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GENETICS

Risk of premature ovarian failure is associated to the *PvuII* polymorphism at estrogen receptor gene *ESR1*

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Abstract
Purpose Estrogen plays an important role in the human reproductive system and it action is mediated mainly by two specific receptors: α (ERα) and β (ERβ). There were described polymorphic variants in *ESR1 and ESR2* genes and studies showed controversial results regarding their association with premature ovarian failure. We aimed to determine the prevalence of *ESR1 and ESR2* polymorphisms in Brazilian patients and controls. After associate the polymorphisms with premature ovarian failure (POF).
Methods Genetic association study was performed with 70 women with POF and 73 normally menopaused controls. Detection of *ESR1* (*PvuII*/and *XbaI*) and *ESR2* (*AluI* and *RsaI*) gene polymorphisms were performed using TaqMan PCR. The single-nucleotide polymorphism (SNPs) and haplotype effects were analyzed by multivariate logistic regression and haplotype analysis and a p-value<0.05 was considered significant.
Results Individual SNP analysis revealed that *PvuII* polymorphism was statistically associated with POF (p=0.034) under a recessive model. Regarding *XbaI*, *AluI* and *RsaI* SNPs, no statistical difference was observed between POF group and controls (p=0.575, p=0.258 and p=0.483, respectively). Combined genotypes of *ESR1 and ESR2* polymorphisms did not identify a risk haplotype associated with POF.
Conclusion In Brazilian population evaluated results have demonstrated that the genetic variation in *ESR1* gene (*PvuII* polymorphism) is associated to POF risk.

Capsule Estrogen and estrogen receptor play an important role in the reproductive system. Impairment in function of the receptor isoforms has been associated with the development of premature ovarian failure.

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Keywords Premature ovarian failure · Genetic polymorphism · Estrogen · Haplotype study · Association study

Introduction

Premature ovarian failure (POF) is a process by which the gradual decline of ovarian function results in failure of folliculogenesis before age of 40. It is characterized by the absence of menstruation for a period longer than 6 months (secondary amenorrhea), but it can occur before menarche, leading to primary amenorrhea ([1–4]).

Due to the number of familial cases with affected individuals in more than one generation it has been suggested that POF has a genetic basis. Many genes have been implicated on POF development as *FMR1*, *inhibin*, *BMP15*, *LHR*, *FSHR* and others related to ovarian function [5, 6].

It is well known that follicular growth and maturation occurs by the synergic influence of the hormones estrogen, FSH and LH on the ovary [6–8]. Sequence variations in genes that encode hormone receptor and binding protein genes could change their function, affecting follicular pool size or rate of follicular recruitment and thus the increasing the risk for premature menopause [7]. Due to their active role in folliculogenesis, variations in genes related to estrogen metabolism can be considered important risk factors to POF development [9].

The physiological estrogen response on diverse tissue and organ occurs by the hormone binding to estrogen receptor (ER), at the hypothalamus-hypophysis-ovarian axis, which stimulate gonadotropins releasing and consequently foliculogenesis [10]. There are two specific estrogen receptors, differentially distributed in the tissues: estrogen receptor α (ERα) encoded by the *ESR1* gene (MIM 133430/ Genbank ID 2099) on chromosome 6q25 [11] and estrogen

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receptor β (ERβ) encoded by the *ESR2* gene (MIM 601663/ Genbank ID 2100) on chromosome 14q23-24 [12].

Several variants or SNPs (single nucleotide polymorphism) in ESR genes have been described as *PvuII* (T-397C) and *XbaI* (A-351G) polymorphisms in the *ESR1* gene, *AluI* (+1730G/A) and *RsaI* (G1082A) polymorphisms in the *ESR2* gene. Researchers search for those SNPs contribution to POF development with conflicting results [7, 13, 14].

We therefore investigated whether the largest studied SNPs in *ESR1 and ESR2* genes are associated with idiopathic POF in our population and if there are interaction between them in the POF susceptibility.

Methods

Two cohorts of women were recruited to participate of this study, selected from December 2008 to December 2011: 1) The case group, composed by 70 women with infertility, secondary amenorrhea and diagnosed with POF in the outpatient clinic of POF in Faculdade de Medicina do ABC (FMABC); 2) The control group, composed by 73 menopaused women, recruited from menopause outpatient clinic of FMABC.

POF clinical criteria were established according to the American Society for Reproductive Medicine. All patients presented less than 40 years old and serum FSH levels >40 IU/L. Serum FSH levels were measured from blood samples in two separate time-points within a period of 2 months [14]. All patients were observed by the same physician and underwent a complete clinical examination, with medical and gynecological history, including the reproductive health of the patient's mother and sisters, consanguinity, and other genetic condition in the family, age of menarche, and age of menopause. In all cases the cause of POF was unexplained. As exclusion criteria we considered the POF caused by medical interventions such as oophorectomy, chemo or radiotherapy or the presence of an abnormal karyotype or *FMR1* premutation. Once all the patients were recruited from an infertility center, the investigation of infertility was performed with all patients according to the minimum propaedeutic for infertile couples: hormonal and biochemical profile, serum testing for infectious diseases, imaging examinations, investigation of genetic and/or immunological abnormalities. Male partners passed through andrological examination including medical history, semen analysis, hormonal profile, karyotype and Y chromosome microdeletion screening.

Control group included women who filled the following conditions: a) undergone physiological menopause after 48 years old, b) fertile, c) with normal menstrual history, d) regular menses (duration 25–35 days), e) no personal or familial history of premature or early menopause and f) no consumption of oral contraceptives or other hormonal medications at the time of recruitment.

The participation in the study was voluntary and all participants signed an informed consent form approved by the Ethics Committee of Faculdade de Medicina do ABC.

Table 1 Description of polymorphic variants of *ESR1* and *ESR2* genes evaluated, considering the nucleotide exchange position, register number of SNP and catalog number of Taqman essay

Gene	Nucleotide Exchange and position/Restriction enzyme recognition	Register number of SNP	Taqman essay
ESR1	T-397C/PvuII	rs2234693	C___3163590_10
	A-351G/XbaI	rs9340799	C___3163591_10
ESR2	A+1730G/AluI	rs4986938	C__11462726_10
	G1082A/RsaI	rs1256049	C___7573265_10

SNP analysis

For SNPs evaluation, 5 mL of peripheral blood were collected in EDTA-coated tubes and used for DNA extraction, performed by salting out method (Lahiri and Nurnberger [15]).

Detection of *ESR1* (*PvuII*; *XbaI*) and *ESR2* (*AluI*; *RsaI*) polymorphisms was performed using real time PCR based on TaqMan system. TaqMan primers and probes were commercially available and provided by Applied Biosystems® (Foster City, CA, EUA), according to the Table 1. Assays were performed with TaqMan Universal Master Mix, as suggested by the manufacturer with 50 ng of DNA per reaction. PCR conditions were provided by the manufacturer: 40 cycles of 95 °C denaturation (15 s) and 60 °C annealing/extension (1 min).

Statistical analysis was performed by SPSS Windows 18.0 (SPSS, Inc., Chicago, IL) and SNPStats (www. http://bioinfo.iconcologia.net/SNPstats). Chi square and binary logistic regression tests were applied. A p value<0.05 was considered

Table 2 Clinical data about POF patients

	Average and SD
IMC	23.6 (3.8)
Duration of infertility	3.69 (3.3) years
Age	36.0 (7.49) years
Menopause age	31.5 (6.59) years
FSH levels	65.64 mUI/mL
Habits and comorbities	number of patients
Smoke habit	6
Menopause symptoms	29
POF in family members	10

Table 3 Analysis of genotype distribution of *ESR1* and *ESR2* polymorphisms under a recessive model for *PvuII*, *XbaI* and *AluI* and a dominant model for *RsaI*

POLYMORPHISM	GENOTYPE	POF	CONTROL	*p*	OR
PvuII C/T	CC+TC	60 (85.7 %)	52 (71.2 %)	0.034	2.42 (1.05–5.61)
	TT	10 (14.3 %)	21 (28.8 %)		
XbaI A/G	AA+AG	64 (91.4 %)	65 (89 %)	0.630	1.31 (0.43–4.00)
	GG	6 (8.6 %)	8 (11 %)		
AluI A/G	AA+AG	61 (87.1 %)	66 (90.4 %)	0.540	0.72 (0.25–2.05)
	GG	9 (12.9 %)	7 (9.6 %)		
RsaI G/A	GG	60 (85.7 %)	62 (84.9 %)	0.890	1.06 (0.42–2.69)
	GA+AA	10 (14.3 %)	11 (15.1 %)		

significant. Statistical power was calculated by the program Genetic power calculator (http://pngu.mgh.harvard.edu).

Results

Patients presented in the moment they were evaluated in Human Reproduction center around 36 years old (±7.49), while menopause age occurred at 31.5 years old (±6.59). The average of serum value of FSH was 65.64 mUI/mL. Concerning habits and comorbidities of the patients we observed that few patients have been or were smokers by the time of research (6 patients), and only one smokes ten cigarettes per day. The other five patients smoke less than this. Twenty nine patients presented menopause symptoms as hot flashes, sweating and dry vagina. Besides, body mass index (BMI) of patients was in average 23.6 (±3.8). Data is available at Table 2.

The results of statistical analysis of the *ESR1* and *ESR2* SNPs for the POF and controls, considering genotype frequency, are summarized in Table 3.

PvuII genotypes polymorphism presented frequency of 14.3 % of TT genotype, 85.7 % of TC+CC genotype considering POF patients. Among the control group, genotypes TT and TC+CC were found in 28.8 % and 71.2 %, respectively. The results showed association of *PvuII* polymorphism and POF (p=0.034, OR=2.42, 95 % IC=1.05–5.61). For *XbaI* polymorphism, among the POF group, the frequencies of AA+AG and GG genotypes were 91.4 % and 8.6 % respectively. In the control group, genotypes AA+AG and GG were observed in 89 % and 11 %, respectively. Statistical analysis did not reveal difference between POF group and controls (p=0.630, OR=1.31, 95 % IC=1.05–5.61).

Regarding *ESR2* polymorphisms, POF group presented respectively 87.1 % and 12.9 % of AA+AG and GG genotypes of *AluI* polymorphism; and control group presented 90.4 % and 9.6 %, respectively. No statistical difference was observed in *AluI* polymorphism among both groups (p= 0.540, OR=0.72, 95 % IC=0.25–2.05). Considering *RsaI* polymorphism, we found in the POF group, 85.7 % of genotype GG and 14.3 of genotype GA+AA, and among control group we found 84.9 % genotype GG and 15.1 % of GA+AA. A statistically significant difference was not observed for POF group compared to controls (p=0.890, OR= 1.06, 95 % IC=0.42–2.69).

For *PvuII*, *XbaI* and *AluI* polymorphisms a recessive model was chosen, considering that the presence of two mutated alleles confers enhanced effect on disease. For *RsaI* polymorphism, was used the dominant model because there were no mutated homozygous in the control group, what became the recessive model inappropriate.

The haplotype analysis was performed by logistic regression in order to evaluate whether the combined genotypes of the two polymorphisms of each gene (*ESR1* - *PvuII* + *XbaI*) or (*ESR2* - *AluI* + *RsaI*) could increase the risk of POF. Results show a susceptibility haplotype CA (C-*PvuII*, A-*XbaI*) associated with *ESR1* (Table 4) but not to *ESR2* (Table 5).

Discussion

The role of sex steroid hormones in reproductive function, especially estrogen, has for long been studied and positive correlations have been found by diverse groups and gynecological diseases [16–19].

Table 4 Haplotype analysis of association between *ESR1* polymorphisms

PvuII C/T	XbaI A/G	Freq	*P*-value	OR (95 % CI)
C	A	0.5501	–	1.00
T	G	0.2704	0.21	1.40 (0.83–2.39)
T	A	0.1632	0.0059	2.76 (1.35–5.62)
C	G	0.0163	0.71	1.48 (0.19–11.17)

Table 5 Haplotype analysis of association between *ESR2* polymorphisms

AluI A/G	RsaI G/A	Freq	P-value	OR (95 % CI)
A	G	0.6101	–	1.00
G	G	0.3025	0.48	0.84 (0.50–1.38)
A	A	0.0717	0.66	1.23 (0.49–3.12)
G	A	0.0157	1	0.00 (−Inf - Inf)

Given that the effect of a hormone is given by the interaction with its receptor, interindividual differences in susceptibility to premature ovarian failure or impaired ovarian function may arise from *ESR1* variation [20]. Indeed, studies with *ESR1* knockout mice showed important evidences of reproductive impairment as anovulation and complete infertility in the absence of this gene [21]. Besides, it has been postulated that allelic variation on *ESR1* gene could regulate *ESR1* expression and function, which may also affect the estrogen biological action and influence reproductive efficiency [22, 23].

Conflicting results were found related to distribution of *ESR1 PvuII* and *XbaI* genotypes in patients with premature ovarian failure. Bretherick et al. [7] associated the polymorphic C allele of *PvuII* polymorphism in *ESR1* gene with secondary POF. Yoon et al. [22] associated the presence of TT genotype of *PvuII* polymorphism and TA haplotype of *PvuII*/*XbaI* polymorphisms with POF. Yang et al. [13] found a marginally reduced risk of POF in the presence of the mutated allele of *XbaI* polymorphism. Recently, M'Rabet et al. [20] found a positive association of CC genotype to POF.

In our case–control study, we found a statistically significant association between POF and the C allele of *ESR1 PvuII* polymorphism (rs2234693). We also found a susceptibility haplotype composed by the C allele of *PvuII* polymorphism and the A allele of *XbaI* polymorphism. None of the *ESR2* polymorphisms evaluated was associated with premature ovarian failure. So, our findings are similar to the ones described by Bretherick et al. [7] and M'Rabet et al. [20].

The presence of C allele in *PvuII* site was associated with decreased ERα transcription and consequently low number of receptors. It impacts in the estrogenic response of the tissue and also results in low levels of nuclear transcription factors. Transcription factors regulate a variety of genes both directly and indirectly and are implicated in oncogenic and apoptotic events in a diverse range of estrogen-responsive target tissues. Then it is possible that the decrease of transcription factors could affect cellular function and be the cause of ovarian impairment. No studies showed the physiological role of *XbaI* mutation.

All patients included in this study had secondary amenorrhea. Besides, none of them had a partner with male factors involved in infertility history, as observed by semen analysis. We believe that the recruitment of patients with all the same condition and carefully chosen of controls, based on gender, age and familial history excluding POF have contributed to the homogeneity of the sample and quality of the results.

In conclusion, the findings suggest that *ESR1* polymorphisms are positively associated with the risk of POF. More robust and maybe internationally collaborative studies are needed to confirm these observations.

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