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ASSOCIATION OF *FSIP2* rs4666689 AND *PON2* rs7493 WITH MALE INFERTILITY IN VIETNAMESE POPULATION

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ABSTRACT

Reproductive impairment in men is a multifactorial disease and is currently considered a global health issue. Previous studies have investigated the correlation between genetic variants and male infertility in different populations. However, such studies have appeared in limited amounts in the Vietnamese population. This study aimed to assess the association of polymorphisms *FSIP2* rs4666689 and *PON2* rs7493 with male infertile susceptibility in the Vietnamese population. Total DNAs were isolated from 376 samples, including 175 males with infertility and 201 controls having at least one child. For *FSIP2* rs4666689, all 376 samples were applied for genotyping using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). For *PON2* rs7493, only 178 samples (80 infertile patients and 98 controls) were used to assess genotype frequencies. By using statistical methods, we showed that the distribution of their genotypes was in accordance with Hardy-Weinberg equilibrium (p-values > 0.05). However, no association between both polymorphisms (*FSIP2* rs4666689 and *PON2* rs7493) and male infertility in the Vietnamese population was detected (p-values > 0.05). This study would help enrich to the knowledge about the effects of hereditary factors on male infertility in the Vietnamese population.

Keywords: Male infertility, *FSIP2*, *PON2*, Vietnam, PCR-RFLP.

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INTRODUCTION

Infertility is a disease featured by the incapability to achieve a clinical pregnancy after at least 12 months of unprotected sexual intercourse or due to the impairment in the reproductive system of either partner or both (Zegers-Hochschild et al., 2017). Male reproductive problems account for about half of the infertile cases, and its incidence was reported to be 7% in the world population (Krausz & Riera-Escamilla, 2018). A multitude of etiologies, including both genetic and non-genetic factors, result in reproductive diseases in men. However, determining the genetic causes of male infertility is challenging due to more than 2,000 genes participating in spermatogenic processes (Krausz & Riera-Escamilla, 2018). Additionally, numerous genes involved in the apoptotic process, DNA repair, foreign chemicals detoxification, and reactive oxygen species response had been suggested to be associated with male infertility, including fibrous sheath interacting protein (*FSIP2*) and paraoxonase 2 (*PON2*).

FSIP2, located on 2q32.1, containing 23 exons and spanning over 94 kb encodes a protein associated with the sperm fibrous sheath and specific to spermatogenic cells (Martinez et al., 2018). *FSIP2* is strongly expressed in the testis, particularly in the cytoplasm of primary germ cells and flagella of spermatids during the spermiogenesis (Brown et al., 2003; M. Liu et al., 2021; Martinez et al., 2018). Mutations in *FSIP2* were reported in male infertile patients, particularly in multiple morphological abnormalities of the sperm flagella (MMAF), a type of infertility in men (W. Liu et al., 2019; Manco et al., 2021).

PON2, located on 7q21.3, containing 9 exons and spanning over 30 kb encodes an enzyme member of the paraoxonase (PON) family including three known members (*PON1*, 2 and 3) located adjacent to each other. *PON2* is the oldest family member, and the other two evolved from it with 70% sequence identity (Manco et al., 2021). It is an intracellular enzyme localized in the endoplasmic reticulum (ER) (Horke et al., 2007) and mitochondria (Devarajan et al., 2011). *PON2* is ubiquitously expressed in the heart, lung, placenta, and testis (Ng et al., 2001). The protein may act as a cellular antioxidant enzyme and plays a role in defense responses to pathogenic bacteria by calcium-dependent hydrolytic activity (Manco et al., 2021). Polymorphisms in *PON2*, especially *PON2* rs7493, have been demonstrated to be associated with numerous diseases such as ischemic stroke (Rodríguez-Esparragón et al., 2017), hemorrhage (Park et al., 2015), hearing loss (Li et al., 2013), and coronary heart disease (Sanghera et al., 1998). This polymorphism was also studied for its correlation with male infertility in the Greek and Slovenia populations, but the results were inconsistent (Lazaros et al., 2011; Volk et al., 2011).

The role of genetic variants of *FSIP2* and *PON2* in reproductive deterioration in males was proposed in various populations with inconsistent outcomes due to different genetic backgrounds. Therefore, to understand whether the polymorphisms in *FSIP2* and *PON2* have a potential correlation with male infertility in Vietnamese individuals, we conducted a case-control study of *FSIP2* rs4666689 and *PON2* rs7493 in the Vietnamese population.

MATERIALS AND METHODS

Study participants and collection of blood samples

A patient group of 175 infertile patients, including idiopathic non-obstructive azoospermia (NOA) and oligospermia (< 15 million sperms/ml) men, were recruited from several hospitals of northern Vietnam. Patients with abnormal karyotype, azoospermia factor (AZF) region disorders and medical history of diseases affecting infertility (mumps, sexually transmitted diseases, and drug addiction) were excluded from the study. The control group includes 201 healthy men who had fathered at least one child without seeking assisted reproductive technology (ART). All participants that met

the requirements above gave informed consent for the blood collection. For *FSIP2* rs4666689, all 376 participants were included in the case-control study. For *PON2* rs7493, only 178 samples (80 cases and 98 controls) were employed. The study was approved by the Institutional Review Board of the Institute of Genome Research, Vietnam Academy of Science and Technology. Blood samples (2 ml) were collected from the patients in EDTA-coated tubes and subsequently stored at -20 ^{o}C.

Methods

SNP genotyping

Genomic DNA was extracted from whole blood samples of participants using Gene JET Whole Blood Genomic DNA Purification Kit (Thermo Fisher). For quality control, genomic DNA was measured by both electrophoresis and spectrophotometry. DNA samples were then diluted from initial concentration to the required concentration (~2.5 ng/μL) and stored at -20 ^{o}C. To genotype polymorphisms, *FSIP2* rs4666689 and *PON2* rs7493, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was employed using specific pairs of primers (Table 1). The primers were designed by Primer blast and checked for dimerization on the IDT website (https://www.idtdna.com/pages). After that, the PCR products were digested with restriction enzymes (RE) *Eco*RI and *Dde*I to determine the genotypes of *FSIP2* rs4666689 and *PON2* rs7493, respectively (Table 1).

Table 1. List of primers used for polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) amplification

SNP/Gene	Primer sequence	PCR product (bp)	PCR-RFLP	
			Genotype	Fragment size
rs4666689/ *FSIP2*	F:5'-ACAATGCAAGGGAAAGCTCA-3' R:5'-AACAGACTGTGCCGAATCCC-3'	350	TT	240, 106
			TA	350, 240 106
			AA	350
rs7493/ *PON2*	F:5'-CCTGTGTTGGCATGGAATAACT-3' R:5'-GGCTCTGTGGTATAAAGTGCCT-3'	359	GG	245, 114
			GC	359, 245 114
			CC	359

Note: SNP: Single nucleotide polymorphism.

Statistical analysis

Data obtained from the above methods were statistically analyzed using Microsoft Excel (Microsoft Corp., Washington, DC, USA) and R version 4.0.3 (R Core Team, 2020). Hardy-Weinberg equilibrium (HWE) of the population was calculated using the Chi-square test ($\chi 2$) of package "Hardy Weinberg" (Graffelman, 2015). Additionally, package "epitools" (Aragon, 2020) was used to assess the correlation between polymorphisms with male infertility in 3 test models: additive, dominant, and recessive. An odds ratio with a confidence interval of 95% was calculated to estimate the association. All the statistical tests were two-sided. The estimation was considered to be statistically significant if p-value < 0.05.

RESULTS

Genetic analysis of *FSIP2* and *PON2* polymorphisms

The targeted DNA regions of *FSIP2* rs4666689 and *PON2* rs7493 were amplified using specific pairs of primers. The results in agarose gel 1.5% showed specific, sharp, and bright DNA bands with the correct molecular weight. After that, PCR products were digested with restriction enzymes *Eco*RI and *Dde*I to determine the genotypes of *FSIP2* rs4666689 and *PON2* rs7493, respectively (Fig. 1).

Six representative *Eco*RI-digested products (1–6) of *FSIP2* rs4666689 (Fig. 1A) indicated that genotype of sample 1 was heterozygous (TA), samples 2–5 were

homozygous (AA), and sample 6 was wildtype (TT). A total of 376 samples (175 cases and 201 controls) were genotyped for polymorphism *FSIP2* rs4666689. The minor allele frequencies in the cases, controls, and the studied population were 0.103, 0.100, and 0.101, respectively (Table 2). Furthermore, using the Chi-square test, the distribution of polymorphism *FSIP2* rs4666689 followed Hardy-Weinberg equilibrium in the cases, controls, and the studied population (*p*-values > 0.05).

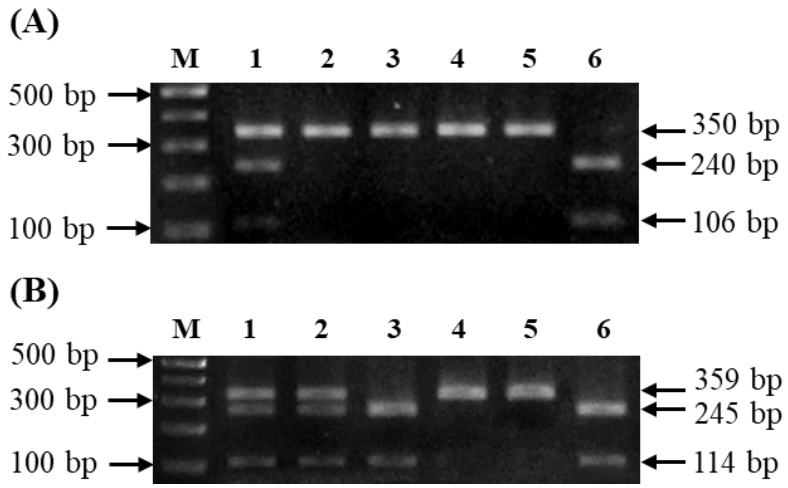

Figure 1. Restriction enzyme-digested PCR products on agarose gel 1.5%. M: Marker 100 bp. **(A)** *Eco*RI-digested PCR products of *FSIP2* rs4666689. 1: Heterozygous TA (3 bands of 350 bp, 240 bp, and 106 bp), 2 - 5: Homozygous AA (1 band of 350 bp), 6: Wildtype TT (2 bands of 240 bp, and 106 bp). **(B)** *Dde*I-digested PCR products of *PON2* rs7493. 1-2: Heterozygous GC (3 bands of 359 bp, 245 bp, and 114 bp), 3 and 6: Wildtype GG (2 bands of 245 bp, and 114 bp), 4-5: Homozygous CC (1 band with 359 bp)

For *PON2* rs7493, six representative samples (1–6) digested with restriction enzyme *Dde*I showed wildtype GG presented in samples 3 and 6, heterozygous GC in samples 1 and 2, and homozygous CC in samples 4 and 5. A total of 178 samples (80 cases and 98 controls) were genotyped for the polymorphism. The minor allele frequencies were summarized in Table 2. Allele C (minor allele) of *PON2* rs7493 appeared in the whole population with a frequency of 0.166. However, no discrepancy was detected between the case (MAF = 0.175) and control group (MAF = 0.158) of the polymorphism. The distribution of polymorphism *PON2* rs7493 was in accordance with Hardy-Weinberg equilibrium in the cases, controls, and the whole population (*p*-values > 0.05).

Table 2. General information on the studied single nucleotide polymorphisms

SNP/Gene	Alleles	MAF in case group	HWE in case group	MAF in control group	HWE in control group	MAF in the whole population	HWE in the whole population
rs4666689/ *FSIP2*	T > A	0.103	0.129	0.100	0.114	0.101	0.928
rs7493/ *PON2*	G > C	0.175	0.727	0.158	0.271	0.166	0.306

Note: SNP: Single nucleotide polymorphism; MAF: Minor allele frequency; HWE: Hardy-Weinberg equilibrium.

Association of *FSIP2* rs4666689 and *PON2* rs7493 with male infertility

To understand more about the correlation of both polymorphisms with the diseases, we performed statistical analysis in 3 test models: additive, dominant, and recessive, and alleles (Table 3). For polymorphism *FSIP2* rs4666689, *p*-values obtained from analysis of the correlation between the identified genotypes with male infertility in 3 models (additive, dominant, recessive) and alleles were 0.096, 0.139, 0.513, and 0.879, respectively, which were higher than 0.05, therefore no significant difference was detected.

Table 3. Association of *FSIP2* rs4666689 with male infertility

SNP/Gene	Test model	Cases (n = 175)	Controls (n = 201)	OR	95% CI	*p*-value
rs4666689/ *FSIP2*	Additive					0.096
	TT	0 (0.00%)	4 (1.99%)	1.000		
	TA	36 (20.57%)	32 (15.92%)	0.200	0.007 - 1.396	0.089
	AA	139 (79.43%)	165 (82.09%)	0.264	0.010 - 1.744	0.156
	Recessive					
	TT	0 (0.00%)	4 (1.99%)	1.000		
	TA + AA	175 (100.00%)	197 (98.01%)	0.250	0.009 - 1.649	0.139
	Dominant					
	TT + TA	36 (20.57%)	36 (17.91%)	1.000		
	AA	139 (79.43%)	165 (82.09%)	1.186	0.707 - 1.991	0.513
	Allele					
	T	36 (10.28%)	40 (9.95%)	1.000		
	A	314 (89.72%)	362 (90.05%)	1.038	0.642 - 1.671	0.879

Note: n: Number of participants; OR: Odds ratio; 95% CI: 95% confidence interval of odds ratio; *p*-value measured using Chi-square test.

Table 4. Association of *PON2* rs7493 with male infertility

SNP/Gene	Test model	Case (n = 80)	Control (n = 98)	OR	95% CI	*p*-value
rs7493/ *PON2*	Additive					0.742
	GG	54 (67.50%)	68 (69.39%)	1.000		
	GC	24 (30.00%)	29 (29.59%)	0.959	0.500–1.849	0.901
	CC	2 (2.50%)	1 (1.02%)	0.425	0.013–5.375	0.441
	Dominant					
	GG	54 (67.50%)	68 (69.39%)	1.000		
	GC + CC	26 (32.50%)	30 (30.61%)	0.916	0.484–1.741	0.787
	Recessive					
	GG + GC	78 (97.50%)	97 (98.98%)	1.000		
	CC	2 (2.50%)	1 (1.02%)	0.429	0.014−5.395	0.446
	Allele					
	G	132 (82.5%)	165 (84.18%)	1.000		
	C	28 (17.5%)	31 (15.82%)	0.886	0.504−1.560	0.671

Note: n: Number of participants; OR: Odds ratio; 95% CI: 95% confidence interval of odds ratio; *p*-value measured by Chi-square test.

For polymorphism *PON2* rs7493, with *p*-values of 3 models and allele forms higher than 0.05, we concluded that no statistical significance was obtained when comparing genotypes and alleles of polymorphism in the case and control groups (Table 4).

In conclusion, genotypes of *FSIP2* rs4666689 (TT/TA/AA) and *PON2* rs7493 (GG/GC/CC), as well as alleles of *FSIP2* rs4666689 (T/A) and *PON2* rs7493 (G/C) were not associated with male infertility in the studied population in any test models.

DISCUSSION

It is estimated that more than 180 million people worldwide are suffering from reproductive deterioration, concentrating in developing nations with a ratio of one in four couples (Inhorn & Patrizio, 2014; Magalhães et al., 2021). Among them, about 30% of the infertile cases are unable to identify the potential causes (Fainberg & Kashanian, 2019), where genetic factors are proposed to play a crucial part. Notably, 15% of infertile cases are caused by genetic factors, ranging from chromosome abnormalities to single nucleotide polymorphisms (Fainberg & Kashanian, 2019; Krausz et al., 2015). *FSIP2* and *PON2* are among the genes that have contributed to male infertility susceptibility.

FSIP2 encodes a protein associated with the fibrous sheath, a cytoskeletal structure in mammalian sperm flagella, transcribes only during the postmeiotic period of spermatogenesis. Mutations in *FSIP2* can lead to abnormalities in the fibrous sheath and were frequently found in patients with multiple morphologic abnormalities of sperm flagella (MMAF), characterized by short, absent, angulated, or irregular caliber flagella of sperm (W. Liu et al., 2019; Nsota Mbango et al., 2019). In 2018, Martinez and colleagues found that 4 out of 78 patients with MMAF, accounting for 5.1%, carried mutations in the *FSIP2* gene, and none of them was detected in the control sequence database (Martinez et al., 2018). They were later indicated to be correlated with disorganization of the FS and axonemal defects. Additionally, in a cohort of 40 unrelated Han Chinese men with MMAF, two patients were diagnosed with loss-of-function mutations in *FSIP2* (W. Liu et al., 2019), suggesting the prominent role of *FSIP2* in male infertility. Recently, a novel compound heterozygous mutation found in the infertile patient was able to abrogate FSIP2 protein expression (M. Liu et al., 2021). Since FSIP2 plays a prominent role in male infertility, in the current study, we aimed to identify the association between *FSIP2* rs4666689 and the disease in the Vietnamese population. However, our results showed that there was no correlation of *FSIP2* rs4666689 with male infertility.

PON2, encoding for an antioxidant enzyme of the paraoxonase protein family, functions to reduce the reactive oxygen species (ROS) levels in the cells (Horke et al., 2007; Ng et al., 2001). Genetic variants in the gene, particularly *PON2* rs7493, potentially alter its functions, resulting in a reduction of serum paraoxonase activity (Mochizuki et al., 1998; Sanghera et al., 1998), and subsequently lead to increased oxidative stress. An excessive amount of ROS can cause defects in sperm functions, damage sperm DNA and are contributed as potential factors in 30–80% of infertile men (Dinesh, 2012; Wagner et al., 2018). However, previous studies on the correlation of polymorphisms in such genes appeared in limited amounts and generated inconsistent results. For example, in the Slovenian population with 187 infertile, including non-obstructive azoospermia (NOA) and oligo-asthenoteratozoospermia (OAT), and 194 healthy men, no association between *PON2* rs7493 and reproductive impairment was observed (Volk et al., 2011). However, in the Greek population, an opposite result was generated when testing genotype frequencies of *PON2* rs7493 in 120 oligospermia and 170 normozoospermic men for their correlation with male infertility (Lazaros et al., 2011). Patients carrying the genotype GG were less common than those in the control group ($p < 0.001$), and allele C (minor allele) was significantly increased in oligospermia men ($p < 0.008$). Additionally, they concluded that the appearance of allele C was associated with

decreased sperm concentration. In accordance with the Slovenia population study, we did not find any correlation of the polymorphism with male infertility in the current study of the Vietnamese population ($p > 0.05$). Such discrepancy in the association of polymorphism in various ethnic cohorts could be explained by differences in genetic background, environmental factors, or lifestyle.

CONCLUSION

In the current study, we analyzed the correlation of two polymorphisms *FSIP2* rs4666689 and *PON2* rs7493 with male infertility using the PCR-RFLP technique in the Vietnamese population. Our statistical analysis indicated that no association between each polymorphism and the disease was identified. The study would help contribute to the knowledge of genetic backgrounds of infertility in men in the Vietnamese population.

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