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GENETICS

Association of *BMP15* and *GDF9* variants to premature ovarian insufficiency

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Abstract

Purpose To identify genetic variation associated to premature ovarian insufficiency (POI).

Methods A total of 74 women with POI (group POI), 45 women with increased FSH levels (group high FSH), and 88 controls (non-POI) were studied. Genotyping of *BMP15*:c.-9C>G (rs3810682), *BMP15*:c.328+905A>G (rs3897937), and *BMP15*:c.852C>T (rs17003221); and *GDF9*:c.134-694G>A (rs4705974), *GDF9*:c.-31-951G>A (rs11748063), *GDF9*:c.-152G>C (rs30177), and *GDF9*:g.1073C>T (rs803224) was performed by the TaqMan methodology. Chi-square and Fisher's exact tests were performed to evaluate the distribution of genotypes, alleles, odds ratio, and the Hardy-Weinberg equilibrium of each variation. Haplotype analysis was performed for each gene considering the case and control groups. Bonferroni's correction was applied to chi-square and Fisher's exact test data, and p values < 0.007 for genotypes and alleles and < 0.006 for haplotypes were considered significant.

Results It was observed a statistically significant difference in genotype distribution of *BMP15*:c.852C>T between group POI and controls ($p < 0.001$). TT and TC genotypes were more frequently observed in group POI. Genotype distribution in case group POI, however, was not in the Hardy-Weinberg equilibrium, due to the increased number of heterozygotes in the sample. Concerning *GDF9*, no association was found among the studied genetic variants and POI or high FSH groups.

Conclusion It is concluded from the present study that the genotypes CT and TT from *BMP15*:c.852C>T variation may be risk factors for the development of POI.

Keywords Premature ovarian insufficiency · *BMP15* · *GDF9* · Infertility · Gene variants

Introduction

Women's reproductive age was postponed due to the changes in professional and social life in the last decades. This fact put in evidence of some gynecological pathology that impact on ovarian reserve decrease and premature ovarian insufficiency (POI) and menopause [1].

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In regular conditions, menopause occurs among 50–52 years old, while premature menopause is considered when menses cessation and ovarian decrease occur before 40 years old [2]. Apart from menstrual irregularity, the other criterion that must be considered to proper diagnosis of POI is FSH (follicle-stimulating hormone) dosage.

Traditionally, POI has been diagnosed through two or more FSH dosages that result in levels above 40 mIU/mL at least 1 month apart. However, in 2013, the ESHRE POI Consensus (that occurred in Utrecht, the Netherlands) determined a new cutoff level for FSH as two dosages of 25 mIU/mL or above, respecting the same period of time [3].

The etiology of POI is complex and can be attributed to autoimmune diseases, infections, iatrogenic exposition, gene mutations, and chromosome aberrations [4–7]. Therefore, adrenocortical (21-OH) and anti-thyroid antibodies (TPO), drugs, and previous surgeries affecting ovaries must be

considered in clinical evaluation. Also, karyotype, *FMR1* gene premutation must be the first investigated when a genetic cause is considered.

However, mutations in other genes have emerged as possible causes of POI, when primary causes were discarded, including genes that exert hormonal effects or affect follicular function as *BMP15* and *GDF9* [8].

BMP15 is a member of the beta-growth factor superfamily (TGFb) and is located in Xp11.2, an important chromosomal region for ovarian differentiation [9, 10]. *GDF9* also is a member of beta-growth factor superfamily (TGFb) and is located in 5q31.1. [11, 12]. The factors secreted by oocytes, BMP15 and GDF9 play an important role in the development of the primordial follicle, ovulation process, corpus luteum formation, proliferation of granulosa cells, and oocyte maturation through paracrine/autocrine signaling pathways [9, 10, 13]. In bovines, the *BMP15* transcript is observed from oocyte maturation to morula stage, after fertilization [14].

In view of the possible role of these genes in POI development and the small number of scientific articles available, most of them performed with European and Asiatic populations, the objective of the present study was to evaluate the association of *BMP15* and *GDF9* variations with POI in a Brazilian population and to evaluate which variants are shared by patients with increased levels of FSH, in order to contribute to elucidation of the etiology of premature ovarian insufficiency.

Methods

Casuistic

The present study is an association study, using a case-control design, developed with patients recruited from the premature ovarian insufficiency outpatient clinic of the Instituto Ideia Fértil (case group) and women recruited from the gynecology and climacteric outpatient clinic of Faculdade de Medicina do ABC (control group). The research project was approved by the FMABC's Ethics Committee and all patients read and signed the informed consent form.

The case group was composed of 119 women and subdivided into two groups:

- The case group POI consisted of 74 women with POI, with FSH levels > 25 mIU/mL and cessation of menstruation before 40 years of age. POI criteria followed The ESHRE Guideline Group on POI [3]. All of them had age up to 40 years old at the time of study inclusion.
- The case group high FSH consisted of 45 women, with FSH levels between 10 and 25 mIU/mL, irregular menses, and age up to 40 years old, possible candidates to ovarian insufficiency development. Sisters or cousins of patients were not included in the sample.

Patients with a history of surgical manipulation of the gonads and gonadal lesions and history of radio- and/or chemotherapy were excluded. Also, patients with abnormal levels of thyroid hormones and reagent anti-TPO were excluded from the sample. As inclusion criteria, all patients (POI and high FSH groups) must have normal karyotype and normal genotype for the *FMR1* gene expansion (alleles with less than 55 CGG repeats.).

The control group consisted of 88 non-POI women with 50 years of age or more, whose menopause occurred after 50 years of age naturally. All of them had at least one pregnancy and parity and never experienced irregular menses. As in menopause increased levels of FSH were normally expected, no FSH dosage was performed for the control group. Women with a familial history of POI were not included in the control group.

Genotyping

The collection of 5 mL of peripheral blood in a tube containing EDTA from all the women in the study was performed. DNA extraction was performed using the salting out methodology [15].

The studied variants of the *BMP15* and *GDF9* genes, described in Table 1, were identified by real-time PCR using the TaqMan SNP genotyping assays (C_27504454_10, C_7455457_10, C_32866291_10, C_27970131_10, C_2563478_10, C_2264894_10, C_2563470_10). PCR conditions were provided by the manufacturer: heating at 60 °C for 30 s, followed by an initial denaturation of 95 °C for 20 s, followed by 40 denaturation cycles of 95 °C for 1 s, annealing at 60 °C for 20 s. Positive controls for each genotype were included in all reactions. Variant nomenclature was described according to HGVS variant nomenclature (https://varnomen.hgvs.org).

Statistical analysis

For quantitative variables as age and FSH levels, Shapiro-Wilk's test was performed in order to identify if the samples have normal distribution. Data with no normal distribution was described by median and percentile. Normal data was described by average and standard deviation.

The distribution data of the genotypes and alleles, odds ratio, and the Hardy-Weinberg (HW) equilibrium were analyzed by the chi-square test and Fisher exact test using BioStat 3.0 software. Bonferroni's correction was applied to genotype and allele distribution analysis and p values < 0.007 were considered statistically significant.

The analysis of the haplotypes for the two genes was also performed using Haploview 4.2 software. The p value < 0.006 was considered statistically significant after Bonferroni's correction.

Table 1 Description of *BMP15* and *GDF9* variations according to dbSNP (accessed January 15, 2019)

***BMP15* (NM_005448.2)**	**rs3810682:C>G**	**rs3897937:A>G**	**rs17003221:C>T**	
Alleles	C>G (rev)	A>G	C>T	
Ancestral allele	G	G	T	
HGVS name	*BMP15*:c.-9 C>G	*BMP15*:c.328+905 A>G	*BMP15*:c.852 C>T	
Functional consequence	5'UTR variant	Intronic variant	p.Ser284=	
GnomAD allele frequency	*G* = 0.16200	*G* = 0.37980	*T* = 0.05660	
AbraOM allele frequency*	*G* = 0.18182	Not reported	*T* = 0.107784	
***GDF9* (NM_001288824.2)**	**rs4705974:G>A**	**rs11748063:G>A**	**rs30177:G>C**	**rs803224:C>T**
Alleles	C>T (fwd)	C>T (fwd)	G>C (rev)	C>T (rev)
Ancestral allele	T	T	C	T
HGVS name	*GDF9*:c.134-694 G>A	*GDF9*:c.-31-951G>A	*GDF9*:c.-152 G>C	rs803224:C>T
Functional consequence	Intronic variant	5'UTR variant	5'UTR variant	Intergenic
GnomAD allele frequency	*T* = 0.1703	*T* = 0.3484	*C* = 0.3082	*T* = 0.1396
AbraOM allele frequency*	Not reported	Not reported	*C* = 0.724315	Not reported

*AbraOM is a database for Brazilian genomic variants [16]

Results

The average ages of patients in case groups POI and high FSH were 38 [35–41] and 37 [35–39] years old, respectively, and in the control group was 56 [53.75–60.5] years old. The average levels of FSH hormone for POI patients were 67.20 mIU/mL [53.23–108.90] in the first dosage and 65.00 [41.19–101.40] in the second dosage and 16.15 [12.26–19.19] and 16.05 [12.65–22.18] mIU/mL for high FSH patients. Additional exams and hormone dosages can be observed in supplementary data as Table 4.

GDF9 and *BMP15* genotype and allele distributions among case groups 1 and 2 and controls were statistically evaluated as well as odds ratio and HW equilibrium data. The results can be observed in Table 2.

The statistical analysis of the *BMP15*:c.852C>T showed a statistically significant difference of group POI results for genotype distribution ($p < 0.001$) when compared with the control group. The CT and TT genotypes were more frequent in the group POI. The other *BMP15* variations evaluated showed no statistically significant difference. *GDF9* variations evaluated showed no statistically significant difference among groups.

No *BMP15* or *GDF9* haplotypes were associated to POI or high FSH. Data is available in Tables 3.

Discussion

The etiology of premature ovarian insufficiency can be attributed to several genetic alterations and is still a subject of research. According to Vilodre et al. (2007), different causes of POI may lead to either reduction in the number of follicles and/or defects in the follicle stimulus mechanism [14]. In this project, we performed the analysis of the variations rs3810682, rs3897937, and rs17003221 of the *BMP15* gene and the variations rs4705974, rs11748063, rs30177, and rs803224 of the *GDF9* gene in two groups of patients: women with established ovarian insufficiency and women with increased FSH values, possible candidates for POI. We compared the findings in these groups to the results of a control group in order to find possible risk factors for POI in our population and to identify women at high risk for developing ovarian failure. Also, we have studied haplotypes associated to the condition to both genes.

The correlation of the *BMP15* gene with POI was observed initially in animal models. Galloway et al. in 2000 [17] performed a study with sheep that had a mutation in *BMP15*. The heterozygous models had an increased rate of ovulation and birth of twins and triplets. The homozygous sheep developed ovarian failure, originated from a compromised follicular development. Besides, a substitution in *BMP15* (Y235C) in heterozygous was identified from sisters with POI leading to further investigation of the importance of the gene for the condition [18]. Since then, some authors have investigated the association of *BMP15* variations with premature ovarian insufficiency in different populations with diverse findings, as described by Qin et al. (2015) [19] and detailed in Table 5 in the Electronic Supplementary Materials (ESM) [18, 20–32]. Some variations seem to be frequent in POI cases while some were exclusive of specific populations.

In the present study, the analysis of *BMP15* genotypes and alleles showed that the CT and TT genotypes for *BMP15*:c.852 C>T were more frequent in the case group POI than in the control group. No difference was observed in the distribution of the *BMP15* variations among patients with high FSH and controls, and no haplotype was associated to POI or high FSH groups.

Table 2 Genotype and allele distributions for *BMP15* and *GDF9* polymorphisms

Gene	SNP	Group	*n*	Genotypes, *n* (%)			*p*	Alleles, *n* (%)		*p*	HWE
BMP15	rs3810682			GG	CG	CC		G	C		
	c.-9C>G	POI	70	46 (66%)	16 (23%)	8 (11%)	0.72	108 (77%)	32 (23%)	0.69	–
		High FSH	42	29 (69%)	8 (19%)	5 (12%)	0.57	66 (79%)	18 (21%)	0.94	–
		Control	88	58 (66%)	23 (26%)	7 (8%)		139 (79%)	37 (21%)		
	rs3897937			GG	GA	AA		G	A		
	c.328+905A>G	POI	71	3 (4%)	47 (66%)	21 (30%)	0.06	53 (37%)	89 (63%)	0.09	–
		High FSH	43	1 (2%)	32 (74%)	10 (23%)	0.26	34 (40%)	52 (60%)	0.28	–
		Control	88	7 (8%)	68 (77%)	13 (15%)		82 (47%)	94 (53%)		
	rs17003221			TT	TC	CC		T	C		
	c.852C>T	POI	70	18 (26%)	45 (64%)	7 (10%)	*< 0.001*	81 (58%)	59 (42%)	0.03	*0.02*
		High FSH	43	15 (35%)	15 (35%)	13 (30%)	0.58	45 (52%)	41 (48%)	0.31	–
		Control	88	23 (26%)	34 (38%)	31 (35%)		80 (46%)	95 (54%)		
GDF9	rs30177			CC	CG	GG		C	G		
	c.-152G>C	POI	74	8 (11%)	22 (30%)	44 (59%)	0.73	38 (26%)	110 (74%)	0.64	–
		High FSH	45	7 (15%)	16 (36%)	22 (49%)	0.67	30 (335)	60 (67%)	0.37	–
		Control	87	9 (10%)	31 (36%)	47 (54%)		49 (28%)	126 (72%)		
	rs11748063			TT	TC	CC		T	C		
	c.-31-951G>A	POI	65	15 (23%)	22 (34%)	28 (43%)	0.033	52 (40%)	78 (60%)	0.02	
		High FSH	41	5 (12%)	14 (34%)	22 (54%)	0.118	24 (29%)	58 (71%)	0.78	–
		Control	87	7 (8%)	34 (39%)	46 (53%)		48 (28%)	126 (72%)		
	rs803224			TT	TC	CC		T	C		
	Intergenic	POI	70	56 (80%)	13 (19%)	1 (15)	0.33	125 (895)	15 (11%)	0.46	–
		High FSH	45	33 (73%)	8 (18%)	4 (9%)	0.72	74 (82%)	16 (18%)	0.35	–
		Control	86	68 (79%)	13 (15%)	5 (6%)		148 (87%)	23 (13%)		
	rs4705974			TT	TC	CC		T	C		
	c.134-694G>A	POI	65	0 (0%)	14 (22%)	51 (78%)	0.31	14 (11%)	116 (89%)	0.49	–
		High FSH	41	1 (2%)	10 (24%)	30 (73%)	0.81	12 (15%)	70 (85%)	0.78	–
		Control	86	3 (3%)	17 (20%)	66 (77%)		23 (13%)	149 (87%)		

After Bonferroni's correction, the *p* value < 0.007 was considered significant for genotypes and alleles. It was not applied to HWE that considered *p* values < 0.05 to evidence disequilibrium

n, number of samples; %, percentage of samples; *HWE*, the Hardy-Weinberg equilibrium; Statistically significant results are in italics

Table 3 Haplotype distribution concerning *BMP15* and *GDF9* variations

SNV	Haplotype associations	Frequency[1]	Case ratios[1]	Control ratios[1]	*p* value[1]	Frequency[2]	Case ratios[2]	Control ratios[2]	*p* value[2]
BMP15 haplotype									
rs3810682 rs3897937 rs17003221	GAC	0.292	0.276	0.304	0.579	0.248	0.212	0.265	0.352
	GGT	0211	0.238	0.189	0.288	0.157	0.166	0.153	0.786
	GAT	0.171	0.202	0.147	0.199	0.199	0.236	0.182	0.310
	GGC	0.108	0.056	0.149	0.008	0.184	0.172	0.190	0.732
	CAT	0.066	0.079	0.055	0.406	0.069	0.091	0.058	0.328
	CGT	0.062	0.060	0.063	0.904	0.055	0.043	0.061	0.541
	CGC	0.047	0.025	0.064	0.099	0.046	0.012	0.062	0.072
	CAC	0.044	0.065	0.028	0.107	0.041	0.068	0.028	0.132
GDF9 haplotype									
rs4705974 rs11748063 rs30177 rs803224	CCGT	0.496	0.447	0.538	0.102	0.527	0.488	0.547	0.367
	CTGT	0.189	0.241	0.144	0.025	0.135	0.135	0.136	0.981
	CCCC	0.105	0.094	0.115	0.557	0.129	0.158	0.115	0.317
	TTCT	0.104	0.094	0.113	0.588	0.116	0.121	0.114	0.875
	CCCT	0.057	0.060	0.054	0.804	0.043	0.043	0.045	0.806
	TTGT	0.024	0.037	0.013	0.167	0.016	0.024	0.012	0.468

After Bonferroni's correction, the *p* value < *0.006* was considered significant

[1] Case group IOP

[2] Case group 2 High FSH

In a previous study performed by our research group, Peluso et al. (2017) studied 186 infertile women with regular FSH levels, in order to correlate the same *BMP15* variations investigated here to ovarian response. It was observed that none of the alleles was associated to oocyte retrieval or oocyte maturation results in their population. Their results reinforce that that the variation is not common in Brazilian population, and the association found in the present research is specific to POI. Peluso found a significant association of TT genotype from *BMP15*:c.852 C>T to increased levels of estradiol, a serum abnormality also observed in patients with POI [33].

The Hardy-Weinberg deviation of genotype distribution was observed for this variation on case group POI, due to an increased number of heterozygotes. Deviation from HWE at a marker locus can be due to diverse reasons as population stratification, inbreeding, selection, nonrandom mating, genotyping error, actual association to the disease or trait under study, or a deletion or duplication variation [34]. In fact, in a study of complex diseases in human populations, none of these reasons can be discharged. Therefore, the equilibrium deviation can be a good fact, reinforcing the association of such genotype to the condition or a bad fact, considering that a methodological error could have occurred. Here, reinforcing the idea of association to the condition, we observed no deviation in the control group.

In the literature data, Ma et al. (2015), Tiotiu et al. (2010), Zhang et al. (2007), and Laissue et al. (2006) observed that the frequencies of the *BMP15* c.852C>T genotypes did not vary significantly between the POF and control groups, while Dixit et al. (2006) showed that the haplotype G-G-C in the three frequent variants c.-9C>G, c.308A>G, and c.852C>T was associated with POI [21, 22, 26, 27, 29].

Concerning *BMP15* c.-9C>G, Leidig et al. (2008) did not find association of with the condition [25]. Ma et al. (2015) observed that the C allele was disproportionally frequent in cases and controls, and Dixit et al. (2006) also observed association of the variant as described previously [21, 29]. Regarding *GDF9*, no difference was observed in the distribution of the *GDF9* variations among patients and controls. Also, in the literature, no association was found between the variants studied with POI.

Voourhuis et al. in 2011 carried out a study with 3616 patients with natural menopause with the objective of studying the association between the 23 single-base variations in five genes, *AMH*, *AMHR2*, *BMP15*, *FOXL2*, and *GDF9* with the timing of menopause. They evaluated rs3810682, rs6521896, rs17249566, rs5961233, and rs3897937 in *BMP15*, of which two of them are common to our study. In *GDF9*, they evaluated six different variations, including rs30177, rs803224, rs11748063, and rs4705974, which are common to the present study. The analysis also showed no significant association of *GDF9* variations to menopause age, but rs6521896 in *BMP15* was associated with later menopause [35].

The divergence of the results of the present study against the results of other populations demonstrates the divergence in the ethnic composition of the Brazilian population, in particular the population of São Paulo's metropolitan area, where

the study was developed. Brazil is a continental country with wide intra-territorial differences. Thus, the application of international models for the attribution of risk factors should be carefully considered. To exemplify, Carvalho-Silva et al. (2001) carried out a genetic study with DNA from Brazilian white population. They found distinct chromosome Y footprints of Italian immigration to southern Brazil, migration of Moroccan Jews to the Amazon region, and Dutch DNA in Brazil northeast. In contrast with the mtDNA data in white Brazilians, which showed that more than 60% of the matrilineages were Amerindian or African, only 2.5% of the Y-chromosome lineages were from Sub-Saharan Africa, and none were Amerindian [36].

One limitation of the present study is the sample size, which despite being larger than that of many POI studies is still small for association analysis. More robust studies could confirm the results observed in the present study. To justify our sample size, we would like to emphasize the rigor of patient selection, excluding genetic alterations common to this group of patients (as *FMR1* premutated alleles and positive anti-TPO). Another limitation was the method of choosing the variations of each gene, which was based on previous association in the literature. It is possible that other variations of the studied genes were associated to the condition, but were not addressed in the present study. Direct sequencing and GWAS studies could evidence different variations associated to POI and are being performed by the research group.

The genetic study and determination of the frequency of gene variants in cases and controls contribute to the understanding of possible risk factors related to the development of premature ovarian insufficiency. To understand these factors is extremely important in a population that has changed their lifestyle, in a population where pregnancies are delayed due to large use of contraceptive methods and the equal insertion of women in universities and in the labor market. Early mapping of risk factors can assist women in reproductive decision-making, such as anticipating motherhood or preserving oocytes or embryos.

Conclusion

The cause of POI has been studied worldwide. The many divergences in results found may be associated with different ethnicities of the populations studied. Variant genotypes from *BMP15* were found at high frequency in women with POI. In view of the important physiological role of this gene in oogenesis, these variants may be associated with the risk of developing POI in Brazilian population.

Compliance with ethical standards The research project was approved by the FMABC's Ethics Committee and all patients read and signed the informed consent form.

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