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GENETICS

"Evaluation of four genes associated with primary ovarian insufficiency in a cohort of Mexican women"

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Abstract

Purpose Primary ovarian insufficiency (POI) is a clinical condition observed in women younger than 40 years of age, characterized by amenorrhea, hypoestrogenism, high levels of follicle-stimulating hormone (FSH), and infertility. Mutations in some master regulators of the development, maturation, and maintenance of ovarian follicles such as *BMP15*, *FSHR*, *FOXL2*, and *GDF9* have been suggested as etiological factors in the development of POI. The aim of this study, the first in the Mexican population, is to evaluate the presence of mutations or polymorphisms in these four candidate genes.

Methods In a sample of 20 Mexican patients with idiopathic POI, we looked for and analyzed genetic variants in *BMP15*, *FSHR*, *FOXL2*, and *GDF9* genes.

Results We observed two polymorphisms: a coding change, c.919G>A (p.Ala307Thr), in the *FSHR* gene and a synonymous variant, c.447C>T (p.Thr149Thr), in the *GDF9* gene. These two variants have been reported previously as polymorphisms (rs6165 and rs254286, respectively). We observed no significant difference associated with POI in the patients when compared with a healthy control group ($p > 0.05$). Also, no exonic variants were found for the genes *BMP15* and *FOXL2* in the individuals tested.

Conclusions The lack of association of the evaluated genes in this sample of Mexican women is consistent with the complex genetic etiology of POI that is observed across cohorts studied thus far.

Keywords Primary ovarian insufficiency · *BMP15* · *FSHR* · *FOXL2* · *GDF9* · Mexican population

Introduction

Primary ovarian insufficiency (POI) is a heterogeneous and multifactorial disorder characterized by absent menarche (primary amenorrhea) or cessation of menses for a period of at least 4–6 months (secondary amenorrhea), hypoestrogenism, infertility, and high FSH levels (> 40UI/L, measured in at least two occasions with a month apart), before the age of 40 [1–3]. This clinical condition affects 1 in 100 women younger than 40 years of age (1%), 1 in 1000 women below 30 years of age (0.1%), and 1 in 10,000 women younger than 20 years of age (0.01%) [4]. Approximately 10–28% of patients present primary amenorrhea and 4–18%, secondary amenorrhea [5].

Based on the presence or absence of follicles, POI is classified as follicular or afollicular. The former has ovarian follicles whose function has been impaired by other pathologies, whereas the latter is characterized by the complete depletion of ovarian follicles [4]. POI is further classified into sporadic (the majority) and familial cases [6]; the latter comprising 4–30% of patients (depending on the studied population), thus suggesting it is a hereditary disorder [7, 8].

POI etiology is not well understood [9], but the condition has been widely reported in association with (1) low initial number of follicles, (2) accelerated follicular atresia, (3) compromised maturation process, or (4) folliculogenesis blockage previous to antral stage and ovulation inhibition [10, 11]. Reports have suggested that such mechanisms can be activated by iatrogenic, metabolic, infectious, chromosomal,

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autoimmune, environmental, or genetic factors [12, 13]. Concerning the latter, and based on murine *knockout* models [14], several candidate genes have been considered for association with the recruitment, development, and maturation of follicles and their oocytes [15]. Some point mutations in autosomal or X-linked genes (*INHA*, *FOXL2*, *ESR*, *FSHR*, *FMR1*, *GDF9*, *BMP15*, and others) have been found in both syndromic and non-syndromic cases [2, 9, 16]. However, < 25% of POI cases show direct association with such mutations, and therefore, POI etiology still remains unexplained in most patients [6, 17]. Among the most studied and proposed candidate genes are *BMP15*, *FSHR*, *FOXL2*, and *GDF9* [7, 9].

BMP15: Bone morphogenetic protein 15, found within the superfamily of transforming growth factors β (TGF-β), is located in Xp11.22 [18] and is expressed in the oocytes of humans and other mammalian species [19]. It stimulates the proliferation, development, and maturation of granulosa cells, follicles, and oocytes and also acts in the regulation of ovulation [20–22]. *BMP15* mutations have been associated with both primary and secondary amenorrhea, with a prevalence of 1.5–15% [7].

FSHR: Follicle-stimulating hormone receptor is located in 2p21-p16 [23]. It participates in normal reproductive function regulating the development and maturation of follicles and oocytes, stimulating the production of estrogens, estradiol, and progesterone by granulosa and thecal cells [14, 16, 23].

FOXL2: Blepharophimosis-ptosis-epicanthus inversus syndrome (BPES) (OMIM 110100) is a hereditary autosomal dominant syndrome characterized by alterations in the eyelids and POI in type I, although POI is absent in type II [24]. BPES, in both types I and II, is caused by *FOXL2* (Forkhead Box L2) mutations; this gene is located in 3q23 [25]. *FOXL2* participates in ovarian and eyelid development, and in the maintenance of female gonads [26, 27]. Further, it intervenes in the differentiation and maintenance of granulosa cells and the development and maturation of oocytes [24, 28]. Mutations in this gene have also been found in cases without BPES and also associated with infertility due to folliculogenesis blockage [29, 30].

GDF9: Growth differentiation factor-9 is found within the TGF-β superfamily, located in 5q31.1, participates in the proliferation and differentiation of granulosa and theca cells, and regulates follicular development and ovulation [20, 23, 31].

The aim of this initial study in a Mexican patient sample is to evaluate the presence of mutations or polymorphisms in the *BMP15*, *FSHR*, *FOXL2*, and *GDF9* genes.

Material and methods

Patients

We recruited 20 Mexican women under 40 years old in a period of 3 years, with idiopathic, sporadic or family IOP, normal karyotype (46,XX), primary or secondary amenorrhea, and elevated FSH (> 40UI/L) in two measurements. Pregnancy was not excluded in the patient group. All participants signed an informed consent letter previously approved by the local ethics committee and the study has been performed in accordance with the ethical standards as laid down in the Declaration of Helsinki. In addition, healthy women with an age range of 15–39 years and unrelated to the patients, participated as a control group ($n = 50$ for *FSHR* and $n = 100$ for *GDF9*). We conducted a descriptive, observational, and comparative study.

Molecular analysis

Genomic DNA was extracted from peripheral blood samples obtained from each patient using standard protocols (Miller et al. 1988) [32]. The exome of each gene (divided into fragments: *BMP15* 1, 2A, and 2B; *FSHR* 7–8 and 10; *FOXL2* 1, 2, and 3; and *GDF9* 1, 2A, and 2B) was amplified by polymerase chain reaction (PCR) (primers, exons, and conditions are presented in Table 1). The amplicons were analyzed by agarose gel electrophoresis and then sequenced by Sanger's method in an automated sequencer (ABI 3130; Applied Biosystems, Foster City, CA). The genetic variants identified were confirmed in three different experiments.

Statistical analysis

Statistical analysis was performed using the software SPSS v.22.0 (SPSS Inc., Chicago, IL). Descriptive statistics were performed for categorical variables expressed as frequencies. Quantitative variables were expressed as mean ± standard deviations (SD). A chi-square test was used to compare allele and genotype frequencies between patients and a healthy control group. A $p < 0.05$ was considered significant.

Results

Twenty patients with idiopathic POI were included in the study. The average age for POI onset was of 24.8 ± 6.8 years; most cases started between the ages of 20 and 25 years ($n = 7$, 35%), 30% before 20 years old ($n = 6$), 30% between 26 and 35 years ($n = 6$), and only 5% after 35 years old ($n = 1$). The average age at diagnosis was of 30.5 ± 6.7 years; most women were diagnosed between 26 and 30 years old ($n = 9$, 45%), 35% after 30 years ($n = 7$), and 20% were diagnosed before 25 years old ($n = 4$). Concerning amenorrhea, most cases presented secondary amenorrhea (85%), with sporadic type POI (80%) and fertility problems (found in 80% of the studied cases) (Table 2).

Molecular analysis revealed no variants in *BMP15* and *FOXL2*, but found variants in exons of two genes, a transition c. 919G>A (p.Ala307Thr) located in exon 10 of the *FSHR* gene, and a transition c.447C>T (p.Thr149Thr) in exon 2A

Table 1 *BMP15*, *FSHR*, *FOXL2*, and *GDF9* primers

Gene	Forward	Reverse	Fragment size	Exon	Conditions
BMP15	AGTGACGTCCCTTGGGCTTG	CAAAGCCTGACAGTAAACCC	477 bp	1	95 °C for 4 min, followed by 28 cycles at 95 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min, and 72 °C for 7 min
	GGGCTGATTATAGCTATCAGTC	GGAAGAGGCAGTAACCTCAGCTG	617 bp	2A	95 °C for 4 min, followed by 28 cycles at 95 °C for 1 min, 65 °C for 1 min, 72 °C for 1 min, and 72 °C for 7 min
	GGGAATCTCTTCTCCGGAGAACC	CTAGCTCACAAGTGGGGGAAGAGAC	555 bp	2B	95 °C for 4 min, followed by 29 cycles at 95 °C for 1 min, 64 °C for 1 min, 72 °C for 1 min, and 72 °C for 7 min
FSHR	CCGTGTATTGTTTGCATCTG	GAAGCTTCTCCCCTAGCTGC	395 bp	7–8	94 °C for 2 min, followed by 33 cycles at 94 °C for 1 min, 60 °C for 30 s, 72 °C for 75 s, and 72 °C for 4 min
	CCTGCACAAAGACAGTGATG	GCCCCAGTTTGCCAGTC	540 bp	10	94 °C for 2 min, followed by 33 cycles at 94 °C for 1 min, 60 °C for 30 s, 72 °C for 75 s, and 72 °C for 4 min
FOXL2	CAGCGCCTGGAGCGGAGAG	CTTGCCGGGCTGGAAGTGC	546 bp	1	95 °C for 10 min, followed by 30 cycles at 95 °C for 1 min, 66 °C for 1 min, 72 °C for 2 min, and 72 °C for 10 min
	GACCCGGCCTGCGAAGACA	GGCCGCGTGCAGATGGTGT	516 bp	2	95 °C for 10 min, followed by 35 cycles at 95 °C for 1 min, 66 °C for 1 min, 72 °C for 2 min, and 72 °C for 10 min
	CGCGGCCGCTGTGGTCAAG	GCTGGCGGCGGCGTCGTC	501 bp	3	94 °C for 2 min, followed by 30 cycles at 95 °C for 30 s, 66 °C for 30 s, 72 °C for 2 min, and 72 °C for 10 min
GDF9	TTCCTCACTAGTTCTCCCAAGC	CATCTTCCCTCCACCCAGT	461 bp	1	95 °C for 4 min, followed by 28 cycles at 95 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min, and 72 °C for 7 min
	TTCAAGCACTACTGGTAG	AGCCTGAGCACTTGTGTCATT	460 bp	2A	95 °C for 4 min, followed by 27 cycles at 95 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min, and 72 °C for 7 min
	ATGAAAGACCAGCTGGAGCA	TTTGCCAAATAGGCTCAAGG	686 bp	2B	95 °C for 4 min, followed by 29 cycles at 95 °C for 1 min, 64 °C for 1 min, 72 °C for 1 min, and 72 °C for 7 min

Primers *BMP15, FSHR*, *FOXL2*, and *GDF9* [33–36]. Amplification conditions were standardized in this work

of the *GDF9* gene. Both have been previously reported as polymorphisms (rs6165 and rs254286). These two genetic variants were observed as either wild type, heterozygous, or polymorphic homozygous (Fig. 1). The allele and genotype frequencies for both polymorphisms are shown in Table 3. The analysis found no significant difference between patients and controls ($p > 0.05$), and the distribution was similar in both patient and control groups ($p = 0.691$ and 0.729, respectively). The population was in Hardy–Weinberg equilibrium. The allele frequencies of the two polymorphisms observed in the Mexican population (rs6165 and rs254286) were compared with those reported in the NCBI-SNPs HapMap project,

Table 2 Clinical characteristics of patients with POI

	Primary amenorrhea	Secondary amenorrhea	Sporadic type	Familiar type	Fertility	Infertility
Number patients	3	17	16	4	4	16
Frequency (%)	15%	85%	80%	20%	20%	80%
	FSH and LH average levels					
FSH	76.4 mUI/ml ± 23.2 (2.5–10.2 mUI/ml normal levels)					
LH	47.2 mUI/ml ± 14.4 (1.9–12.5 mUI/ml normal levels)					

Frequencies obtained with 20 patients. Quantitative variables are expressed as mean ± SD and categorical variables as frequencies (in percentage)

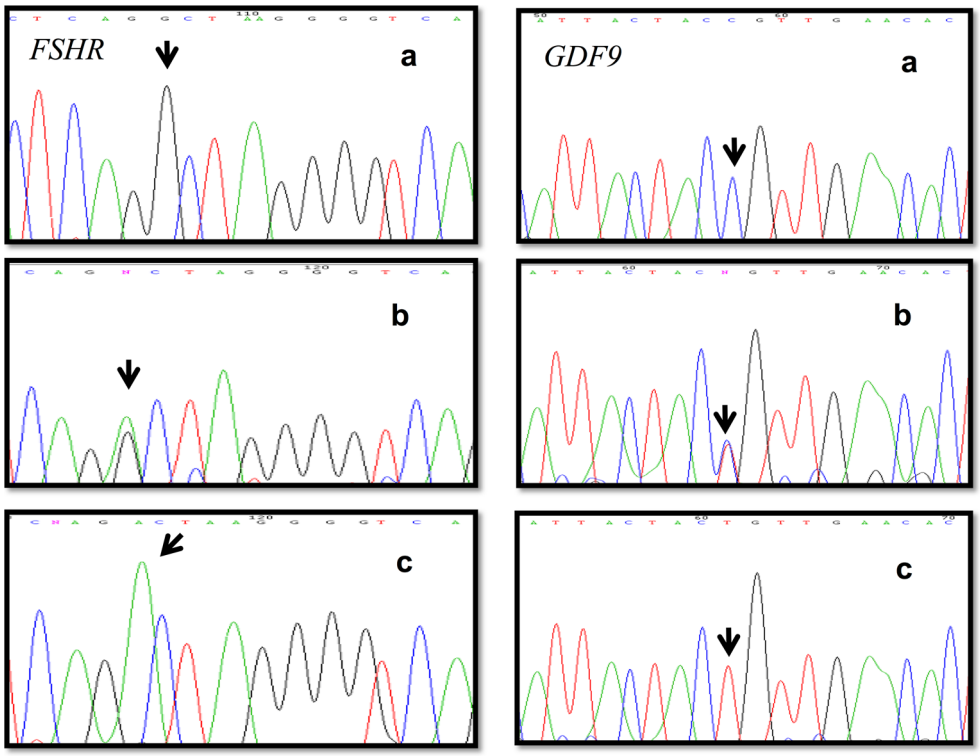

Fig. 1 Electropherogram showing the polymorphisms c. 919G>A (p.Ala307Thr) in the *FSHR* gene and c.447C>T (p.Thr149Thr) in the *GDF9* gene in the three evaluated conditions: (a) wild type, (b) heterozygous, and (c) polymorphic homozygous

finding that the allele frequencies of the rs6165 polymorphism were similar to the Sub-Saharan African population ($G = 25\%$ and $A = 76\%$) and the allele frequencies of the rs254286 polymorphism were similar to the Japanese population ($C = 60\%$ and $T = 40\%$).

Discussion

POI candidate genes have been evaluated in diverse world populations [37]; mutations in these genes could modify the amino acid sequence affecting the function of the coded protein and resulting in amenorrhea and infertility. However, the genetic variants identified and associated with POI are somewhat disparate from one population to another, and it has been suggested that differences could be due to the heterogeneity in the design of the studies or of the analyzed populations [16, 29].

This is the first study conducted in the Mexican population concerning the genes *BMP15*, *FSHR*, *FOXL2*, and *GDF9*. All of which have been reported to be mutated in at least some cases of POI in other populations [7]. We found two genetic variants, c.919G>A (p.Ala307Thr) in exon 10 of the *FSHR* gene (extracellular domain) and c.447C>T (p.Thr149Thr) in exon 2 of the *GDF9* gene (pro-region), but found no significant difference for either of them in cases vs. control ($p > 0.05$). Regarding the *FSHR* gene, past reports are varied,

Table 3 Frequency of the polymorphisms c. 919G>A and c.447C>T

Gene	Polymorphism	AA change	Exon	Genotype	Genotype frequency (%) (POI patients)	Genotype frequency (%) (control population)	*p*	Allele	Allele frequency (%) (POI patients)	Allele frequency (%) (control population)	*p*
FSHR	c.919G>A	p.Ala307Thr	10	G/G	2 (10%)	7 (14%)	0.46	G	14 (35%)	31 (31%)	0.691
				G/A	10 (50%)	17 (34%)		A	26 (65%)	69 (69%)	
				A/A	8 (40%)	26(52%)					
				Total	20 (100%)	50 (100%)			40 (100%)	100 (100%)	
GDF9	c.447C>T	p.Thr149Thr	2A	C/C	6 (30%)	26 (26%)	0.88	C	23 (58%)	107 (54%)	0.729
				C/T	11 (55%)	55 (55%)		T	17 (42%)	93 (46%)	
				T/T	3 (15%)	19 (19%)					
				Total	20 (100%)	100 (100%)			40 (100%)	200 (100%)	

$p < 0.05$ was considered significant

e.g., Sundblad (2004), Woad (2013), Ma (2015), and Ghezelayagh (2018) found similar results to ours, meaning that though they observed the polymorphism c.919G>A (p.Ala307Thr), they did not find any significant difference in 20 Argentinean women with POI, 80 from New Zealand, 63 from China, and 84 from Iran, respectively [38–41]. Contrary to these findings, the genetic variant was associated with POI in a Brazilian population ($n = 100$) [16], but in countries including Japan, Greece, India, Germany, Denmark, and Singapore, no changes were identified in this gene; and thus the genetic variants associated with POI are geographically restricted [42, 43].

As for *GDF9* gene, like *FSHR*, the reported results differ depending upon the population studied. For example, whereas we found the synonymous variant c.447C>T (p.Thr149Thr), in either wild type, heterozygous, or polymorphic homozygous configurations in both patients and control subjects, Kumar (2017) observed no variation in 54 women from India, nor has it been found in women from New Zealand ($n = 38$) [44]. However, in USA, China, and Caucasian populations, this and other variants have been reported [29, 36], although mostly in heterozygous state but only in POI patients, with none seen in the controls [7, 15].

Despite the differences found in diverse populations of the world, we observed that the allele frequencies of the rs6165 and rs254286 polymorphisms identified in the Mexican population were similar to those reported in the Sub-Saharan African and Japanese populations respectively (NCBI-SNPs HapMap project), although this has been mainly attributed to a coincidence, given that the Mexican population has greater European ancestry, than Asian or African.

Mexican patients with POI showed no variations in the *BMP15* or *FOXL2* genes. Similarly, neither Takebayashi (2000) nor Ni (2010) found any *BMP15* gene variants in 15 Japanese and 118 Chinese women, respectively [33, 45]. Nevertheless, Laissue (2009) showed evidence implicating *FOXL2* gene variants in non-syndromic POI (without BPES) in a Tunisian population [46]. Further, Kumar (2017) recently identified diverse *BMP15* gene variants in a sample of 53 women with idiopathic POI, although with no clear association [44]. Taking into account the previous data, the overall sample size that has been explored in various groups is sufficient to indicate that ethnic differences must be considered in the search of genetic variants linked to POI.

As for clinical characteristics, the average age for symptom onset in the patients from our population was 24.8 ± 6.8 years of age, but most started before 25 ($n = 13$, 65%); in 80% of these cases, POI was sporadic type ($n = 16$) and 80% could not achieve pregnancy ($n = 16$). This is of particular concern now that women are increasingly delaying the age for first pregnancy and in this initial study, we did not find variants in any of the four proposed genes in the Mexican population as a common cause for POI, although the results are consistent with the complex genetic etiology of POI that is observed across cohorts studied thus far. However, other candidate genes could be studied as a cause of POI in the Mexican population.

Compliance with ethical standards

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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