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Research Article

The *FMR1* premutation as a cause of premature ovarian failure in Brazilian women

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Abstract

The loss-of-function mutation of the *FMR1* gene due to expansion of the 5' UTR CGG repeat causes the fragile X syndrome, the most frequent form of inherited mental retardation. On the other hand, the *FMR1* premutation, which is transcriptionally active and produces the protein, confers an increased risk for premature ovarian failure (POF) to carrier females. Among 41 unrelated Brazilian women with idiopathic POF, we found three carriers of premutations (CGG expansionse ≥ 59 repeats) and two carriers of high-intermediate alleles (50-55 repeats). Two premutations and two intermediate alleles were detected among the 16 familial POF cases, and one premutated woman, among the 25 sporadic cases. The premutation frequency among the familial cases (12.5%) differed significantly from that found in a control group of 96 unrelated Brazilian women aged ≥ 47 years, who had not experience POF and in which no premutations or high-intermediate alleles were detected. In the search for factors influencing the probability of a premutation carrier presenting POF, another 20 unrelated premutated women with POF, from fragile X families, were included in the study. The analysis of the *FMR1*-linked loci DXS548 and FRAXAC1 did not indicate any association of a particular haplotype with the occurrence of POF. An effect of X-inactivation skewing was not apparent in blood cells, and POF-associated premutations showed a wide range of repeat sizes, from 59, the smallest known to expand to full mutations upon transmission to offspring, to approximately 200.

Key words: premature ovarian failure, menopause, *FMR1* premutation, fragile X syndrome.

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Introduction

The fragile X syndrome is the most frequent single cause of inherited mental retardation, with an estimated incidence of 1 in 4,000 males and 1 in 8,000 females (Crawford *et al*., 2001). In most cases, the loss-of-function mutation, which causes this syndrome, consists of an expansion of the polymorphic CGG trinucleotide repeat in the 5' untranslated region of the *FMR1* gene at Xq27.3. This fully mutated allele is abnormally hypermethylated and is not transcribed (Fu *et al*., 1991; Oberlé *et al*., 1991; Pieretti *et al*., 1991; Verkerk *et al*., 1991; Nolin *et al*., 2003). Alleles with 6 to ~55 triplets are considered to be normal, those with 29-30 triplets being the most common in the general population. Alleles with repeats in the ~55-200 range, the premutations, are transcribed and the protein (FMRP) is produced, but these alleles are unstable and may expand to full mutations upon maternal transmission. The smallest allele known to have expanded to a full mutation had a 59-triplet repeat (Nolin *et al*., 2003). However, the boundary between normal and premutation alleles is not well defined, and constitutes a "gray zone" containing high-normal and low-premutation alleles (Eichler *et al*., 1994). These intermediate alleles are defined as those varying between 41~60 repeats, which may or may not be transmitted in an unstable manner (Murray *et al*., 1997). Their tendency to expand is directly related to the size of the repeat (Nolin *et al*., 2003).

Premature ovarian failure (POF), defined as the cessation of ovarian function before the age of 40, affects approximately 1% of women in the general population (Coulam *et al*., 1986). It has been described both in patients with X chromosome abnormalities, mostly deletions, and with a normal karyotype. A familial pattern of premature ovarian failure, suggesting autosomal or X-linked inheritance, has been reported, but few genes causing POF have

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been identified so far. *DIAPH2* at Xq22, one of the human homologues of the *Drosophila melanogaster* diaphanous gene, was found disrupted by a balanced translocation in a mother and a daughter with POF (Bione *et al*., 1998). Mutations of *FSHR*, the follicle-stimulating hormone receptor gene at 2p21, which led to partial loss of function of the protein, were described in secondary amenorrhoea (Beau *et al*., 1998). Mutations of this receptor gene were previously reported as the cause of primary ovarian failure (Aittomaki *et al*., 1996). *FOXL2,* the forkhead transcription factor gene at 3q23, which is mutated in blepharophimosis, ptosis and epicanthus inversus syndrome with or without ovarian failure, had novel mutations described that caused only POF (Harris *et al*., 2002). Ovarian failure was also associated to mutations in *E1F2B*, the eukaryotic translation initiation factor 2B, in patients with leukodystrophy, a condition termed ovarioleukodystrophy (Fogli *et al.*, 2003). However, given that more than 30% of all POF cases are familial, there must be many more POF-causing alleles awaiting discovery. Accordingly, it was a breakthrough when a significant association between the fragile X premutations and POF was demonstrated both by the analysis of women carrying premutations, and by the screening of women affected by POF (Cronister *et al*., 1991; Schwartz *et al*., 1994; Conway *et al*., 1995, Vianna-Morgante *et al*., 1996; Murray *et al*., 1998; Allingham-Hawkins *et al*., 1999; Vianna-Morgante *et al*., 1999; Marozzi *et al*., 2000).

Although no doubt remains that the fragile X premutation is associated with POF, it has been evident since the first studies that the presence of the premutation is not deterministic for POF to occur and, according to The International Collaborative POF in Fragile X Study (Allingham-Hawkins *et al*., 1999), about 24% of premutation carriers in fragile X families experience POF. However, some data suggest that POF is at the extreme of the spectrum of the premutation effects on ovarian function. The age at menopause of premutated women who did not experience POF was shown to be significantly lower than the menopausal age of their non-carrier relatives (Vianna-Morgante *et al*., 1999). Pointing in the same direction are the increased serum follicle-stimulating hormone concentrations, a marker of late ovarian failure, observed in premutation carriers (Braat *et al*., 1999; Murray *et al*., 1999), even in those which are on oral contraceptive (Hundscheid *et al*., 2001). Factor(s) causing a woman to exceed a threshold and manifest POF are presently unknown. In fact, the nature of the POF/premutation association remains elusive. The absence of FMRP does not make a woman more likely to present POF, since fully mutated women do not tend to experience POF (Allingham-Hawkins *et al*., 1999; Vianna-Morgante *et al*., 1999). Premutation carriers produce an excess of *FMR1* mRNA, probably as a response to the impairment of FMRP synthesis that seems to be correlated with the size of the repeat expansion (Tassone *et al*., 2000). It is speculated that this mRNA excess could be toxic to the cells and interfere with normal ovary function. A direct effect of the expansion itself abnormally recruiting RNA-binding proteins, thus generally impeding the availability of these factors, has been considered as an explanation for the neurological impairment (FXTAS syndrome) presented by some male carriers of the *FMR1* premutation (Hagerman and Hagerman, 2004). The same mechanism is apparently at the basis of another trinucleotide-expansion disease, myotonic dystrophy (Day and Ranum, 2005). Another possibility is that the premutation expansion affects the expression of neighboring genes involved in ovary function (Vianna-Morgante *et al*., 1996). Under these hypotheses, the size of the expanded repeat could be a factor influencing the manifestation of POF. Indeed, a significant positive association of the *FMR1* repeat size with ovarian dysfunction was detected in a recent study of a large sample of women with repeats ranging from common to premutation sizes (Sullivan *et al*., 2005). Another possibility was raised some years ago, in a study of Dutch fragile-X families showing that POF occurred predominantly in carrier women who inherited the premutation from their fathers (Hundscheid *et al*., 2000). However, this parent-of-origin effect was not confirmed, either in the United Kingdom (Murray *et al.*, 2000), or by us in Brazil (Vianna-Morgante and Costa, 2000). It has been suggested that reduced fitness of premutated women with POF could have influenced these studies differently, selection being more effective against mother-daughter pairs in a population where women reproduce later (Sherman, 2000), and actually at the time Hundscheid *et al*. (2000) conducted their study, the Netherlands had the oldest maternal age of any other country.

Herein we report the investigation of the premutation/POF association in 41 unrelated Brazilian women ascertained because they presented with POF, and in a further 20 premutated women belonging to Brazilian fragile X families, who had experienced POF. We determined the frequency of the premutation among women ascertained by POF, and showed that the *FMR1* premutation is an important single cause of POF in our population, especially in POF familial cases. We failed to identify predisposing factors influencing POF manifestation in *FMR1* premutation carriers.

Subjects and Methods

Patients and controls

Premature ovarian failure (POF) was considered as the cessation of menstruation for at least one year before the age of 40. A total of 41 unrelated women ascertained by idiopathic POF were studied to determine the frequency of the *FMR1* premutation among them. Sixteen of them were familial cases of POF and 25 occurred sporadically. Thirty-two of the women were attending the Outpatient Climacteric Clinic, at the Department of Obstetrics and Gy-

necology of the University of São Paulo Medical School. The nine other women with menopause were referred from different medical services in the State of São Paulo. The control group included 96 normal unrelated women who had not experienced POF before age 47, who attended the same clinic at the Medical School, on a routine basis. In the search for factors related to the manifestation of POF, we also included in the analysis 20 unrelated premutation carriers with POF, ascertained in fragile X families referred to the Genetic Counseling Service of the Department of Genetics and Evolutionary Biology at the University of São Paulo. For X-inactivation studies, a control group formed by 53 premutated women without POF belonging to the same fragile X families was used. This study was approved by the Ethics Committee of the Hospital das Clínicas, University of São Paulo Medical School (269/99).

Methods

DNA was extracted from peripheral blood lymphocytes. Screening for FRAXA premutations was performed by PCR, with primers **c** and **f** (Fu *et al*., 1991), followed by electrophoresis on sequencing acrylamide gels, as previously described by Kenneson *et al*. (1996), with slight modifications (Mingroni-Netto *et al*., 2002). When only one allele was detected, in order to distinguish between homozygosis and heterozygosis for a non-amplified expanded allele, Southern blotting was carried out using doubly digested *Eco*R I/*Eag* I fragments probed with StB12.3, as previously described (Mingroni-Netto *et al*., 1994). The X-inactivation patterns were studied by determining the activation ratios (the proportion of active normal alleles) through densitometry comparison of the allele bands on the auto-radiographs (BIORAD GS 700 Image Densitometer, Molecular Analyst/BC). In order to investigate if a particular subgroup of premutations was associated with POF, the microsatellite *loci* DXS548 and FRAXAC1, which are tightly linked to the *FMR1* gene (Richards *et al*., 1991; Riggins *et al*., 1992), were genotyped by PCR, according to previously described procedures (Mingroni-Netto *et al*., 1994). The allele nomenclature followed Macpherson *et al*. (1994).

Results

No premutations or intermediate alleles in the upper range of the distribution (≥ 50 repeats) were found in the control group of 96 women. Among the 41 unrelated women referred because of POF, three (7.32%) were diagnosed as premutation carriers ($p = 0.03$; Fisher's exact test). Among the 16 familial cases of POF, two women had the premutation (12.5%; $p = 0.02$), and one premutated woman was detected among the 25 isolated cases (4%; $p = 0.21$) (Table 1). Two high-intermediate alleles, with 50 and 52 repeats, were found in the familial cases.

In the study of factors influencing POF manifestation, we included 20 unrelated women with a premutation and POF, who were detected in fragile X families (Table 2). So, a total of 23 women carrying the premutation were genotyped for the *FMR1*-linked microsatellite loci DXS548 and FRAXAC1. The haplotype linked to the premutation could be identified in women from the fragile X families by genotyping affected males in the families: the haplotypes 2-1 and 6-4, the most frequently linked to fragile X chromosomes, were found in eight and four women, respectively (Table 2). In the three carriers ascertained through POF, the DXS548 and FRAXAC1 alleles were determined, but haplotypes could not be inferred (Table 1).

We determined the activation ratios - the proportion of active normal alleles - in blood cells in all 23 premutated women with POF, and in 53 premutated women who did not experience POF. The mean activation ratios were 48.59 and 45.60, respectively, not differing statistically ($p = 0.56$; Mann-Whitney test).

Discussion

Among the 41 women ascertained by POF, the frequency of the *FMR1* premutation (7.3%) was significantly higher than in the control group, in which premutations were not detected. The difference was also significant when we analyzed the 16 familial cases separately (12.5%). On the other hand, the 4% frequency of premutations among the 25 sporadic cases did not reach significance relative to the controls. These results confirm the *FMR1* premutation as an important causative factor of POF. Two previous studies on series of women with POF point in the same direction. In the United Kingdom, the screening of 147 women with idiopathic POF for the fragile X premutation, revealed six premutation carriers (4.1%), four among 25 women with familial POF (16%), and two others among 122 women with sporadic POF (1.6%) (Murray *et al*., 1998). In Italy, six carriers (6%) were found among 106

Table 1 - Premutation carriers ascertained by POF.

Patients	Menopause age (years)	CGG-repeat sizes		DXS548	FRAXAC1
		Premutated alleles	Normal alleles	Alleles	
Familial POF	35	86	23	2-7	1-3
Familial POF	30	87	30	6-7	3-4
Sporadic POF	39	59	29	2-7	1-3

Table 2 - Premutation carriers with POF ascertained in fragile X families.

Patients	Age at menopause (years)	Recurrence of POF	$(CGG)_n$		DXS548/FRAXAC1 haplotypes	
			Normal alleles	Premutated alleles*	Fra(X) chromosome	Normal chromosome
F1	31	Familial	23	*	8-3	8-4
F2	28	Sporadic	30	*	7-4	7-3
F3	35	Sporadic	20	*	7-4	7-3
F4	30	Sporadic	20	*	5-3	7-3
F5	29	Sporadic	29	*	6-4	9-3
F6	30	Sporadic	30	*	6-4	9-3
F7	13	Sporadic	20	*	6-4	7-3
F8	37	Sporadic	26	75	2-1	7-3
F9	34	Sporadic	20	*	6-4	6-3
F10	17	Familial	42	99	2-3	2-1
F11	35	Familial	21	*	2-1	6-3
F12	38	Sporadic	30	*	2-3	7-3
F13	14	Familial	30	*	2-1	7-3
F14	35	Familial	30	*	2-1	2-3
F15	28	Sporadic	29	*	2-1	2-4
F16	37	Familial	30	*	2-1	7-3
F17	38	Familial	30	*	?-3	7-3
F18	34	Sporadic	32	*	2-1	7-3
F19	25	Familial	20	*	2-1	7-3
F20	36	Sporadic	23	110	7-4	7-3

*Premutation diagnosed by Southern blotting.

women with POF, four among 33 familial cases (12%), and two among 73 sporadic cases (3%) (Marozzi *et al*., 2000). On the other hand, in a study conducted in the USA (Kenneson *et al*., 1997), none of 33 women who experienced ovarian failure under the age of 40 years (17 familial and 16 sporadic POF cases) carried the premutation. Taken together, these studies point to the significance of the *FMR1* premutation as a cause of POF, especially when this condition is familial. Indeed, the overall frequency of the premutation in these series of POF women is clearly dependent on the proportion of familial cases.

We also investigated some factors that could influence the manifestation of POF associated with the *FMR1* premutation. The pattern of silencing of the premutation by X-inactivation could influence the occurrence of POF in carriers, the amount of active premutations having to reach a threshold for the effect to appear. We did not observe a significant difference between the activation ratios in blood cells of premutated women who experienced POF and of those who did not. However, this possibility cannot be ruled out, since the pattern of X-inactivation in the ovary might not be the same as in blood. The size of the premutation could also influence the manifestation of POF. In our sample, the measure of the expansion was tentative, from Southern blots, since the size of the repeat could not be precisely determined in every premutation carrier, due to the inherent difficulties in amplifying these GC-rich segments. This prevented the performing of a correlation analysis between premutation sizes and ages at menopause. However, it is noteworthy that the sizes of the premutations in women experiencing POF encompass a wide range, from as small as 59 up to near 200 trinucleotides.

POF associated with the *FMR1* premutation appears to cluster in some families (Vianna-Morgante *et al*., 1996), while in others none of the carrier women have POF. Considering that peculiarities of certain premutations could explain these clusters, we genotyped the DXS548 and FRAXAC1 loci in the search for a linkage disequilibrium involving certain haplotypes and the POF-associated premutations. No trend in this direction was observed, since the distribution of the haplotypes in premutation women with POF did not characterize their families as a subset of fragile X families. Indeed, 60% of the premutation carriers with POF had the haplotypes most commonly observed on the fragile X chromosomes in Brazilian families (Mingroni-Netto *et al*., 1999) as well as in other populations (Chiurazzi *et al*., 1996). Therefore, no indication was found that the POF-associated premutations had a specific origin or resided on a particular haplotype background.

In conclusion, this work confirms in Brazilian women the *FMR1* premutation as the most frequent single cause of POF identified to date and should be considered in the diagnosis of women experiencing POF. It should be noted that all the premutations that we ascertained through POF were detected in families without any history of mental retardation. Genetic counseling and diagnostic tests for possible carriers could be offered, aiming at the prevention of mental retardation.

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