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Influence of intermediate and uninterrupted *FMR1* CGG expansions in premature ovarian failure manifestation

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BACKGROUND: Studies attempting to precisely define the range of fragile mental retardation 1 (*FMR1*) expansions and its influence in premature ovarian failure (POF) manifestation are partially lacking. To this aim, we evaluated a large cohort of POF patients for the size and, in selected cases, for the sequence of the CGG expansion. Furthermore, the correlation between POF and X-inactivation was investigated in FRAXA families. METHODS: By fluorescent PCR, 190 POF and 200 control women were sized for the CGG tract; some subjects were also characterized by sequencing and for the *FMR1* activation ratio. RESULTS AND CONCLUSION: We found a significant association (19/190, 10%, $P < 1 \times 10^{-6}$) between POF and *FMR1* premutation (range 63–163 repeats) and a significant enrichment (9/190, 4.7%, $P = 0.021$) of POF carriers of intermediate expansions (range 41–58 repeats). Interestingly, intermediate alleles were entirely composed of CGG repeats. Furthermore, the analysis of three pairs of siblings with similar *FMR1* expansions and discordant for the POF phenotype showed a direct correlation between the expression of the intermediate/premutated allele and POF manifestation. The results obtained strengthen the correlation between *FMR1* expansion and POF and suggest that the manifestation of the ovarian dysfunction could be influenced both by the pattern of interruption of the CGG repeat and by X-inactivation.

Key words: AGG interruption/CGG length/*FMR1* expansion/POF/X-inactivation pattern

Introduction

Premature ovarian failure [POF (MIM: 311360)], defined as secondary hypergonadotropic (FSH ≥ 40 IU/l) amenorrhoea occurring before the age of 40, affects approximately 1% of females (Coulam, 1982), and its aetiology is still unknown in most cases. Previous reports indicate a high incidence of POF among *FMR1*-premutation carriers (Allingham-Hawkins *et al*., 1999; Sullivan *et al*., 2005); similar results were obtained analysing the prevalence of *FMR1* premutation in a POF population. Moreover, several POF families showed the co-segregation of *FMR1* premutation and POF, thus strengthening the observed association (Marozzi *et al*., 2000).

Fragile X syndrome, a form of X-linked mental retardation, is caused by more than 200 CGG repeats (full mutation) in the 5′ untranslated region (UTR) of the fragile mental retardation 1 gene [*FMR1* (MIM: 309550)] (Fu *et al*., 1991; Warren and Ashley, 1995). Premutation alleles, ranging from 59 to 199 repeats, are identified as those showing a high probability to expand to over 200 repeats in one generation (Nolin *et al*., 2003). However, alleles ranging from 41 to 58 CGGs, defined as intermediate alleles, have the potential for being unstable when transmitted from parent to child, leading to a full mutation in several generations.

The association between *FMR1* premutation and POF has been previously investigated at a molecular level by analysing *FMR1*-related factors, such as the repeat tract size, both among FRAXA carriers and a small POF population. These studies reported a significant positive association of repeat size with ovarian dysfunction. Particularly, they demonstrated a linear effect of repeat size on the age at menopause among carriers of low and medium repeat size alleles (59–99 repeats) (Sullivan *et al*., 2005) and a significant increase of *FMR1* alleles in the range of 35–54 CGG repeats in a POF population (Bretherick *et al*., 2005). However, these investigations suffered some limitations, such as the use of survey in which the inclusion criterion was the *FMR1* premutation and not POF and the analysis of a reduced number of POF cases. Furthermore, previous studies did not investigate the possible correlation between the sequence organization of the CGG repeat tract and POF manifestation. To overcome these limitations, we investigated a large cohort of POF women (190 patients). We evaluated by PCR the size of the CGG repeat tract and the prevalence of

FMR1 premutation in regard to a control population of 200 women experiencing physiological menopause. Furthermore, individuals showing similar CGG repeat sizes but discordant phenotypes (POF and non-POF) were analysed for other *FMR1*-related factors, such as the sequence of the repeated array and the X-chromosome inactivation pattern.

Materials and methods

Patient population and controls

A total of 190 unrelated patients affected by POF were recruited by Reproductive Endocrinology Services of the Department of Obstetrics and Gynecology in Milan. The POF status was defined as the cessation of ovarian function for a period of >6 months, before or at the age of 40 years, and FSH (40 ≥ IU/l detected on two different occasions. Subjects underwent complete medical assessment, which included gynecological (age of menarche and previous menses) and obstetric history, with previous pregnancy outcome. Probands underwent clinical and gynecological examination, ultrasound pelvic evaluation and karyotyping as previously described (Vegetti *et al*., 1998). Family history was reviewed during genetic counselling. All the patients included in our study were phenotypically and cytogenetically normal and considered idiopathic because they did not show any POF-related conditions (ovarian surgery, previous chemo- or radiotherapy, autoimmune diseases or metabolic disorders such as galactosaemia). The median age at menopause for the POF group was 33.2 ± 6.9. The analysis was performed also on a control population of 200 women above the age of 50, with normal menstrual history and not experiencing premature menopause. The mean age at menopause for the control group was 51.2 ± 3.2, and menopause occurs as a natural process.

All subjects included in the study gave their informed consent to review their medical history and to collect peripheral blood samples suitable for molecular analysis. All the samples were collected at or before 1 year from the diagnosis.

Sizing of premutated allele

FMR1 CGG repeat number was determined by a fluorescent sequencer method. Primers for the *FMR1* gene were C: 5′-Cy5GCTCAGCTCCGTTTCGGTTTCACTTCCGGT-3′ and F: 5′-AGCCCCGCACTTCCACCAGCTCCTCCA-3′ (Fu *et al*., 1991). These primers amplified a PCR product containing the CGG repeat and 222 bp of flanking sequences. The DNA samples were amplified using the GC-Rich PCR System (Roche Diagnostics, Milan, Italy). PCRs were carried out using 200 ng of genomic DNA as a template in a final volume of 50 µl containing the following: 1.5 M GC-Rich Resolution Solution, 200 µM dACT, 100 µM dGTP and 100 µM 7-deaza-GTP, 0.6 µM of each primer, 2U GC-Rich enzyme and 1X PCR buffer containing 1.5 mM $MgCl_2$. Thermal cycling conditions were as follows: 95°C, 10 min; denaturation (95°C, 45 s), annealing (63°C, 45 s), elongation (72°C, 45 s) (10 cycles); and denaturation (95°C, 45 s), annealing (63°C, 45 s), elongation (72°C, 45 s, with auto-extension of 5 s per cycle) (25 cycles).

All fluorescent PCR products were analysed using the Applied Biosystem model 3100 Genetic Analyzer with a Genescan capillary. The results were elaborated with the Genescan 3.1 software (Applied Biosystem, Foster City, CA, USA).

Validation of the FMR1 premutation condition

To validate the premutated alleles previously detected by PCR, genomic DNA was digested with *EcoRI/EagI*, blotted onto Hybond N+ (Amersham, Milan, Italy) and hybridized to the StB 12.3 probe as previously described (Rousseau *et al*., 1991).

Sequence analysis of the premutation allele

The PCR products were separated on 3% agarose gel for 6–8 h at 3.3 V/cm. Once the fragments were well resolved, the premutation band was excised and DNA was isolated from the gel slice, using a Qiaquick DNA gel extraction kit (Qiagen, Milan, Italy).

The DNA was reamplified using 5 µl PCR product as a template in a 50 µl reaction as previously described, and the PCR products were sequenced using primers F and C. Sequencing reactions were performed following the Big Dye terminator protocol (Applied Biosystem), and all fluorescent traces were analysed using the Applied Biosystem model 3100 DNA Sequencing System. All the sequencing reactions were performed two times.

X-inactivation analysis

The X-inactivation pattern was determined by calculating the '*FMR1* activation ratio'; in Southern blot analysis, a female will show an unmethylated band of 2.8 kb (normal active allele) and a methylated band of 5.2 kb (normal inactive allele). The intensity of the 2.8 kb band divided by the sum of the intensities of 2.8 kb and 5.2 kb bands, ascertained by densitometry using the Image Quant analysis software (Amersham), will reflect the number of cells in which the normal *FMR1* gene is active (*FMR1* activation ratio). To validate the X-chromosome inactivation pattern, the results of the AR (CAG)n polymorphism assay (Allen *et al*., 1992) were compared with the *FMR1* activation ratio. The primers used for the amplification were AR1: 5′-CyTCCAGAATCTGTTCCAGAGCGTGC-3′ and AR2: 5′-GCTGT GAAGGTTGCTGTTCCTCAT-3′. All fluorescent PCR products were analysed using the Applied Biosystem model 3100 Genetic Analyzer with a Genescan capillary. The results were elaborated with the Genescan 3.1 software (Applied Biosystem).

Results

Sizing of FMR1 alleles in POF women

We evaluated by fluorescent PCR the size distribution of the CGG repeat tract in a POF population of 190 patients and in a control group of 200 women experiencing physiological menopause, finding a similar distribution in the range of non-expanded alleles (1–40 repeats) (Figure 1). The frequency of *FMR1*-premutation carriers was 19 of 190 patients with CGG expansions ranging from 63 to 163; 9 of 190 patients showed intermediate expansions ranging from 43 to 52 repeats (Figure 1 and Table I). In the control population, no premutated alleles were observed, and two women were carriers of 51 and 53 *FMR1* intermediate alleles (Table I).

The obtained results indicate a very high prevalence of *FMR1* premutation [19 of 190, 10%, 95% confidence interval (CI) 5.8–14.2%, Fisher's exact test $P < 1 \times 10^{-6}$] than expected by evaluating the control population (0/200). We also found a significant prevalence of intermediate alleles in the POF population (9 of 190, 4.7%, 95% CI 1.7–7.7%, Fisher's exact test $P = 0.021$) with respect to control group (2/200). The age at menopause and the length of intermediate and premutated alleles of POF (28) and non-POF (2) individuals are reported in Table I.

Sequencing of intermediate CGG alleles

FMR1 intermediate carriers affected or not by POF status (9 affected by POF and 2 non-POF) were further characterized by the sequencing of the *FMR1*-expanded allele (Figure 2). Interestingly, all the POF patients showed an intermediate allele

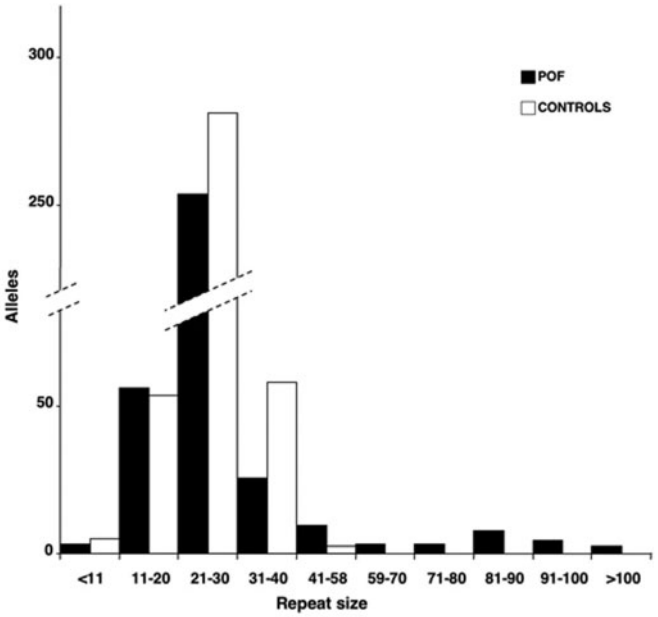


Figure 1. Size distribution of the *FMR1* CGG repeat tract in a POF population of 190 women (black bars) in respect to a control population of 200 women experiencing physiological menopause (white bars).

Table I. Age at menopause and CGG repeat size in carriers of premutated and intermediate *FMR1* alleles (subjects 1–28, POF patients; NC1 and NC2, controls)

Subjects	Age at menopause	Repeat number
1	36	43
2	40	43
3	39	43
4	35	43
5	31	43
6	38	46
7	28	46
8	40	50
9	33	52
10	26	63
11	31	64
12	34	65
13	27	71
14	31	72
15	38	77
16	40	81
17	22	81
18	30	83
19	37	85
20	32	87
21	23	87
22	24	90
23	32	92
24	32	94
25	39	96
26	38	97
27	32	123
28	38	163
NC1	53	51
NC2	54	53

entirely composed by CGG triplets, whereas the two women who underwent physiological menopause showed an AGG-interrupted pattern of sequence organization, with 1 and 2 AGG interruptions, respectively (Table II).

Then, by using the Mfold software (Zuker, 2003), we derived the predicted RNA secondary structure of the sequenced CGG regions. The analysis also included the 5′ UTR non-repeated flanking sequences. All the uninterrupted alleles of the POF patients formed a hairpin build by the whole CGG expansion, whereas the two women who underwent physiological menopause, although possessing expanded regions of 51 and 53 repeats, showed branched hairpins of 40 and 33 repeats, respectively, due to the existence of AGG interruptions (data not shown).

Analysis of X-inactivation pattern

The availability of three FRAXA families, in which siblings carrying the *FMR1* premutation were discordant for the POF manifestation, allowed investigation of the involvement of the X-inactivation in premature menopause. All the subjects included in the analysis were cytogenetically normal. The X-inactivation analysis was performed by calculating the *FMR1* activation ratio and by the androgen receptor assay; examples of both analyses are shown in Figure 3.

The pedigree of the three families (A-B-C) is shown in Figure 4. The figure also includes the size of *FMR1* expansion and the percentage of active premutated allele for each of the analysed individuals. Expanded alleles of individuals A II-1, A II-2, C II-1 and C II-2 were also sequenced. Both individuals A II-1 and A II-2 showed one AGG interruption (A II-1: 9 CGG + 1 AGG + 62 CGG; A II-2: 9 CGG + 1 AGG + 67 CGG), whereas the two 50 CGG alleles of family C were both uninterrupted (individual C II-1 corresponds to patient number 8 in Table II). In each family, POF patients (A II-1, B I-3, C II-2) always showed a percentage of activation of the intermediate/premutated allele at least twofold higher than exhibited by the corresponding healthy sister (A II-2, B I-2, C II-1) (Figure 4).

Discussion

Our study was focused on the correlation between POF manifestation and the FRAXA carrier status; to this aim, we determined by PCR the size distribution of the CGG array in one of the largest available cohorts of POF patients (190 subjects) and in a control population of 200 women experiencing physiological menopause. Nineteen POF patients carried CGG premutations (from 63 to 163 repeats), while another group of nine POF patients carried intermediate expansions (from 43 to 52 repeats). No carriers of canonical *FMR1* premutations were identified in the control population, whereas two women experiencing physiological menopause showed CGG alleles of intermediate size. These data confirmed that the POF population is characterized by a high prevalence of *FMR1*-premutation carriers (19/190, 10%, Fisher's exact test $P < 1 \times 10^{-6}$) and by a significant enrichment of carriers of intermediate CGG expansion (9/190, 4.7%, Fisher's exact test $P = 0.021$). The frequency of intermediate alleles in our control group was lower than that previously reported (Bretherick *et al.*, 2005; Sullivan *et al.*, 2005); this difference might be attributed to the inclusion criteria (i.e., women who certainly showed physiological menopause).

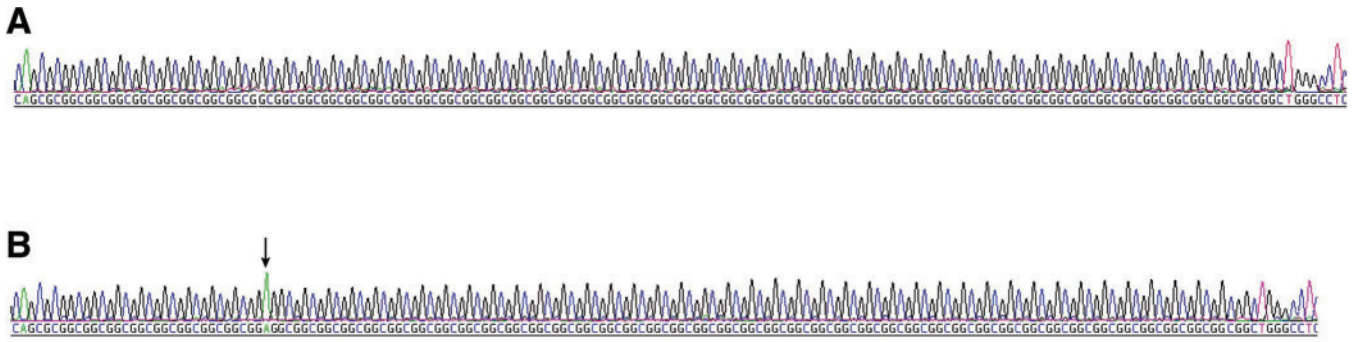


Figure 2. An example of two electropherograms showing 52 uninterrupted (**A**) and 51 interrupted (**B**) *FMR1* CGG triplets. The arrow indicates the position of the AGG interruption.

Table II. AGG interspersion patterns of intermediate *FMR1* alleles in POF women (subjects 1–9) and controls (NC1 and NC2)

Subjects	Repeat number	AGG interspersion pattern
1	43	Uninterrupted
2	43	Uninterrupted
3	43	Uninterrupted
4	43	Uninterrupted
5	43	Uninterrupted
6	46	Uninterrupted
7	46	Uninterrupted
8	50	Uninterrupted
9	52	Uninterrupted
NC1	51	9 CGG + 1 AGG + 40 CGG
NC2	53	9 CGG + 1 AGG + 9 CGG + 1 AGG + 33 CGG

To correlate the POF manifestation to *FMR1*-related factors other than the size, we investigated by sequencing the molecular feature of the *FMR1* expansion in individuals affected or not by POF and characterized by a similar intermediate length of the CGG repeated array. Interestingly, all the analysed POF patients were carriers of intermediate and uninterrupted CGG tracts, whereas non-POF women showed a CGG array interrupted by one or two AGGs. The predicted secondary structure of the corresponding 5′ UTR *FMR1* mRNA was markedly influenced by the sequence organization of the repeated tract. Uninterrupted CGG array (carried by POF patients) showed perfectly paired hairpin structures, whereas AGG interruptions (carried by non-POF individuals) drastically reduced the length of the hairpin. Although interesting, this observation should be

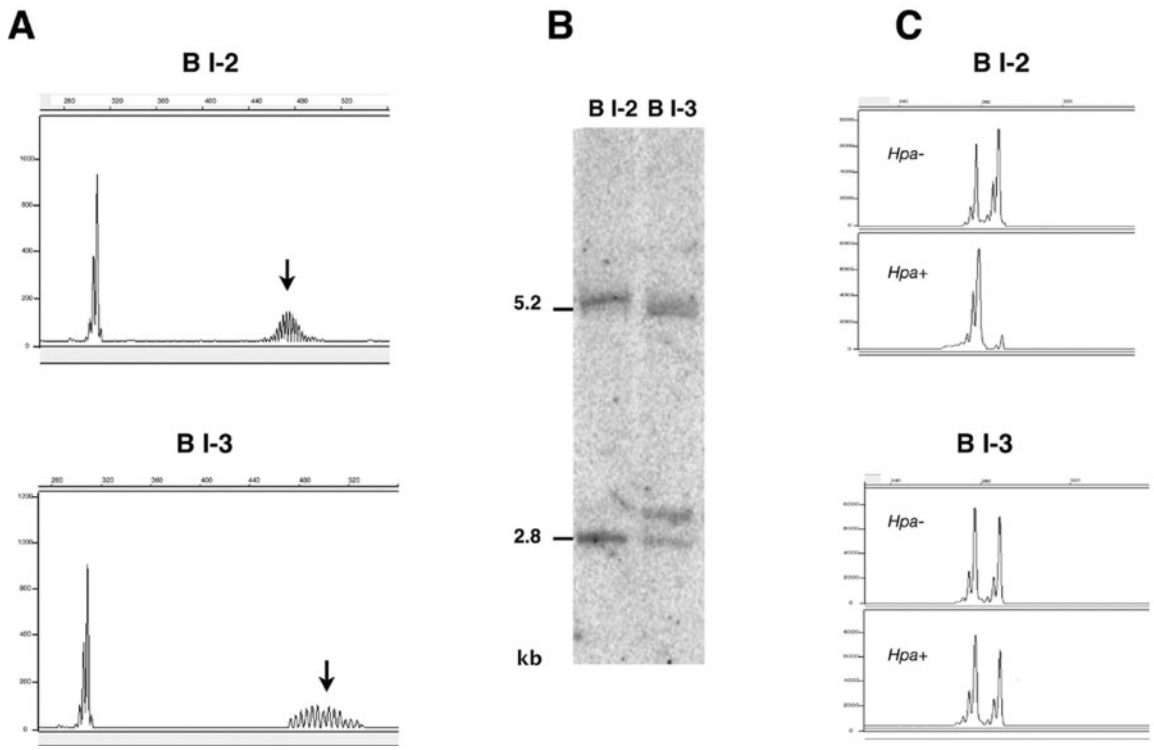
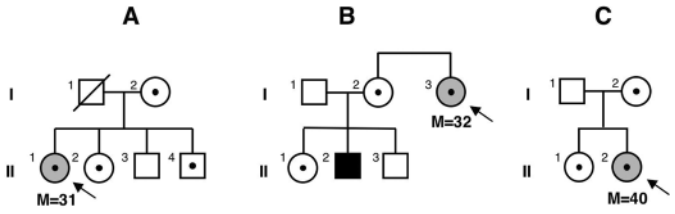


Figure 3. Derivation of X-inactivation and of CGG repeat size in two sisters discordant for the POF manifestation (pedigree B in Figure 4). Individuals B I-3 and B I-2 were, respectively, affected or not by POF (**A**) determination of the CGG repeat length by fluorescent PCR; arrows indicate the *FMR1*-premutated allele; (**B**) evaluation of the *FMR1* activation ratio by Southern blot; the normal active and inactive *FMR1* alleles are indicated by 2.8 and 5.2 kb, respectively; and (**C**) androgen receptor methylation assay.



Subjects	Presence of POF	Repeat Number	% of Active Intermediate/Premutated Allele
A II-1	Yes	72	70%
A II-2	No	77	28%
B I-3	Yes	91	52%
B I-2	No	83	8%
C II-2	Yes	50	94%
C II-1	No	50	53%

Figure 4. Pedigrees of three FRAXA families showing a correlation between POF manifestation and X-inactivation pattern; patients affected by POF are indicated in grey, whereas patients affected by fragile X syndrome are represented in black; the presence of the FMR1 expansion is indicated by a black dot; M indicates the age at menopause. At the time of this analysis, individuals A II-2, B I-2 and C II-1 had more than 40 years; the table reports the molecular features of the *FMR1* intermediate or premutated alleles (number of trinucleotide repeats and the percentage of active expanded alleles).

confirmed by a larger sample of POF patients and controls. We also determined the sequence organization of some CGG alleles from premutated POF patients (data not shown); these alleles, analysed by Mfold software, showed hairpins with a length similar to or greater than that shown by POF patients carrier of intermediate *FMR1* alleles.

Although preliminary, the above findings might suggest a mechanism by which female carriers of the *FMR1* expansion are at risk to manifest the POF. The brain post-mortem analysis of male carriers of *FMR1*-premutation alleles (affected by Fragile X-associated Tremor/Ataxia Syndrome or FXTAS) (Hagerman *et al*., 2001; Berry-Kravis *et al*., 2003) identified intranuclear inclusions in neurons and astrocytes (Greco *et al*., 2002). Furthermore, a detailed molecular analysis of *FMR1*-premutated male revealed a direct correlation between increased *FMR1* mRNA level and the molecular features of the *FMR1* array (size and sequence organization) (Tassone *et al*., 2000a,b; Tassone and Hagerman, 2003; Allen *et al*., 2004; Napierala *et al*., 2005). Thus, the development of FXTAS might be due to the accumulation of the premutated mRNA in the nucleus by aberrant protein binding to specific hairpin structures within the 5′ UTR of *FMR1* RNA. These RNA–protein complexes could interfere with the normal cell functioning and finally yield neuronal cell death. Interestingly, the level of *FMR1* mRNA is also elevated in females carrying the *FMR1*-premutated allele, and thus an FXTAS-like mechanism can also be hypothesized for the POF manifestation.

A direct demonstration of the involvement of *FMR1*-premutated alleles in POF manifestation certainly requires functional studies, particularly regarding the correlation between specific molecular features of the expanded repeated array (minimal size and sequence feature) and the capability to originate a cytotoxic effect. However, at the moment, these studies are difficult to design, essentially due to the lack of available tissue-specific and developmental stage-specific cell lines.

One type of analysis that can give further information on the involvement of the premutated *FMR1* gene in POF manifestation may be represented by X-inactivation studies. If one supposes that the *FMR1*-premutated mRNA is involved in the POF manifestation, the modulation of gene expression by X-inactivation could have an effect on the phenotype. Previous reports suggest that, in *FMR1*-premutation carriers, the X-inactivation pattern does not influence the POF manifestation (Murray *et al*., 2000; Sullivan *et al*., 2005). Conversely, other studies suggest that, in siblings with a common genetic background, *FMR1*-related symptom severity may be dictated by the X-inactivation pattern (Heine-Suner *et al*., 2003; Berry-Kravis *et al*., 2005). On the basis of these observations, we investigated the X-inactivation pattern in three sib pairs, carriers of intermediate or premutated alleles comparable in size and sequence organisation, but discordant for POF phenotype. In each sib pair, the POF phenotype correlates with the higher extent of intermediate or premutated *FMR1* gene expression. Conversely, the individuals from different families, manifesting or not the POF phenotype, showed a random X-inactivation pattern (B I-3 and C II-1, 52 and 53%, respectively). Although this analysis has been carried out on only three families, the results obtained suggest the importance of the familial background in investigating the effect of this chromosomal mechanism on the ovarian failure pathogenesis. This observation also suggests that the underlying mechanism probably involves other genetic factors than merely the extent of *FMR1* gene expression. Conversely, it could be argued that the blood cells X-inactivation ratio does not resemble that of the ovarian tissue.

The derived high prevalence of *FMR1*-premutation carriers within the POF population, in conjunction with X-inactivation experiments, reinforces the proposed correlation between premutated *FMR1* mRNA and POF manifestation. Furthermore, the finding that a significant number of POF patients are carriers of an uninterrupted intermediate CGG allele suggests that alleles shorter than the canonical FMR1-premutation range, but characterized by specific molecular features, could also play a role in POF manifestation.

In conclusion, women who carry the *FMR1*-premutation allele have an increased risk of POF, suggesting that not only genetic counselling but also fertility counselling might be required for the evaluation of family planning.

Electronic-database information

The URLs for data presented herein are as follows: Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for *FMR1* gene and POF) and Mfold, http://www.bioinfo.rpi.edu/applications/mfold/ (for RNA structure prediction).

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