loesch et al., 2004], or behavioural conditions, ADHD [Farzin et al., 2006], and ASD [Clifford et al., 2007], all occurring predominantly in males. Some behavioural and cognitive problems were also reported in apparently unaffected adult premutation carriers, such as attention deficits in females [Hunter et al., 2008], and

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impairment of executive control of memory, in males [Cornish et al., 2009]. The intermediate length or grey zone (GZ) FMR1 alleles have been relatively poorly defined, with the recommended upper boundary of ~ 54 CGG repeats, and the lower boundary of 45 repeats, the latter based on the claim that the alleles below this size showed 'no meiotic or mitotic instability' [Maddalena et al., 2001]. However, both boundaries for this range varied between individual studies, extending from 34 CGG repeats, for the lower boundary, to 60 repeats, for the upper boundary [reviewed in: Loesch et al., 2007; Mitchell et al.2005]. Resolving an issue of phenotypic effects associated with GZ alleles is important considering their high population frequency of ~3-4 per 100 [Youings et al., 2000]. This implies that even a small effect, if substantiated, is likely to be borne by a considerable number of individuals. However, the results of rare studies based on special educational needs (SEN) students have been controversial, with only some proportion of studies reporting a significant excess of carriers of GZ alleles in these populations [reviewed in: Mitchell et al., 2005].

The molecular investigations of the functional status of GZ alleles have been more informative. We showed a significant elevation of FMR1 m RNA in 43 young male carriers identified amongst SEN students in the 2005 survey [Mitchell et al., 2005], compared with normal controls of similar age [Loesch et al., 2007]; this elevation was proportional to the size of CGG expansions within the GZ (with the lower bound of 41) and the lower premutation range. The late onset fragile X associated neurodegeneration has been attributed to the RNA 'toxicity' [Greco et al., 2002]. However, the applicability of this model to neurodevelopmental conditions which may be attributed to either PM or GZ alleles, is still unresolved.

Here we conducted comprehensive molecular testing in 26 consenting Caucasian male carriers of GZ alleles ages 7-19 years from the total of 43 carriers identified in our 2005 [Mitchell et al.] study. The CGG repeat number ranged from 41 to 54, with one subject with 55 repeats also included as borderline. Most (16) came from the SEN population, 5 carriers were from a non-SEN population from the same schools, 5 were brothers of the identified carriers. We adopted 40 CGG repeats as the lower boundary of GZ range based on our earlier finding that the onset of an elevation of mRNA levels was recorded in FMR1 alleles with 40 repeats [Loesch et al., 2007]. We have also included 4 normal repeat size controls in molecular analyses. The neuropsychological testing was conducted in all but two GZ carriers (N=24), and the individual results are available from the authors. Six participants had their FSIQ scores assessed by the *Wechsler Intelligence Scale* (appropriate for age) < 1 SD, but nearly one half (11/24) showed deficit in executive skills assessed by *The Controlled Oral Word Association Test* [Spreen & Benton, 197], 7 of 20 showed impairment in adaptive skills assessed by the *Vineland Adaptive Behaviour Scales Interview* [Sparrow & Cicchetti, 1984], and one half showed concentration deficits assessed by *The Conner's Global Index-Parent version* [Conners et al., 1996]. One third of the sample was classified as ASD using the *Autism Diagnostic Observation Schedule-Generic, ADOS-G* [Lord et al. 1999]. This research was prospectively reviewed and approved by Human Research Ethics committees of La Trobe University and Royal Hobart Hospital.

The major aim of this study was to get a better insight into the mechanisms involved in the FMR1 mRNA 'toxicity' in GZ carriers identified in the Tasmanian study, by relating the levels of mRNA transcript to the expression of a prominent epigenetic marker, DNA (cytosine-5-)-methyltrasferase 1 (DNMT1). This marker is known to regulate FMR1 transcription in full mutation subjects [Pietrobono et al., 2002], and to play a major role in regulation of global methylation coupled to DNA replication [Biniszkiewicz et al., 2002]. We have also investigated the relationship between levels of these two transcripts and cytochrome c-1 (CYC1), a nuclear oxidative-phosphorylation gene expressed during periods of mitochondrial expansion [Li et al., 1996]. This was to explore the possibility that epigenetic mechanisms are associated with the FMR1 mRNA toxicity of GZ alleles, leading to CYC1 over-expression associated with mitochondrial dysfunction [Li et al., 1996]. The methods of CGG repeat sizing and FMR1mRNA assessments were reported in: Loesch et al. [2007]. Here we validated the assays for FMR1mRNA levels using a relative standard curve method, and found that the results closely corresponded to those earlier estimates in a larger sample, which included the present participants [Loesch et al., 2007].

The results of correlations between the molecular measures assessed using linear regression showed significant relationship between the levels of FMR1 mRNA and CGG size p=0.046 (Fig. 1A), DNMT1 p=0.022 (Fig. 1B), and CYC1 p=0.045 (Fig. 1C). The DNMT1 expression was also significantly correlated with CYC1 expression (Fig. 1D). The relationship between CGG size and DNMT1 or CYC1 levels was not significant, but an extensive variability and the small sample size should be considered. The correlations between neuropsychological scores and molecular measures, using regression models appropriate for the shape of distribution of individual variables, were not significant in the reduced sample of 24 (or less for some neuropsychological measures).

Since ASD was one of the commonest diagnoses in the identified SEN carriers, we screened an independent sample of ASD children, diagnosed using ADOS-G [Lord et al., 1999], for the size of CGG repeats in the FMR1 gene. This sample comprised 42 Caucasian male children and adolescents aged 5-20 years, recruited via advertisements placed in publications of autism specific organizations in Victoria. Potential heterogeneity of the sample was reduced by selecting higher functioning individuals based on their performance on a Wechsler intelligence test, with the median PIQ of 89, and omitting individuals with co-morbid diagnoses. All participants provided cheek swab samples. Genomic DNA was obtained by placing a buccal brush in a labelled tube containing 400 μL of 50 mM NaOH, rotating, and incubating at 95°C for 15 minutes. 80 μL of 1M Tris-HCl pH 7.5 was added, mixed and centrifuged, and then stored at 4°C. The supernatant was removed and stored at −20°C until testing. The CGG repeat sizing was performed using a fully validated PCR assay [Khaniani et al., 2008], and was assessed with precision of +/- one triplet repeat across the normal and GZ ranges, using a fragment analyser (MegaBace, GE Healthcare). The distribution of the number CGG repeats from the autism sample was compared with a distribution in a population of Tasmanian newborns [described in Mitchell et al. 2005], where DNA was extracted from autoclaved Guthrie cards following the procedure described by Holden et al. [1996]. Scoring of the CGG repeat size was as in: Holden et al. [1996] using the same primers *c* and *f* [Fu et al., 1991] in a radioactive reaction. PCR products,

together with allele ladders of known sizes, were run on a 6% polyacrylamide gel. The results (triplet repeat number) of this test correlated very highly with the results obtained from the saliva samples (r^2=0.974, y=0.999x-0.108), which shows the robustness of the analyses. We found a significant excess of GZ carriers (N=5, with repeat size ranging from 41-52) in the autism sample of 42, compared with a normal control sample of 576, with 19 carriers identified (two sided Fisher's exact test p-value =0.018).

Our results suggested that GZ alleles might be associated with some behavioural phenotypes, including ASD, and that epigenetic dysregulation, triggered by elevated FMR1 transcript, might be involved in the origin of these phenotypes. Moreover, we replicated the results of our previous study in a larger sample [Loesch et al., 2007], showing a significant elevation of the FMR1 transcript, and the linear relationship between the levels of this transcript and the number of CGG repeats in alleles within the GZ range. The findings from those two studies are consistent with the earlier data based on human cell lines transfected with the FMR1 5'-UTR containing CGG repeat lengths ranging 0 to 99, and a downstream reporter gene, which demonstrated an increase in transcription levels for constructs possessing either PM or GZ alleles [Chen et al., 2003].

Further, we found that the over-expression of FMR1 was significantly correlated with the expression of DNMT1, and there was a marginally significant relationship between the expression levels of DNMT1 and CYC1, the latter being a marker of mitochondrial dysfunction [Li et al.,1996; Ayub & Hallett, 2004]. These findings are suggestive of epigenetic involvement in GZ alleles, and are supportive of the results obtained for PM alleles from a human kidney fibroblast cell line (HEK293), where insertion of 176 CGG repeats 3' of the FMR1 promoter induced over-expression of FMR1 mRNA, and altered expression of major epigenetic factors, which was associated with increased apoptosis [Handa et al., 2005]. Considering small sample size, and only a marginally significant correlation between the FMR1 and DNMT1 transcripts in our study, the validity of this hypothesis warrants confirmation using larger independent samples of both controls and carriers including PM, as well as GZ, carriers, and verifying the observed increase of DNMT1 transcript by assessment of the levels of this gene's protein product by Western analyses.

We have characterized cognitive and behavioural status of 24 GZ carriers included in this study and found that the most common manifestations were impaired executive skills and attention problems, and one third fell into the ASD category. Although these findings are suggestive of the role of GZ alleles in the origin of these phenotypes, we need to emphasize that they were based on a sample largely pre-selected for behavioural or cognitive problems. A similar argument applies to the data in: Aziz et al. [2003], where the main manifestations in 6 PM and 4 GZ carriers aged 4-15 years comprised ASD, hyperactivity, and minor cognitive impairments. The only statistical evidence so far for the influence of small CGG repeat expansions on cognitive status was based on the finding of a significant link between the GZ alleles and reduced cognitive abilities in a large sample of male students aged 4-7 years [Loat et al., 2006]. In our data, we did not find significant correlations between neuropsychological and molecular measures, but considering small sample size the power of the test may have been inadequate. However, our results in boys with ASD, though they

need to be confirmed, suggest that GZ alleles may be associated with an increased risk of autistic behaviours. An increased prevalence of ASD has already been reported in the carriers of PM alleles [Clifford et al., 2007]. That the involvement of epigenetic component suggested by our results from the total sample of SEN children carrying GZ alleles may also apply to the carriers with ASD has been indicated by dysregulation of UBE3A, associated with the elevation of FMR1 transcript in the PM alleles [Handa et al., 2005]. A concept of disturbance of epigenetic regulation leading to aberrant gene expression and abnormal neurodevelopment is just emerging [Zhao et al., 2007], and there is ample evidence that some forms of co-morbid autism have strong epigenetic links.

In summary, our data suggest that epigenetic component might be involved in a 'toxicity' of excessive FMR1 transcript in small expansion alleles, leading to decreased cell survival and thus contributing to the increased risk of neurodevelopmental conditions, including autism. If this hypothesis is confirmed in future studies based on larger numbers and the whole range of small CGG expansions, it will create opportunities for epigenetic therapy [Guy et al., 2007].

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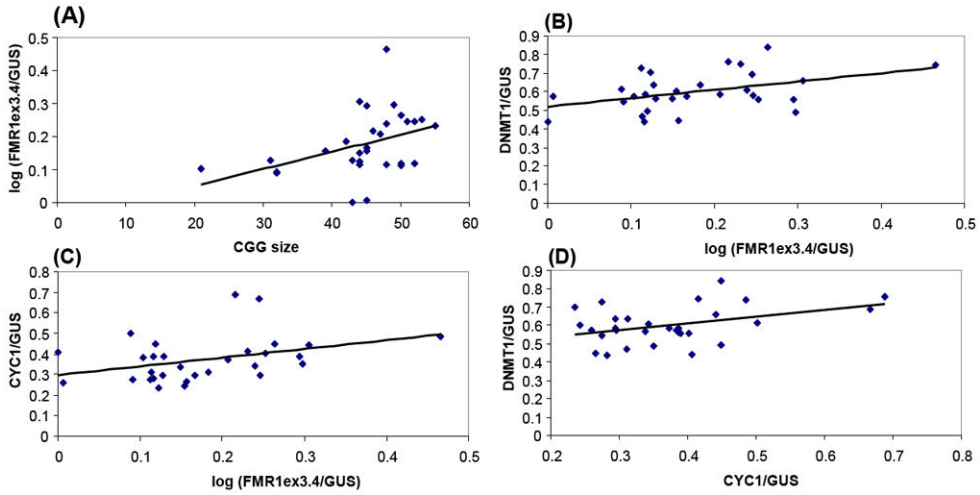

Figure 1.

*. Association of FMR1 mRNA with CGG expansion size and expression of DNMT1 and CYC1 in the whole blood of 26 GZ carriers and 4 normal controls. Statistical analysis was conducted using the R statistical computing package version 1.9 (R development Core team, 2004).

*FMR1, DNMT1 and CYC1 mRNA levels examined using reverse real-time PCR. An aliquot of total RNA was isolated from 3 ml of peripheral blood using Tempus Blood RNA tubes [Loesch, et al. 2007]. All samples were diluted to 6ng/ul, and reverse transcription was performed using the Multiscribe Reverse Transcription System (Applied Biosystems), 50 units/ul, as per manufacturer's instructions. The 7900HT Fast Real Time PCR System (Applied Biosystems) was then utilized to quantify FMR1, DNMT1 and CYC1, where all target genes were standardized GUS using the relative standard curve method as previously described [Godler et al. 2009]. Samples were quantified in arbitrary units (au) in relation to the standard curves performed on each plate.