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Research Article

ESR1 rs9340799 Is Associated with Endometriosis-Related Infertility and *In Vitro* Fertilization Failure

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Estrogen receptor alpha has a central role in human fertility by regulating estrogen action in all human reproductive tissues. Leukemia inhibitory factor (LIF) expression, a cytokine critical for blastocyst implantation, is mediated by estrogen signaling, so we hypothesized that *ESR1* gene polymorphisms might be candidate risk markers for endometriosis-related infertility and *in vitro* fertilization (IVF) failure. We included 98 infertile women with endometriosis, 115 infertile women with at least one IVF failure and also 134 fertile women as controls. TaqMan SNP assays were used for genotyping *LIF* (rs929271), *MDM2* (rs2279744), *MDM4* (rs1563828), *USP7* (rs1529916), and *ESR1* (rs9340799 and rs2234693) polymorphisms. The SNP *ESR1* rs9340799 was associated with endometriosis-related infertility ($P < 0.001$) and also with IVF failure ($P = 0.018$). After controlling for age, infertile women with *ESR1* rs9340799 GG genotype presented 4-fold increased risk of endometriosis (OR 4.67, 95% CI 1.84–11.83, $P = 0.001$) and 3-fold increased risk of IVF failure (OR 3.33, 95% CI 1.38–8.03, $P = 0.007$). Our results demonstrate an association between *ESR1* rs9340799 polymorphism and infertile women with endometriosis and also with women who were submitted to IVF procedures and had no blastocyst implantation.

1. Introduction

Endometriosis is a benign gynecological estrogen-dependent inflammatory condition defined by the presence of endometrial-like tissue in extrauterine locations [1]. Endometriosis affects up to 10% of women of reproductive age and is responsible for infertility and pelvic pain [2]. Due to its complexity, endometriosis is usually referred to as exhibiting a polygenic and multifactorial basis [3]. Estrogen plays a significant role in the pathogenesis of the disease by promoting endometriotic tissue cell survival, maintenance, and differentiation [2, 3]. Estrogen activates a wide array of tissue- and organ-specific physiological responses by binding to its receptor ESR1, mostly located at the thecal layer, and modulating uterine events preparing the endometrium for embryo attachment and implantation [4].

Though many studies suggest that genetic polymorphisms of estrogen receptor α gene (*ESR1*) modify susceptibility to women's disorders including osteoporosis, preeclampsia, and breast cancer, limited studies have demonstrated associations of *ESR1* polymorphisms in women with endometriosis-related infertility [5–7]. Previous reports have shown associations of *ESR1* genetic variants with susceptibility to endometriosis and fertility status [6, 8–13], but many

studies failed to achieve an association regarding *ESR1* variants and endometriosis-related infertility [9, 14–16]. Interestingly, Lamp et al. linked *ESR1* SNPs only to endometriosis without infertility [12], while Wang et al. associated *ESR1* rs3798573 with risk of both endometriosis and infertile endometriosis in Han Chinese women [13]. *ESR1* rs2234693 (*PvuII)* polymorphism was significantly more prevalent in infertile women at premature ovarian aging [17] and was predictive of an improved controlled ovarian stimulation [18]. Both rs9340799 (*XbaI*) and *ESR1* rs2234693 (*PvuII*) polymorphisms are associated with differences in the response to ovarian stimulation bestowing an indirect role that might affect implantation rates [19].

In a recent investigation, gene-array analysis revealed more than 300 genes downregulated in patients with repeated *in vitro* fertilization (IVF) failure, with at least 8% of them being estrogen dependent [20]. Numerous factors as folliculogenesis, endometrial receptivity, and oocyte maturation have been associated with failure of *in vitro* fertilization (IVF) failure, but the lack of estrogen responsiveness might be a great challenge in these situations [20]. The embryonic implantation process requires a receptive endometrium and both estrogen and TP53 present essential roles during implantation through the regulation of leukemia inhibitory factor (*LIF*), a polyfunctional glycoprotein cytokine critical for blastocyst implantation [21]. LIF expression is continuous in the uterus; however, it shows a transient expression peak during pregnancy and this peak coincides with the onset of implantation at the 12th day after fertilization in humans [22]. *LIF* has been described as an important gene in differentiation, proliferation, and cell survival pathways [23] and its expression is reduced in endometrium from women with unexplained infertility [24].

To our knowledge, no study has focused on *ESR1* polymorphisms and infertile women who were submitted to conventional *in vitro* fertilization (IVF) procedures with unsuccessful blastocyst implantations. Meanwhile estrogen functions are so important to blastocyst implantation and to the pathogenesis of endometriosis; *ESR1* gene variants might be one of the causative factors for these conditions in infertile women. We then hypothesized that genetic variants in *ESR1*, *MDM2*, *MDM4*, *USP7*, and *LIF* genes may differ between fertile women and two groups of infertile women: first, women with endometriosis-related infertility and second, women with failure of *in vitro* fertilization procedures.

2. Material and Methods

2.1. Subjects. Patients and subjects were invited to participate and signed a consent form at inclusion. The research project was approved by the Hospital de Clínicas de Porto Alegre (HCPA) Ethics Committee (GPPG 05-182; GPPG 09-430). Infertile patients with and without endometriosis and controls were divided into three study groups as previously described [25]. Infertility was defined as the inability of a couple to achieve pregnancy after 1 year of regular unprotected sexual intercourse [26]. The IVF Failure Group consisted of 115 infertile women with at least one IVF failure, submitted to conventional IVF with 35 years or less. Patients with endometriosis, previous thyroid disease, positive antilupus or anticardiolipin antibodies, and thrombophilias were excluded from our sample. Controlled ovarian hyperstimulation was performed with the use of recombinant human FSH and pituitary suppression with GnRh antagonist (fixed day-6 protocol). Ovulation was induced by 6500 IU recombinant hCG when at least three follicles had reached a diameter of 17 mm, and transvaginal follicle aspiration was performed 36 hours later under ultrasound guidance. Embryos were classified according to the cumulative embryo classification, taking into account cleavage speed, blastomere symmetry, extent of fragmentation, and the presence or absence of multinucleated blastomeres. The Endometriosis Group comprised 98 infertile women with minimal or mild endometriosis as diagnosed by laparoscopy according to the classification proposed by the American Society for Reproductive Medicine recruited at the Gynecology Service of HCPA, in Southern Brazil [26]. Other causes of infertility were excluded by hysterosalpingography, sperm evaluation, and hormonal measurements whenever necessary. The Fertile Group consisted of 134 women with no history of infertility, who already had two or more children without any difficulties or assisted reproduction and underwent laparoscopy for tubal ligation at HCPA.

2.2. Genotyping. Genomic DNA was extracted from peripheral blood leukocytes using the Illustra blood genomic Prep Mini Spin Kit (GE Healthcare, Piscataway, NJ, USA) as described by the manufacturer. DNA concentration was measured with Nano-Drop 1000 (Thermo Scientific, Wilmington, USA) and diluted to a final concentration of 10 ng/μL.

TaqMan allelic discrimination analyses were performed according to Applied Biosystems standard protocols (Applied Biosystems, Carlsbad, USA). The analyzed SNPs were as follows: *MDM4* rs1563828 (C_9493064_10), *USP7* rs1529916 (C_9688119_1), *LIF* rs929271 (C_7545901_10), *ESR1* rs9340799 (C_3163591_10), *ESR1* rs2234693 (C_3163590_10) (Applied Biosystems), and *MDM2* rs2279744 for which a custom-made TaqMan assay was made, using forward primer 5′-CGGGAGTTCAGGGTAAAGGT-3′, reverse primer 5′-ACAGGCACCTGCGATCATC-3′, VIC probe 5′-CTCCCG-CGCCGAAG-3′ and FAM probe 5′-TCCCGCGCCGCAG-3′ (Applied Biosystems). PCR cycling reactions were performed on an ABI StepOne System (Applied Biosystems) and consisted of initial denaturation at 95°C for 15 min, 40 cycles with denaturation 95°C for 15 s, and then annealing and extension at 60°C for 1 min.

2.3. Statistical Analysis. Clinical features of women in all study groups were compared by t-test. Differences in genotype distribution were assessed by chi-square analysis, which was also used to test for Hardy-Weinberg equilibrium. Logistic regression analysis was carried out to estimate the odds ratios with 95% confidence intervals (CIs) in order to assess the influence of *ESR1* rs9340799 genotypes on endometriosis-related infertility and IVF failure. Statistical analyses were performed using the SPSS 20.0 statistical package. All reported P values are two-tailed and were considered statistically significant when equal to 0.05 or less.

Table 1: Characteristics of controls and patients.

Characteristics*	Fertile $n = 134$	Endometriosis $n = 98$	P value	IVF Failure $n = 115$	P value
Age	42.68 ± 12.88	32.87 ± 4.70	$P < 0.001$	31.65 ± 3.24	$P < 0.001$
Pregnancies	3.62 ± 1.94	0.34 ± 0.92	$P < 0.001$	0.17 ± 0.51	$P < 0.001$
Spontaneous abortions	0.45 ± 1.04	0.16 ± 0.63	$P = 0.015$	0.13 ± 0.39	$P < 0.001$
Caesarean sections	0.65 ± 0.95	0.07 ± 0.33	$P < 0.001$	0 ± 0	$P = 0.005$

*Mean ± SD.

3. Results

The clinical and demographic characteristics of the women enrolled in the study are shown in Table 1. Mean age at recruitment was higher in the Fertile Group (42.6 ± 12.88 years) than in both the Endometriosis (32.87 ± 4.7 years) and IVF Failure (31.65 ± 3.24 years) groups since only women of 35 years or less were included in these two latter groups. The population-based fertile control women presented a mean of 3.62 ± 1.94 pregnancies reflecting the average number of pregnancies in the normal population from Southern Brazil. Both Endometriosis and IVF Failure groups presented low frequencies of pregnancy, abortion, and caesarean due to their infertility status. Patients and healthy study subjects did not differ significantly regarding self-attributed skin color as a self-denomination of "white" color predominated in all study groups as previously described in [25].

Hardy-Weinberg equilibrium was achieved for all SNPs in the three study groups (data not shown). Table 2 presents genotype frequencies of the SNPs included in the study. No association was found between *LIF*, *MDM2*, *MDM4*, and *USP7* SNPs and endometriosis-related infertility or *in vitro* fertilization failure. However, a strong association was found between the *ESR1* rs9340799 polymorphism and clinical phenotype in both case groups (Endometriosis, $P < 0.001$ and IVF Failure, $P = 0.018$) when compared with the Fertile Group. Interestingly, no association was found between *ESR1* rs2234693 and the outcomes.

To evaluate the effects of the *ESR1* rs9340799 polymorphism, we carried out a logistic regression analysis, controlled by age, with endometriosis-related infertility and IVF failure as outcomes. Results are summarized in Table 3 and show a statistically significant effect of AG (OR 2.67, 95% CI 1.49–4.78, $P = 0.001$) and GG (OR 4.67, 95% CI 1.84–11.83, $P = 0.001$) genotypes with endometriosis-related infertility. Regarding the IVF Failure Group, genotype GG contributed significantly to the outcome as women with genotype GG had 3-fold-increased risk of IVF failure (OR 3.33, 95% CI 1.38–8.03, $P = 0.007$).

4. Discussion

In the present study, we have analyzed common SNPs in *ESR1*, *MDM2*, *MDM4*, *USP7*, and *LIF* genes in infertile women with endometriosis or failure of *in vitro* fertilization procedures. Our results demonstrate an association between *ESR1* rs9340799 polymorphism with infertile women with endometriosis and also with women who were submitted to IVF procedures and had no embryo implantation.

TP53 regulates maternal reproduction through the expression of *LIF* [27]. At 12 days of pregnancy, *LIF* is expressed at high levels making the uterus receptive to the blastocyst [27]. Both *TP53* and estrogen are essential for *LIF* expression in the endometrial glands, and impaired function of these proteins are clearly associated with failure of blastocyst implantation [27]. Different studies have demonstrated that SNPs modulate the activity of *TP53*, and also in its regulators *MDM2*, *MDM4*, and *USP7* are more frequent in IVF patients [25, 28]. We have previously shown that *TP53* polymorphisms are associated with both endometriosis-related infertility and IVF failure in patients from Southern Brazil [25]. Using the same cohort, we expanded the analysis to other *TP53* signaling network genes [29], and in contrast with previous findings, our results demonstrated no association of *MDM2*, *MDM4*, *USP7*, and *LIF* polymorphisms with endometriosis-related infertility or IVF failure patients.

LIF is regulated by both *TP53* and estrogen. Estrogen signaling is mediated through its nuclear receptor alpha. Studies have demonstrated an association between *ESR1* polymorphisms and endometriotic women with and without infertility [13, 17], but to our knowledge, no study has evaluated *ESR1* polymorphisms in IVF failure. Our results demonstrate an association between *ESR1* rs9340799 polymorphism (also known as *ER-α Xbal*) and endometriosis-related infertility. In regard to the association found here, a previous meta-analysis performed to derive a more precise association between the *ESR1* polymorphisms and risk of endometriosis found no obvious associations [30]. However, it is important to note that even though the authors indicate that ethnicity (Caucasian or Asian), country (Japan, China, Korea, Germany, and Italy), and sample size could not explain heterogeneity across the fifteen studies included in the meta-analyses, only two studies included Caucasian populations (totalizing only 111 cases and 146 controls from a total of 1349 cases and 1411 controls). In addition, there was no uniformity in the classifications regarding "endometriosis" among the different studies. To minimize bias towards endometriosis classification, we only included in the present study infertile women with minimal or mild endometriosis as diagnosed by laparoscopy according to the classification proposed by the American Society for Reproductive Medicine [26]. The classification of endometriosis is changing from a local disorder to a complex disease as new molecular mechanisms are being

Table 2: Genotype and allele frequencies of *TP53* signaling pathway gene polymorphisms.

	Fertile *n* (%)	Endometriosis *n* (%)	*P* value*	IVF Failure *n* (%)	*P* value**
MDM2 rs2279744					
TT	57 (42.5)	41 (41.8)	0.824	48 (41.7)	0.918
TG	64 (47.8)	45 (45.9)		54 (47)	
GG	13 (9.7)	12 (12.2)		13 (11.3)	
G	0.67	0.65	0.765	0.65	0.765
T	0.33	0.35		0.35	
MDM4 rs1563828					
CC	34 (25.4)	34 (34.7)	0.268	40 (34.8)	0.141
CT	71 (53)	43 (42.9)		59 (51.3)	
TT	29 (21.6)	21 (21.4)		16 (13.9)	
C	0.52	0.57	0.477	0.6	0.254
T	0.48	0.43		0.4	
HAUSP rs1529916					
CC	73 (54.5)	53 (54.1)	0.977	53 (46.1)	0.224
CT	52 (38.8)	39 (39.8)		48 (41.7)	
TT	9 (6.7)	6 (6.1)		14 (12.2)	
C	0.74	0.74	1	0.67	0.277
T	0.26	0.26		0.33	
LIF rs929271					
TT	57 (42.5)	47 (48)	0.702	46 (40)	0.784
TG	60 (44.8)	39 (39.8)		51 (44.3)	
GG	17 (12.7)	12 (12.2)		18 (15.7)	
T	0.65	0.68	0.653	0.62	0.659
G	0.35	0.32		0.38	
ESR1 rs9340799					
AA	71 (53)	27 (27.6)	<0.001	45 (39.1)	0.018
AG	54 (40.3)	55 (56.1)		51 (44.3)	
GG	9 (6.7)	16 (16.3)		19 (16.5)	
A	0.73	0.55	0.008	0.61	0.071
G	0.27	0.45		0.39	
ESR1 rs2234693					
CC	27 (20.1)	18 (18.4)	0.861	17 (14.8)	0.105
CT	69 (51.5)	54 (55.1)		51 (44.3)	
TT	38 (28.4)	26 (26.5)		47 (40.9)	
C	0.46	0.46	1	0.37	0.196
T	0.54	0.54		0.63	

*Chi-square analysis for the difference between Fertile and Endometriosis groups. **Chi-square analysis for the difference between Fertile and IVF Failure groups.

discovered [2]. The endometriotic process is classified as an estrogen-dependent inflammatory disease similar to cancer due to its capability to invade surrounding tissues, to promote angiogenesis, inflammation, and apoptosis in favor of the new endometriotic tissue survival [31–36]. Estrogen production plays a central role in the pathology of endometriosis enhancing the survival of the endometriotic tissue, and together with prostaglandins and cytokines, mediating pelvic pain and infertility [37, 38]. The fact that estrogen inhibitors such as GnRh analogues, oral, and aromatase inhibitors are used to

Table 3: Logistic regression model for *ESR1* rs9340799 using the Fertile group as reference.

ESR1 rs9340799	OR (95% CI)	*P* value
Endometriosis		
AA	—	—
AG	2.67 (1.49–4.78)	0.001
GG	4.67 (1.84–11.83)	0.001
IVF Failure		
AA	—	—
AG	1.49 (0.87–2.54)	0.114
GG	3.33 (1.38–8.03)	0.007

OR (95% CI) was calculated by binary logistic regression analysis. IVF Failure: women with recurrent failure of IVF; Endometriosis: infertile women with minimal or mild endometriosis.

reduce pelvic disease and pain also corroborates conceptive to the fact that estrogen signaling is critical for endometriosis [39].

Estrogen receptor polymorphisms have been associated with ovarian response to follicle stimulating hormone in IVF patients [40], with poor responders to IVF [41], with IVF parameters such as the number of follicles and collected oocytes, maturation, pregnancy rates, and embryo quality in women with unexplained infertility [42] and with the outcome of ovarian stimulation in IVF [43]. This is the first time that an association between *ESR1* rs9340799 and failure of IVF is demonstrated. Remarkably, we did not find any association between *ESR1* rs2234693 polymorphism (also known as *PvuII*) and endometriosis-related infertility or failure of IVF. Both rs9340799 (A-351G) and rs2234693 (C-397T) SNPs are localized in intron 1 of the *ESR1* gene in chromosome 6q25 and are in linkage disequilibrium [42]. Although both SNPs are present in intron 1 and do not lead to any amino acid change, it is plausible that they may directly influence *ESR1* gene expression or alternatively could be linked to some unidentified causative DNA sequence variants. Introns can significantly affect gene expression in a variety of ways, as they may contain enhancer elements or promoters that might control alternative splicing, as well as various *cis*- and *trans*-regulatory elements that may lead to different proteins isoforms [44–46].

Although, to our knowledge, this is the first study to report an association between *ESR1* genetic variants and failure of *in vitro* fertilization, our study had limitations. First, only two common *ESR1* polymorphisms were investigated, so haplotype analysis was not performed. Second, examination of endometrial tissue to evaluate the effect of the analyzed SNPs regarding *TP53* and *LIF* expression was not performed. Endometrial samples are being collected at this time, so analyses of protein response at the implantation stage are underway. Lastly, it is known that allele frequencies are greatly affected by racial and ethnic backgrounds. Although ancestral informative markers were not used to infer individual ancestry, we used self-reported skin color as a control for ethnic background, and no significant difference in the distribution of self-denominated skin color was observed among the study groups as the majority of individuals self-denominated them as "white."

5. Conclusion

Our results reveal a potential novel candidate biomarker for the diagnostic and prognostic assessment of endometriosis-related infertility and IVF failure. Our results demonstrate a 4-fold increased risk of endometriosis and a 3-fold increased risk of IVF failure in infertile women with *ESR1* rs9340799 GG genotype. Further studies exploring a haplotype analysis of the *ESR1* gene will help to clarify the role of *ESR1* genetic variants in infertile women. Along with that, functional studies are needed to elucidate the possible effect that *ESR1* rs9340799 might have on ESR1 and LIF expression.

Conflict of Interests

The authors report that they have no conflict of interests.

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