Intermediate sized CGG repeats are not a common cause of idiopathic premature ovarian failure

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Introduction

The Fragile X mental retardation 1 (FMR1) gene is on the X chromosome and contains a polymorphic 5′ CGG-triplet repeat which is transcribed into mRNA but not translated into a protein. Full mutations (≥200 repeats) cause Fragile X syndrome (FXS) predominantly in males (Hagerman and Hagerman, 2004), although premutations (59–200 repeats) are associated both with Fragile X Tremor and Ataxia Syndrome (FXTAS; Hagerman and Hagerman, 2004) and premature ovarian failure (POF; Allingham-Hawkins et al., 1999).

POF results in amenorrhoea, the cessation of ovarian function and elevated FSH levels (≥40 IU/l) and affects ~1% of women before the age of 40 years (Coulam et al., 1986; Conway, 2000). In females from families with FXS ~23% of premutation carriers had POF (Allingham-Hawkins et al., 1999). In women with a history of idiopathic POF ~5% were found to be premutation carriers (Murray et al., 1998). The definition of a premutation is somewhat arbitrary and is generally based on the stability of the repeat during meiotic transmission and the likelihood that the repeat will expand to a full mutation: the smallest repeat that has been shown to expand to a full mutation in one generation is 59 CGGs (Nolin et al., 2003), and therefore the boundary of premutation and normal is usually taken at about 55 CGGs. Large repeats sizes around the boundary of normal and premutation are termed intermediate or grey zone. Two studies have reported an association between idiopathic POF and intermediate sized repeats: the first, a Canadian study, defined intermediate as 35–54 CGG repeats (Bretherick et al., 2005) and the second, an Italian study, defined intermediate as 41–58 CGG repeats (Bodega et al., 2006).

We aimed to replicate these findings in an independent, large cohort of women with idiopathic POF.

Materials and Methods

Cases

POF was defined as absent menstruation for a period of at least 6 months before the age of 40 years: we excluded cases with known causes, e.g. hysterectomy, pelvic surgery, chemotherapy or cytogenetic abnormality (determined by G-banded karyotype analysis) and we included cases of primary amenorrhoea. Participants were from one of two sources: endocrinology/gynaecology clinics at the Middlesex Hospital, London (n = 154) or patients with POF who were sent to the Wessex Regional Genetics Service, Salisbury for chromosome analysis (n = 212; Conway et al., 1998; Table I). A proportion of these cases have been included in previously reported series (Murray et al., 1998). In most cases an exact age
at menopause was known, reported by either the patient or the referring clinician. However, in approximately one-third of cases the exact age of menopause was not known but patients were classified as having POF because they were known to be under the age of 40 when they were ascertained as having ovarian failure.

Controls
Controls were from a Fragile X screening survey, in which 2779 mothers of boys with learning difficulties were recruited from the Southwest UK region (Youings et al., 2000). Twenty of the boys with learning difficulties had a full mutation expansion, but also there was an increased incidence of alleles containing >40 repeats, leading us to suggest that intermediate expansions had an effect on cognition. Thus, the allele each mother transmitted to her son could not be used as a control. However, the allele on her other chromosome, which was not passed on to the son with learning difficulties, was ascertained without bias and the repeat size of the untransmitted allele is completely independent of the repeat size of the transmitted allele, because they were inherited from different parents. Hence alleles that were not transmitted to the boys with learning difficulties were used as controls. We had no information about menopause or reproductive history for the control group, except that they had to have had at least one child to be included in the Fragile X screening survey. The control chromosomes represent a random unbiased set of chromosomes from the population and therefore we can assume that the frequency of POF associated with these chromosomes would be ~1%. Power calculations based on the method by Edwards et al. (2005; Gordon et al., 2002), which accounts for the presence of phenotype misclassification, demonstrate that the cost of misclassifying a case as a control has an almost negligible affect on our power to detect the allele frequency differences observed by Brethertick et al. (2005) and Bodega et al. (2006). We had >95% power at the 1% probability level, assuming 1% of controls were affected with POF (http://linkage.rockefeller.edu/pawe/paweph.htm). To enable comparison of the POF group with the control group of single chromosomes, allele frequencies were determined per chromosome rather than per individual.

Genotyping
The FMR1 CGG repeat number was determined as previously described (Ennis et al., 2006), using primers c and f (Fu et al., 1991) and fluorescent labelled PCR, followed by size separation on an ABI 3730 instrument (Applied Biosystems, Warrington, UK). Females with a single allele were followed-up with Southern blot analysis because the PCR method is not sensitive enough to detect large expansions. Therefore, females with a single allele by PCR could either be homozygous for that allele or have a cryptic expansion that can only be detected by the Southern blot method. The POF and control groups were compared for the incidence of three categories of FMR1 repeat: 51–200 repeats, 35–54 CGG repeats (Bretherick et al., 2005), and 41–58 CGG repeats (Bodega et al., 2006). Statistical analyses were performed in Statistics Package for the Social Sciences (SPSS) V15, using Fisher’s exact test.

Results
Incidence of FMR1 premutations in women with idiopathic POF
Women with POF from both centres were combined (n = 366) and the incidence of premutation-sized repeat alleles was compared with controls (Table II: Analysis 1). As 2.5% of chromosomes from females with idiopathic POF had a premutation allele (defined as >50 and <200 CGG, unmethylated repeats), ~5% of women with POF were carriers. Consistent with our previous studies, there was a significant excess of FMR1 premutation carriers in women with POF compared with controls (OR = 9.98, P = 5 × 10⁻⁸, Murray et al., 1998).

Incidence of FMR1 intermediate alleles in women with idiopathic POF
In order to exactly replicate the methodology of the two other studies, intermediate alleles were defined in two ways. Analysis 2 used the definition of 35–54 repeats (Brethertick et al., 2005), and found no association between repeats in this range and POF (Table II: Analysis 2, P = 0.48). Analysis 3 used the definition of 41–58 repeats (Bodega et al., 2006), and found only a very modest increase in the OR which was not significant (Table II: Analysis 3, P = 0.23).

Discussion
In a cohort of 366 females diagnosed with idiopathic POF, ~5% were carriers of an FMR1 premutation allele, nearly 10 times greater than the incidence in controls. These data represent, to our knowledge, the largest series reported to date and confirm previous evidence that FMR1 premutations, but not full mutations, are a significant cause of ovarian failure before the age of 40 years.

Gene transcription from premutation alleles is significantly increased in proportion to CGG repeat number, leading to suggestions that the accumulation of FMR1 mRNA may have a detrimental effect on cells expressing the gene (Tassone et al., 2000). It has been demonstrated that premutation FMR1 mRNA forms part of intranuclear inclusions found in neurones of FXTAS patients (Greco et al., 2006), supporting the hypothesis that FMR1 expansion mutations may have a toxic gain-of-function effect. However, it is currently not known how premutation mRNA may lead to early menopause.

In addition to POF, it has also been suggested that intermediate-sized FMR1 repeats may also be associated with developmental disabilities (Youings et al., 2000; Aziz et al., 2003). Intermediate FMR1 alleles within the range of 41–60 repeats have been shown to generate increased amount of FMR1 transcripts in males compared with alleles in the 5–40 repeat range (Loesch et al., 2007). Although the amount of mRNA produced from intermediate alleles is less than for premutation alleles, it is possible that the increase in mRNA levels from intermediate alleles is sufficient to have a detrimental effect. Sequence analysis of intermediate sized repeats in women with POF found that in the majority of cases, the repeats lacked the AGG interspersions that usually occur at every 10 CGG repeats (Bodega et al., 2006). Thus, purity of the CGG repeat may also contribute to any phenotypic effect of FMR1 expansions. However,
further functional analyses are required to provide stronger evidence to support this hypothesis.

We aimed to replicate previous studies by examining the association between intermediate repeat size and ovarian failure in our series of women ascertained because of POF. Two groups have reported an increased incidence of large normal or intermediate alleles in women diagnosed with idiopathic POF. The incidence of intermediate sized repeats of 41–58 CGGs was assessed in 190 POF and 200 control individuals in an Italian cohort and there was a significant excess in the POF group, with an OR \( P = 0.02; \) Bodega et al., 2006. Similarly, intermediate sized repeats of 35–54 were more common in 53 Canadian POF cases compared with 182 controls, with an OR \( P = 0.01; \) Bretherick et al., 2005. Our analyses tested both repeat ranges defined in the previous publications in a cohort of 366 women from the UK ascertained because of POF. Despite having nearly twice the number of individuals in our study, we failed to replicate the association with either category of intermediate alleles reported previously. It is possible that the association with intermediates is population specific, perhaps driven by an increased prevalence of alleles devoid of CGG repeats in the Italian and Canadian cohorts. Unfortunately we do not have data on the purity of the repeats in our study and therefore were unable to determine the effect of CGG repeat purity. There have also been recent well-publicized reports of an effect of intermediate sized repeats on Anti-Mullerian hormone levels, suggesting increased risk of infertility prior to overt menopause in women with intermediate alleles (38–55 CGGs; Glei- cher et al., 2009, a, b). However, these studies included only 316 individuals of all repeat sizes and have yet to be replicated. The reported associations may represent publication bias in favour of positive associations. It is important to confirm the prevalence of intermediate alleles in other POF cohorts and also to determine the likelihood of ovarian failure in carriers ascertained without prior knowledge of menopausal status.

The prevalence of intermediates in the general population is far higher than that of premutation \( \text{FMR1} \) alleles, with \( \sim 6\% \) of women carrying alleles of 40–60 repeats. Therefore, if such intermediate alleles are associated with a disadvantageous phenotype they may carry a significant burden in the population. Equally, if such reported associations are exaggerated they may cause unjustified anxiety. Although it is unlikely that alleles of this size will expand during meiotic transmission, the identification of a genetic mutation contributing to or causing a phenotype has implications for the family. Additionally, if indeed elevated mRNA levels are the basis for a detrimental effect on ovarian function, it may be that the slightly raised \( \text{FMR1} \) levels in intermediate allele carriers could lead to a low-penetration POF phenotype. However, if true, this would likely affect only a small proportion of intermediate allele carriers, possibly depending upon the number of repeats within the intermediate range as well as other unrelated genetics factors.

In conclusion, our data suggest that intermediate sized \( \text{FMR1} \) alleles should not be considered a high-risk factor of POF based on current evidence, but we advocate further studies to confirm what role, if any, expansions in this category play.

### Authors’ roles


### Funding

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### References


Bretherick KL, Fluiker MR, Robinson WP. \( \text{FMR1} \) repeat sizes in the gray zone and high end of the normal range are associated with premature ovarian failure. Hum Genet 2005; 117:376–382.


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**Table II** \( \text{FMR1} \) repeat sizes in women with idiopathic POF compared with controls.

<table>
<thead>
<tr>
<th>Analysis</th>
<th>CGGn</th>
<th>POF chromosomes</th>
<th>Control chromosomes</th>
<th>OR (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0–50</td>
<td>714 (97.5%)</td>
<td>2772 (99.7%)</td>
<td>9.98 (4.15–23.99)</td>
<td>5 ( \times 10^{-4} )</td>
</tr>
<tr>
<td></td>
<td>51–200</td>
<td>18 (2.5%)</td>
<td>7 (0.3%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0–34</td>
<td>663 (92.9%)</td>
<td>2553 (91.9%)</td>
<td>0.86 (0.64–1.21)</td>
<td>0.48</td>
</tr>
<tr>
<td></td>
<td>35–54</td>
<td>51 (7.1%)</td>
<td>223 (8%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0–40</td>
<td>688 (96.2%)</td>
<td>2697 (97.1%)</td>
<td>1.3 (0.84–2.04)</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>41–58</td>
<td>27 (3.8%)</td>
<td>81 (2.9%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


Loesch DZ, Quang BM, Huggins RM, Mitchell RJ, Hagerman RJ, Tassone F. Transcript levels of the intermediate size or grey zone *FMR1* alleles are elevated, and correlate with the number of CGG repeats. *J Med Genet* 2007;44:200–204.


