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Role of polymorphisms 919A>G and 2039A>G of FSH receptor (FSHR) gene in premature ovarian failure (POF) development

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ABSTRACT

Although the impact of polymorphisms 919A>G and 2039A>G of FSHR gene in normal ovarian function is almost clear, in Premature Ovarian Failure (POF) it still remains elusive. To analyse the putative association of these polymorphisms with POF development, 97 POF patients and 72 women over 40 years of age with normal menstrual record were genotyped for 919A>G and 2039A>G variants. No association was found between genotype GG of each polymorphism and the risk of POF. Nevertheless, ten POF patients and only one control presented the less common combinations 919G-2039A and 919A-2039G ($p<0.05$). In addition, the frequency of primary amenorrhoea and the occurrence of familial POF were significantly increased among patients with 919GG-2039GG genotype. In 45 normally menstruating women, no significant differences were found among different FSHR genotypes in FSH, E_2, Inhibin A and Inhibin B levels. In conclusion, FSHR polymorphism genotypes were not associated either to the risk of POF or to serum hormone levels of control women. However, 919GG-2039GG genotype might be associated to relatively more severe symptoms of POF. On the other hand, the differences found between controls and patients in the prevalence of the cross haplotypes, may suggest that these rare allelic variants might possibly influence POF development.

KEYWORDS: premature ovarian failure, FSHR polymorphisms, hormonal serum levels

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INTRODUCTION

The screening of the FSH receptor (FSHR) coding region evidenced the presence of two polymorphisms within exon 10: 919A>G (Thr307Ala), and 2039A>G (Asn680Ser) [1, 2], which come in two major allelic variants in Caucasians: 919A-2039A and 919G-2039G, the latter occurring in about 40% of the alleles in the population worldwide. The other two combinations, 919A-2039G and 919G-2039A are less common and constitute <5% of FSHR alleles [3].

Even though studies in women with conserved ovarian cycles demonstrate that polymorphisms in exon 10 modulate FSHR function and the ovarian response to FSH [4-7], the impact of these polymorphisms in impaired ovarian function remains controversial [3] Despite the occasional description of an association between the 2039G genotype and amenorrhoea/anovulation [8, 9], the possible association between FSHR polymorphisms and specifically Premature

Ovarian Failure (POF), has been analysed only slightly. POF is a heterogeneous syndrome of multicausal pathogenesis, that affects approximately 1% of women in reproductive age [10]. It is characterized by primary or secondary amenorrhoea before the age of 40, hypoestrogenism and elevated gonadotropin serum levels [11].

The aim of this study was to analyse the putative association of polymorphisms 919A>G and 2039A>G of FHSR gene with POF development, in a cohort of patients and controls from Argentina.

MATERIAL AND METHODS

We studied 97 patients with POF, all with 46,XX karyotype, who met the diagnostic criteria already detailed previously [12, 13]. Briefly, patients had been characterized as POF due to amenorrhoea for over a year starting before the age of 40 (range: 15-39 years old), and serum FSH level above 40 mIU/ml (normal follicular phase levels: 2-9 mIU/ml) in two consecutive determinations. Among these patients, 22 presented POF in association with autoimmune disease/s (AAD-POF patients). None of the AAD-POF patients presented associated Addison's disease. The remaining 75 POF patients were considered idiopathic (I-POF) since they did not show any other POF-related condition (i.e.: ovarian surgery, previous chemo- or radiotherapy, or metabolic disorders such as galactosemia). We also studied 72 control women over the age of 40 with normal menstrual record and without premature menopause. All but one were of proven fertility.

In addition, the implication of FSHR polymorphisms in serum hormone levels were studied in 45 control women selected according to the following inclusion criteria: a) age under 40; b) regular menses (duration: 25-35 days); c) no family record of premature or early menopause; d) no family record of autoimmune disorders; e) no consumption of oral contraceptives or other hormonal medications at the time of inclusion in the study. Weight and height of each subject was recorded, and body mass index (BMI) was estimated according to the formula weight/(height)^2 [13]. Two blood samples were taken from each volunteer, and FSH, oestradiol (E_2), and inhibin B values were measured on mid-follicular phase serum samples, whereas progesterone (P_4) and inhibin A levels were determined on mid-luteal phase serum samples, as described [13].

Genomic DNA was isolated from peripheral blood leukocytes, and two regions encompassing 919A>G and 2039A>G polymorphisms were amplified by polymerase chain reaction (PCR). Polymorphisms were studied by digestion with restriction enzymes as previously described [12]. All samples in which polymorphism 919A>G did not cosegregate with 2039A>G were amplified again from a different aliquot of genomic DNA, and the new PCR product was analysed by direct sequencing.

Odds ratio (OR) and 95% confidence interval (CI 95%) were calculated to estimate the relative risk of the genetic variants. Fisher exact test was used to evaluate differences in prevalence of rare allelic variants between patients and controls, and in clinical manifestations among POF patients with different genotypes. One-way Anova test was used to compare hormonal values in different genotype control groups. The Protocol was approved by the Institutional Review Board of the Instituto de Biología y Medicina Experimental. Informed consent was obtained from all patients and controls.

RESULTS

Polymorphisms 919A>G and 2039A>G were studied in 97 patients with POF and 72 control women over the age of 40 (C>40).

Genotype frequencies of each polymorphism are shown in panels A and B of Table 1. For OR calculations, 919GG and 2039GG genotypes were considered the putative genetic risk factors. We were unable to demonstrate any association between GG genotypes and POF for any of the two found when only I-POF patients were compared to controls (data not shown).

Restriction enzyme analysis evidenced that 919G and 919A were in linkage disequilibrium with

Table 1. Genotype counts of polymorphisms 919A>G and 2039A>G of FSHR gene in controls (C>40) and patients.

A. Polymorphism 919A>G.

Genotype	Controls n/total	POF n/total
A/A	23/72	39/97
A/G	33/72	40/97
G/G	16/72	18/97

OR $_{GG\ vs.\ (AA+AG);\ POF\ vs\ Controls}$ = 0.80; IC $_{95\%}$= 0.35-1.81.

B. Polymorphism 2039A>G.

Genotype	Controls n/total	POF n/total
A/A	23/72	38/97
A/G	34/72	38/97
G/G	15/72	21/97

OR $_{GG\ vs.\ (AA+AG);\ POF\ vs\ Controls}$ = 1.05; IC $_{95\%}$= 0.47-2.37.

C. Combined genotypes.

	Genotype	Controls n/total	POF n/total
Commom genotype frequencies	$AA^{919}\ AA^{2039}$	23/72	35/97
	$AG^{919}AG^{2039}$	33/72	34/97
	$GG^{919}\ GG^{2039}$	15/72	18/97
	Total	**71/72**	**87/97**
OR $_{GG\ vs.\ (AA+AG);\ POF\ vs\ Controls}$ = 0.97; IC $_{95\%}$= 0.42-2.25.			
Rare genotype Frequencies	$AA^{919}\ AG^{2039}$	0/72	4/97
	$AG^{919}\ AA^{2039}$	0/72	3/97
	$AG^{919}\ GG^{2039}$	0/72	3/97
	$GG^{919}\ AG^{2039}$	1/72	0/97
	Total	**1/72** [a]	**10/97** [b]

Fisher exact test a vs. b: p = 0.017.

2039G and 2039A, respectively. All but one control subject and 87 POF patients presented the two major allelic variants 919G-2039G and 919A-2039A. No association was found between 919GG-2039GG genotype and POF (Table 1), also when considering both polymorphisms in combination. Similar results were found when only I-POF patients were compared to controls (data not shown). Nevertheless, the two less common combinations, 919G-2039A and 919A-2039G, were found in 10 POF patients (8 I-POF and 2 AAD-POF) and in only one control. These differences in frequencies are statistically significant ($p<0.05$) (Table 1, panel C). Due to methodological limitations, genotypes 919A-2039A/ 919G-2039G and 919A-2039G/ 919G-2039A could not be told apart. Nevertheless, considering the prevalence of the rare 919A-2039G and 919G-2039A alleles found in our population, the likelihood of finding an individual with such a "double-crossed" genotype would be close to 0.025%.

Potential associations between FSHR polymorphisms and the severity of POF syndrome were also analysed (Table 2). Only patients presenting the two major allelic variants 919G-2039G and 919A-2039A were included, when the combined genotypes were analysed (Table 2, panel c). The frequency of primary amenorrhoea and the occurrence of familial POF were significantly different when comparing patients with GG genotype to patients with AA and AG genotypes, either when analysing each polymorphism individually (Table 2, panels a and b), or in combination (Table 2, panel c). In addition, though not statistically significant, the number of patients with secondary amenorrhoea presenting POF onset before 25 years of age was increased among patients with GG genotype.

The implication of FSHR polymorphisms in hormone serum levels wasanalysed in subjects not affected by the disease. We evaluated FSH, E2, inhibin A and B levels in 45 cycling women under 40years of age (C<40), and correlated these data to FSHR haplotype. Only women in whom 919A>G polymorphism

Table 2. Clinical characteristics of POF patients studied to analyse the implication of 919A>G and 2039A>G polymorphisms in POF severity.

A. Polymorphism 919A>G.

		Amenorrhoea		Familiar history of POF[a]		Age at POF onset (2° amenorrhoea)	
Genotype	n	1°	2°	Yes (Familiar POF)	No (Sporadic POF)	≤25 yrs old	>25 yrs old
AA	39	5	34	9	30	13	21
AG	40	5	35	6	32	14	21
GG	18	7	11	8	9	6	5
Fisher exact test $_{GG\ vs.\ AA+AG}$		p= 0.015		p<0.05		n.s.	

B. Polymorphism 2039A>G.

		Amenorrhoea		Familiar history of POF[a]		Age at POF onset (2° amenorrhoea)	
Genotype	n	1°	2°	Yes (Familiar POF)	No (Sporadic POF)	≤25 yrs old	>25 yrs old
AA	38	4	34	7	31	11	23
AG	38	5	33	7	29	13	20
GG	21	8	13	9	11	6	7
Fisher exact test $_{GG\ vs.\ AA+AG}$		p< 0.01		p<0.05		n.s.	

C. Combined genotypes.

		Amenorrhoea		Familiar History of POF[a]		Age at POF onset (2° amenorrhoea)	
Genotype	n	1°	2°	Yes (Familiar POF)	No (Sporadic POF)	≤25 yrs old	>25 yrs old
AA^{919} AA^{2039}	35	4	31	7	28	10	21
AG^{919} AG^{2039}	34	4	30	5	27	10	20
GG^{919} GG^{2039}	18	7	11	8	9	6	5
Fisher exact test $_{GG\ vs.\ AA+AG}$		p= 0.012		p=0.015		n.s.	

cosegregated with the 2039A>G allelic variant were further considered (n=44). Samples were divided into three groups: 1) 919G-2039G/919G-2039G genotype; 2) 919A-2039A/919G-2039G genotype; and 3) 919A-2039A/919A-2039A genotype. Clinical characteristics and P_4 hormone levels of control subjects from all three groups were similar (Table 3). No significant differences ($p>0.05$) were found between groups in mid-follicular phase FSH, E_2 and Inhibin B levels, and mid-luteal phase Inhibin A levels (Table 3).

Table 3. Clinical characteristics and hormonal profile of control subjects (C<40) studied to analyse the implication of 919A>G and 2039A>G polymorphisms in serum hormone levels.

	A-A/A-A (n=8)	**A-A/G-G** (n=27)	**G-G/G-G** (n=9)
Age (years)	28.7 ± 0.6 (27.0 – 31.0)	30.0± 0.7 (26.0 – 35.0)	27.2 ± 0.5 (25.0 – 30.0)
BMI	21.4 ± 0.9 (18.5 – 27.0)	20.9 ± 0.6 (17.9 – 27.2)	20.3 ± 0.6 (17.2 – 21.6)
Cycle length (days)	27.7 ± 0.8 (26.0 – 32.0)	28.2 ± 0.3 (26.0 – 31.0)	27.0 ± 1.1 (24.0 – 30.0)
P_4 (ng/ml)	10.1 ± 1.6 (2.0 – 16.9)	10.9 ± 0.8 (2.1 – 20.0)	11.7 ± 1.9 (2.0 – 18.7)
FSH (mIU/ml)	4.3 ± 0.3 (3.3 – 5.6)	4.7 ± 0.3 (1.6 – 7.4)	4.7 ± 0.4 (3.3 – 6.1)
E_2 (pg/ml)	79.9 ± 23.3 (32.0 – 235.0)	68.2 ± 7.8 (29.0 – 183.0)	88.8 ± 16.3 (27.0 – 150.0)
Inhibin A (pg/ml)	43.1 ± 5.9 (17.1 – 70.0)	41.3 ± 5.3 (7.8 – 104.5)	32.4 ± 6.2 (7.8 – 57.3)
Inhibin B (pg/ml)	161.7 ± 30.2 (16.0 – 267.1)	135.3 ± 12.2 (37.1 – 287.4)	123.5 ± 31.4 (50.6 – 328.0)

Data are expressed as Mean ± SEM (range). BMI: Body Mass Index. Numbers into brackets represent the range.

DISCUSSION

Several polymorphisms have been reported in the FSHR gene (http://www.ncbi.nlm.nih.gov/SNP); nevertheless, only two, 919A>G (Thr307Ala) and 2039A>G (Asn680Ser) both within exon 10, have been consistently found to modulate FSHR function and the ovarian response to FSH [3, 14]. Although almost clear in women with conserved ovarian cycles, the impact of these polymorphisms in impaired ovarian function remains controversial [3]. In addition, only three previous studies -including a previous one by our own research group- aimed at analyzing the presence of mutations in the FSHR gene as a likely cause of POF, secondarily addressed the distribution of these polymorphisms in POF patients, compared to controls [12, 15, 16]. Consequently, and notwithstanding the fact that such previous studies were unable to find differences in FSHR variant distribution between patients and controls, further extensive trials were necessary aimed specifically at addressing the role of FSHR allelic variants in POF. Indeed, a recent study aimed at investigating the association of FSHR polymorphisms with POF phenotype, suggested that 919A>G polymorphism may be associated with the onset of clinical disease [17].

Our present study, which to our knowledge represents the largest cohort studied to date, was unable to demonstrate any association between GG genotypes and the risk of idiopathic POF for any of 919A>G and 2039A>G polymorphisms. These results might indicate that none of these genetic variants are associated to the risk of POF development, in accordance with a work by Théron-Gérard *et al.* [18]. Noteworthily, we nevertheless found statistically significant differences between patients and controls, in the number of subjects in whom polymorphism

at position 919 did not cosegregate with polymorphism at position 2039. These differences in prevalence may suggest that these rare isoforms might possibly influence POF development. Interestingly, even though previous reports do not focus their work on addressing the presence of the rare cross-genotypes in patients compared to controls [3, 15, 16, 19], the detailed analysis of two of them [15, 16] revealed the presence of the rare allelic variants in POF patients, but not in control subjects. Moreover, a high prevalence of cross-genotypes can be inferred from a report on 36 POF patients from South Brazil, though the control population was not analysed [17]. Presence of the rare isoforms was also described both in anovulatory patients and in controls from The Netherlands [9], nevertheless, populationdifferences could account for these discrepancies. Consequently, we suggest that differences in the prevalence of rare genotypes between POF and controls should not be overlooked. New cohorts of POF patients and controls should be studied in order to further analyse the biological implication of these rare genotypes. In addition, *in vitro* experiments aimed at analysing receptor trafficking, binding characteristic, receptor activation and signal transduction of these isoforms seem necessary.

Even though neither 919A>G nor 2039A>G genetic variants were found to represent genetic risk factors for POF development, the analysis of potential associations between clinical characteristics of POF patients and FSHR polymorphisms arose interesting results.The frequency of primary amenorrhoea and the occurrence of familial POF were significantly increased in patients with GG genotype. In addition,though no statistically significant, the number of patients with secondary amenorrhoea presenting POF onset before 25 years of age, was also increased among these patients. In contrast with our results, presence of allele A at position 919, but not at 2039, was recently found to be associated with a more precocious onset of clinical disease [17]. Nevertheless, the number of POF patients (32) analysed in that opportunity could account for the discrepancies. Our study, in 80 POF patients with secondary amenorrhoea, suggests that 919GG and 2039GG genotype may be associated to a more severe POF manifestation. Considering that 2039GG genotype was found to result in a higher ovarian threshold for FSH and decreased negative feedback to the pituitary (Greb *et al*., 2005), it can be hypothesized that, in the presence of other risk factors that lead to POF development, those patients carrying the “good responder” FSHR, would benefit from some functional activity of the remaining follicles, delaying for a longer period of time the appearance of clinical manifestation of POF

Studies in women undergoing controlled ovarian hyperstimulation indicate that the ovarian FSH threshold is influenced by polymorphisms of exon 10 of FSHR gene [4-6, 20-22]. Greb *et al.* (2005) suggested that polymorphism at position 2039 influences the hormonal dynamics of the natural menstrual cycle. In this work, we found no significantly different levels of mid-follicular phase FSH in control women with different FSHR haplotype, in line with the observations by Behre *et al.* [6] for basal FSH levels in women with conserved ovarian function undergoing controlled ovarian hyperstimulation. On the other hand, we found no significant differences among groups inthe levels of follicular phase E_2 and Inhibin B, and in those of luteal phase Inhibin A. Nevertheless, it cannot be ruled out that the eventual different responsiveness capacity of each FSHR genotype becomes evident only under supraphysiological stimulation. Alternatively, a more complexscenarioshould be considered where the different FSHR variants may interact not only with diverse genetic variants of FSH, but also with differently glycosylated isoforms of the hormone, to fine–tune FSH ovarian responsiveness.

In conclusion, and bearing in mind the limitations due to possible sampling biases, our results suggest that while genotype at positions 919 and 2039 of FSHR gene may not represent a risk factor for POF, it could be associated to severity of syndrome. Contrary, the rare cross-genotypes might possibly represent a genetic risk factor for POF development.

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