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ASSESSING THE ASSOCIATION OF *ESR1* POLYMORPHISMS WITH MALE INFERTILITY IN VIETNAMESE POPULATION

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SUMMARY

Male infertility is a prevalent global concern, impacting an estimated 7% of the male population. Many single nucleotide polymorphisms (SNPs) linked to idiopathic male infertility have been discovered since the 1990s, including two intronic SNPs of the estrogen receptor α gene (*ESR1*), namely rs9340799 and rs2234693. However, the associations of the two SNPs with male infertility have yielded inconsistent results across different populations. In this study, we aimed to assess the association of *ESR1* rs9340799 and *ESR1* rs2234693 with male infertility in a cohort of Vietnamese individuals consisting of 154 fertile men and 146 infertile men. Genotyping *ESR1* rs9340799 and *ESR1* rs2234693 for 300 samples was performed using polymerase chain reaction-restriction fragment length polymorphism (PCR- RFLP). The frequencies of genotypes and alleles were analyzed using bioinformatic tools. The genotype distributions of *ESR1* rs9340799 and *ESR1* rs2234693 followed Hardy-Weinberg equilibrium, but no association of the two SNPs with male infertility was established ($p > 0.05$) in the additive, dominant, recessive, and allele models. Notably, the haplotype analysis revealed a strong association between the haplotype GT and a decreased risk of male infertility (OR = 0.226; 95% CI = 0.106 - 0.484; $p < 0.001$). The findings provide additional knowledge about the roles of single nucleotide polymorphisms in male infertility in the Vietnamese population.

Keywords: Estrogen receptor α, haplotype, idiopathic male infertility, PCR-RFLP, rs9340799, rs2234693

INTRODUCTION

Infertility, as per the World Health Organization's (WHO) definition, refers to a medical condition characterized by the inability to achieve a clinical pregnancy following a period of 12 months of regular, unprotected sexual intercourse (Zegers-Hochschild *et al.*, 2017). It has been estimated that 8-12% of couples worldwide are affected by infertility, and male infertility contributes solely to roughly 30% of the cases, however, this figure varies worldwide (Vander Borght, Wyns, 2018). Additionally, Sun and colleagues documented a global increase in the age-standardized prevalence of male infertility at a rate of 0.291% per year throughout the period spanning from 1990 to

2017 (Sun *et al.*, 2019). Particularly, in Asia, the infertility rate among men reached 37% in 2015 (Agarwal *et al.*, 2015). As the etiology of approximately 15% of male infertility cases is related to genetic abnormalities, they are perceived as potential risk factors that require further investigation (Krausz, Riera-Escamilla, 2018). Thus, numerous genes associated with male infertility have been discovered by utilizing advanced sequencing technologies (Xavier *et al.*, 2021). Among them, the *ESR1* gene has been investigated in several case-control studies (Ge *et al.*, 2014; Kukuvitis *et al.*, 2002; Safarinejad *et al.*, 2010; Zalata *et al.*, 2014).

ESR1 is located on chromosome 6q25 and consists of eight exons and seven introns, with an overall length of 140 kb (Ponglikitmongkol *et al.*, 1988). It encodes for the estrogen receptor α (ERα), which is responsible for mediating physiological responses to estrogen. In recent years, there has been clear evidence that this estrogen receptor is present in the male testis (Carreau, Hess, 2010). ERα has been reported to be present in Leydig cells and efferent ductule epithelium across a wide range of species (Hess, 2002). Expression of ERα has also been found in Sertoli cells and several rat germ cells (Lucas *et al.*, 2008). Moreover, multiple studies have pointed out the presence of both mRNA and the product of the *ESR1* gene in spermatozoa (Durkee *et al.*, 1998) and in human ejaculate (Lambard *et al.*, 2004), suggesting a potential role of ERα in spermatogenesis and sperm functionality. ERα is found at the highest concentration in the efferent ductule epithelium, a region with the main function of reabsorbing luminal fluid as well as increasing the concentration of sperm (Hess, 2002).

Multiple polymorphisms have been identified within the *ESR1* gene (Keene *et al.*, 2008). Two polymorphisms, rs9340799 and rs2234693, are among the most widely studied *ESR1* SNPs in association with male infertility (Ge *et al.*, 2014). Both SNPs are located at intron 1 of the *ESR1*gene. *ESR1* rs9340799 polymorphism is caused by an A/G transition, while *ESR1* rs2234693 is located just 46 bp upstream and driven by a T/C transition. The GG genotype of *ESR1* rs9340799 and CC genotype of *ESR1* rs2234693 were correlated with lower sperm count, sperm concentration, and percentage of normal sperm morphology (Zalata *et al.*, 2014; Lazaros *et al.*, 2010). Concerning the risk of male infertility, the G allele and C allele were more frequent in the patient group (Kukuvitis *et al.*, 2002; Zalata *et al.*, 2014); however, other studies have produced inconsistent results (Bianco *et al.*, 2011; Lazaros *et al.*, 2010; Meng *et al.*, 2013; Safarinejad *et al.*, 2010). Therefore, this study aims to conduct a case-control association study of *ESR1* rs9340799 and *ESR1* rs234693 with male infertility in the Vietnamese population.

MATERIALS AND METHODS

Study subjects

The study consisted of 300 Vietnamese participants, including 154 controls and 146 infertile men. Patients were individuals who had non-obstructive azoospermia or oligospermia while having normal karyotypes and no anomalies in the AZF region. The exclusion criteria for affected men include the usage of male hormone-affecting medication, congenital absence of the testes, varicocele, history of mumps infection, testicular trauma, ejaculatory duct obstruction, and presence of sexually

transmitted infections (STIs). Controls with normal spermiograms and having at least one child without using assisted reproductive technology were accepted into the study. The research was approved by the Institutional Review Board of the Institute of Genome Research, Vietnam Academy of Science and Technology. All participants gave informed consent to be involved in the study. Blood samples collected from participants above (2 mL) were stored in EDTA tubes at -20°C.

SNP genotyping

Total DNA was extracted from blood samples using the Exgene Whole Blood DNA Extraction Kit (GeneAll, Korea) following the manufacturer's instruction. The extracted DNA was subsequently diluted to a concentration of approximately 10 ng/µL for PCR amplification. A pair of primers was custom-designed to amplify the DNA region containing both polymorphisms *ESR1* rs9340799 and *ESR1* rs2234693 (Table 1) using the Primer-BLAST tool (NCBI). To avoid dimerization, both primers were evaluated by the OligoAnalyzer tool (https://sg.idtdna.com/pages/tools/oligoanalyzer). The total volume of the PCR reaction was 10 µL, containing the following reagents: 6.25 µL of nuclease-free water; 1 µL of DreamTaq buffer (10X); 0.6 µL of dNTPs (2.5 mM); 0.2 µL each of forward and reverse primer (10 pmol); 0.05 µL DreamTaq DNA polymerase (5 U/µL) and 1.7 µL of DNA template (10 ng/µL). The optimized PCR program consists of a denaturation step at 95°C for 4 minutes, followed by 35 cycles of 95°C for 40 seconds, 56°C for 30 seconds, 72°C for 40 seconds, and an extension step at 72°C for 5 minutes. Two enzymes were used for DNA genotyping by RFLP, including *Xba*I (*ESR1* rs9340799) and *Pvu*II (*ESR1* rs2234693). The DNA samples were digested with each enzyme separately for 4 hours at 37°C. Afterward, the digested products were separated then visualized on agarose gel. Based on the DNA fragments shown on agarose gels, the genotypes of the study subjects were then determined, using Table 1 as a reference.

Table 1. Genotyping information of *ESR1* rs9340799 and *ESR1* rs2234693.

Polymorphism	Primers (5' - 3')	PCR product (bp)	Genotype	Fragments(bp)
ESR1 rs9340799 A>G (*Xba*I)	Forward: CCAGGGTTATGTGGCAATGAC Reverse: CACAGTAGCGAGTCTCCTTG	600	AA	143; 457
			AG	143; 457; 600
			GG	600
ESR1 rs2234693 T>C (*Pvu*II)			TT	98; 502
			TC	98; 502; 600
			CC	600

Statistical analysis

Data retrieved from PCR-RFLP results were analyzed using R programming software version 4.2.3 (https://www.r-project.org) and Microsoft Excel (Microsoft Corp., USA). The Chi-square test of the package "HardyWeinberg" was used to

determine whether the genotype frequencies of the population followed Hardy-Weinberg equilibrium (Graffelman, 2015). The R package "epitools" (https://CRAN.R-project.org/package=epitools) was used to calculate the odds ratio and its 95% confidence interval to examine the relationship of each SNP with male infertility under three models: additive, dominant, and recessive. Haplotype analysis was performed using the SHEsis tool (Li *et al.*, 2009). All statistical tests were two-sided, and a p-value less than 0.05 was considered statistically significant.

RESULTS

Genetic analysis of *ESR1* polymorphisms

The region containing the SNPs of interest was successfully amplified with the designed primers and visualized on 1% agarose gel. The bands were bright and sharp, with no non-specific bands, and an expected molecular size of 600 bp (data not shown). The PCR products were then digested with restriction enzymes to obtain the genotypes. Ten representative samples (1-10) were shown (Figure 1).

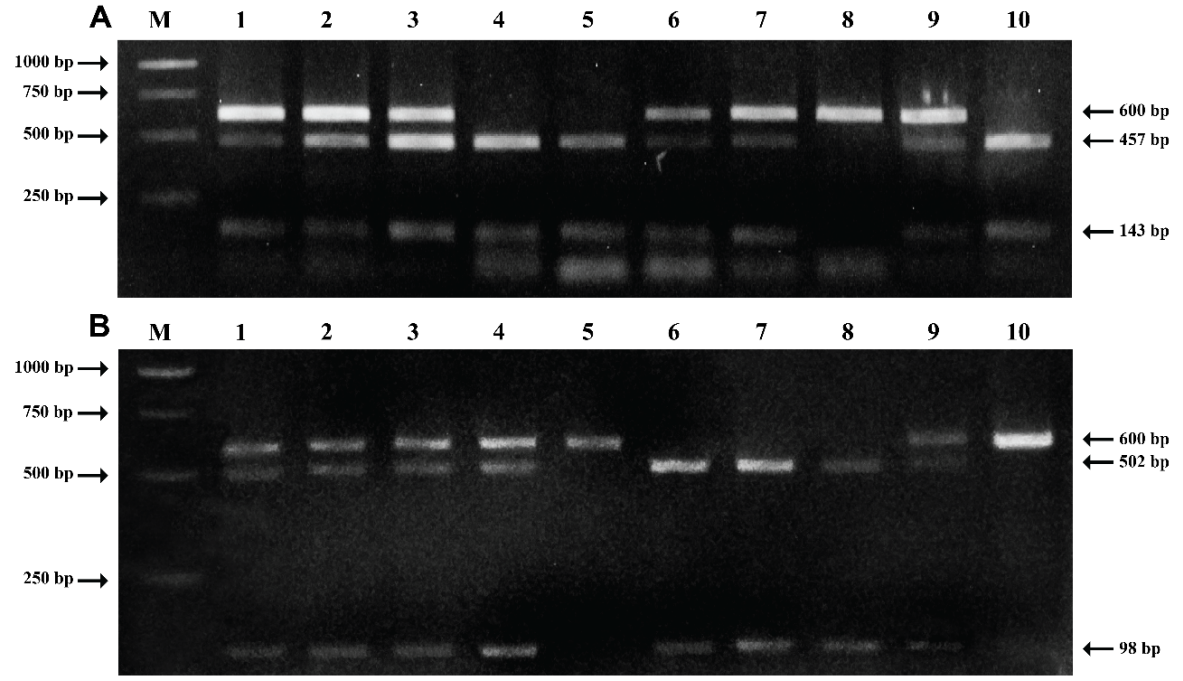

Figure 1. Analysis of digested PCR products by electrophoresis on agarose gels. (A) XbaI-digested products on 1.5% agarose gel. M: Marker 1kb; 4, 5, 10: Homozygous samples AA (2 bands of 457 bp and 143 bp); 1-3, 6, 7, 9: Heterozygous samples AG (3 bands of 600 bp, 457 bp, and 143 bp); 8: Homozygous samples GG (1 band of 600 bp) (B) PvuII-digested products on 2.5% agarose gel. M: Marker 1kb; 6-8: Homozygous samples TT (2 bands of 502 bp and 98 bp); 1-4, 9: Heterozygous samples TC (3 bands of 600 bp, 502 bp, and 98 bp); 5, 10: Homozygous samples CC (1 band of 600 bp).

Both polymorphisms of the *ESR1* gene were genotyped for all 300 subjects. The genotype distributions of *ESR1* rs9340799 and *ESR1* rs2234693 (Table 2) in the case, control, and whole population all followed Hardy-Weinberg equilibrium ($p > 0.05$).

Table 2. Genotype frequencies of *ESR1* rs9340799 and *ESR1* rs2234693.

SNP	Group	Genotypes			Allele frequencies		HWE (*p*-value)
		AA	**AG**	**GG**	**A**	**G**	
rs9340799	Control (n=154)	84	53	17	0.718	0.282	0.061
	Case (n=146)	82	55	9	0.75	0.25	0.956
	Total (n=300)	166	108	26	0.733	0.267	0.168
		TT	**TC**	**CC**	**T**	**C**	
rs2234693	Control (n=154)	65	67	22	0.640	0.360	0.485
	Case (n=146)	49	72	25	0.582	0.418	0.869
	Total (n=300)	114	139	47	0.612	0.388	0.669

Note: HWE: Hardy-Weinberg equilibrium; n: number of participants.

Table 3. Association of *ESR1* rs9340799 and *ESR1* rs2234693 with male infertility.

SNP	Test model	Control (*n*=154)	Case (*n*=146)	OR	95% CI	*p*-value
rs9340799	**Additive**					
	AA	84 (54.6%)	82 (56.1%)	1.000		
	AG	53 (34.4%)	55 (37.7%)	1.063	0.653-1.730	0.805
	GG	17 (11.0%)	9 (6.2%)	0.548	0.220-1.283	0.160
	Dominant					
	AA	84 (54.6%)	82 (56.1%)	1.000		
	AG+GG	70 (45.4%)	64 (43.9%)	0.937	0.593-1.479	0.778
	Recessive					
	AA+AG	137 (89.0%)	137 (93.8%)	1.000		
	GG	17 (11.0%)	9 (6.2%)	0.535	0.219-1.224	0.134
	Allele					
	A	221 (71.8%)	219 (75.0%)	1.000		
	G	87 (28.2%)	73 (25.0%)	0.847	0.588-1.218	0.369
rs2234693	**Additive**					
	TT	65 (42.2%)	49 (33.6%)	1.000		

TC	67 (43.5%)	72 (49.3%)	1.423	0.864-2.351	0.162
CC	22 (14.3%)	25 (17.1%)	1.502	0.757-3.003	0.237
Dominant					
TT	65(42.2%)	49 (33.6%)	1.000		
TC+CC	89 (57.8%)	97 (66.4%)	1.443	0.903-2.317	0.123
Recessive					
TT+TC	132 (85.7%)	121 (82.9%)	1.000		
CC	22 (14.3%)	25 (17.1%)	1.238	0.661-2.332	0.499
Allele					
T	197 (64.0%)	170 (58.2%)	1.000		
C	111 (36.0%)	122 (41.8%)	1.273	0.916-1.771	0.149

Note: n: number; OR: odds ratio; 95% C: 95% confidence interval; *p*-value was calculated using Chi-squared test.

Table 4. Haplotype analysis of *ESR1* rs9340799 and *ESR1*rs2234693.

Haplotype	Control (Frequency)	Case (Frequency)	OR	95% CI	*p*-value
AT	0.521	0.553	1.000		
AC	0.197	0.197	1.004	0.672 - 1.502	0.983
GC	0.164	0.220	1.445	0.959-2.176	0.077
GT	0.119	0.030	0.226	0.106-0.484	< 0.001

Note: n: number; OR: odds ratio; 95% C: 95% confidence interval; *p*-value was calculated using Chi-squared test.

Associations of *ESR1* polymorphisms with male infertility

To investigate the associations between *ESR1* polymorphisms with male infertility, statistical analyses were performed with each SNP under three test models: additive, dominant, and recessive (Table 3). There was no significant difference observed in the genotype distribution of both polymorphisms and *p*-values were higher than 0.05 in all models, indicating that *ESR1* rs9340799 and *ESR1* rs2234693 were not associated with male infertility in the studied population.

ESR1 polymorphisms haplotype analysis

Two SNPs, *ESR1* rs9340799 and *ESR1* rs2234693, are closely located and are in

linkage disequilibrium, thus, we performed haplotype analysis (Table 4). Amongst the three haplotypes containing at least one minor allele, the GT haplotype showed a significantly lower frequency in the case group (0.030) compared to the control group (0.119). Using the AT haplotype as the reference, the GT haplotype exhibits a strong association with a decreased risk of male infertility (OR = 0.226; 95% CI = 0.106 - 0.484; $p < 0.001$).

DISCUSSION

Although estrogen was initially perceived as the "female hormone" and caused abnormalities in male fertility, an extensive research provided evidence that the hormone, along with its receptors, plays an important role in male fertility and reproductive function (Hess, Cooke, 2018). To understand the complex role of estrogen, various ER knockout mouse models have been recruited. In general, male mice lacking the *ESR1* gene were infertile. They showed increased testicular weight, dilation of the seminiferous tubules, luminal dilation and dysmorphogenesis of the efferent ductules, and decreased sperm motility (Antonson *et al.*, 2012). Hence, the effect of *ESR1* polymorphisms on male infertility has been investigated in recent years. In particular, the rs9340799 and rs2234693 variants were the most commonly examined SNPs (Bianco *et al.*, 2011; Kukuvitis *et al.*, 2002; Lazaros *et al.*, 2010; Meng *et al.*, 2013; Safarinejad *et al.*, 2010; Zalata *et al.*, 2014). However, the results of the associations of *ESR1* rs9340799 and *ESR1* rs2234693 with male infertility were discordant.

The G allele of *ESR1* rs9340799 was associated with a higher risk of male infertility in Greek and Egyptian populations ($p < 0.04$) (Kukuvitis *et al.*, 2002; Zalata *et al.*, 2014). Whereas, in the Iranian and Chinese populations, the minor allele was significantly less frequent in the infertile group ($p = 0.032$, $p = 0.003$, respectively), suggesting a protective effect against infertility in men (Meng *et al.*, 2013; Safarinejad *et al.*, 2010). In contrast, other studies showed minimal discrepancy in genotype frequencies between the control and case groups (Bianco *et al.*, 2011; Lazaros *et al.*, 2010). In agreement with these results, there was no significant association between *ESR1* rs9340799 and male infertility in the Vietnamese population ($p > 0.05$). Zalata and colleagues reported that there was a statistically significant association ($p = 0.05$) between the presence of the C allele of *ESR1* rs2234693 and an increased susceptibility to male infertility in the Egyptian population (Zalata *et al.*, 2014). According to Safarinejad, the presence of the CC genotype in the Iranian population was linked to a reduced likelihood of male infertility ($p = 0.011$) (Safarinejad *et al.*, 2010). In contrast, the Brazilian, Chinese, and Greek groups did not show an association between *ESR1* rs2234693 and male infertility (Bianco *et al.*, 2011; Kukuvitis *et al.*, 2002; Lazaros *et al.*, 2010; Meng *et al.*, 2013). Similarly, the present study showed no significant association of *ESR1* rs2234693 with male infertility. It is possible that the differences in the relationship of both SNPs with male infertility in each population could be the results of not only genetic but also environmental and lifestyle factors of each ethnicity.

The results of our haplotype analysis on *ESR1* rs9340799 and *ESR1* rs2234693 indicated a significant correlation between the GT haplotype and a reduced likelihood of

male infertility (OR = 0.226; 95% CI = 0.106 - 0.484; $p < 0.001$). The haplotype under investigation was also examined in the populations of Iran and Brazil; yet no statistically significant correlation was found in either community (Bianco *et al.*, 2011; Safarinejad *et al.*, 2010). Regarding the other haplotypes, it was found that the GC haplotype had a protective association in the Iranian population (OR = 0.49; 95% CI: 0.28 - 0.72; $p = 0.003$), whereas the AC haplotype was associated with an increased risk of male infertility (OR = 1.82; 95% CI: 1.28 - 2.67; $p = 0.032$) (Safarinejad *et al.*, 2010). In contrast, the Brazilian group under investigation exhibited no statistically significant variations in the frequencies of AC, and GC haplotypes between the control and case groups (Bianco *et al.*, 2011). Previous *in silico* analysis determined that although the splicing of *ESR1* mRNA could be slightly altered by these two polymorphisms, there were no possible cryptic splice sites derived from them (Salimi *et al.*, 2018). Despite the lack of a clear explanation for the effect of these SNPs, there is evidence that the enhancer activity differs across *ESR1* haplotypes (Maruyama *et al.*, 2000). Furthermore, these *ESR1* polymorphisms maybe in linkage disequilibrium with other SNPs that affect the function of ERα, or with common structure variants (Frazer *et al.*, 2009). Based on the available information, our findings suggest evidence of the association between the haplotype of *ESR1* rs9340799 and *ESR1* rs2234693 with the susceptibility of male infertility in the Vietnamese population.

CONCLUSION

This study investigated the association of the polymorphisms *ESR1* rs9340799 and *ESR1* rs2234693 with the risk of male infertility in the Vietnamese population. The genotype frequencies of both SNPs in the case, control, and whole group followed Hardy-Weinberg equilibrium; however, statistical analysis using the additive, dominant, recessive, and allele models shows no significant association of the SNPs with male infertility. A significant association between the GT haplotype and a decreased risk of male infertility was discovered. This study is the first to examine the relationship between *ESR1* polymorphisms and male infertility in Vietnam. Thus, it provides additional knowledge about the roles of single nucleotide polymorphisms in Vietnamese infertile men.

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