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

Tran Huu Dinh¹, Dinh Thanh Thao¹, Luong Thi Lan Anh², Nong Van Hai¹3, Nguyen Thuy Duong¹3*

¹Institute of Genome Research, VAST, Vietnam
²Hanoi Medical University, Ministry of Health, Vietnam
³Graduate University of Science and Technology, VAST, Vietnam

Received 11 June 2021, accepted 9 September 2021

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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*Corresponding author email: tdnguyen@igr.ac.vn

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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 °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
diluted from initial concentration to the
required concentration (~2.5 ng/μL) and
stored at -20 °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) EcoRI and DdeI to
determine the genotypes of FSIP2 rs4666689
and PON2 rs7493, respectively (Table 1).

<table>
<thead>
<tr>
<th>SNP/Gene</th>
<th>Primer sequence</th>
<th>PCR product (bp)</th>
<th>PCR-RFLP</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs4666689/</td>
<td>F:5'-ACAATGCAAGGGAAAGCTCA-3' R:5'-AACAGACTGTGCCGAATCCC-3'</td>
<td>350</td>
<td>Genotype Fragment size</td>
</tr>
<tr>
<td>FSIP2</td>
<td></td>
<td></td>
<td>TT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>240, 106</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>350, 240</td>
</tr>
<tr>
<td>rs7493/</td>
<td>F:5'-CCTGTGTTGGCATGGAATAACT-3' R:5'-GGGCTCTGTGGTATAAAGTGCCCT-3'</td>
<td>359</td>
<td>AA</td>
</tr>
<tr>
<td>PON2</td>
<td></td>
<td></td>
<td>350</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>245, 114</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>359, 245</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>359</td>
</tr>
</tbody>
</table>

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 (χ2) of package “Hardy
Weinberg” (Graffelman, 2015). Additionally,
package “epi_tools” (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 EcoRI and
DdeI to determine the genotypes of FSIP2
rs4666689 and PON2 rs7493, respectively
(Fig. 1).

Six representative EcoRI-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](image_url)

**Figure 1.** Restriction enzyme-digested PCR products on agarose gel 1.5%. M: Marker 100 bp. (A) EcoRI-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) DdeI-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 DdeI 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>
<thead>
<tr>
<th>SNP/Gene</th>
<th>Alleles</th>
<th>MAF in case group</th>
<th>HWE in case group</th>
<th>MAF in control group</th>
<th>HWE in control group</th>
<th>MAF in the whole population</th>
<th>HWE in the whole population</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs4666689/FSIP2</td>
<td>T &gt; A</td>
<td>0.103</td>
<td>0.129</td>
<td>0.100</td>
<td>0.114</td>
<td>0.101</td>
<td>0.928</td>
</tr>
<tr>
<td>rs7493/PON2</td>
<td>G &gt; C</td>
<td>0.175</td>
<td>0.727</td>
<td>0.158</td>
<td>0.271</td>
<td>0.166</td>
<td>0.306</td>
</tr>
</tbody>
</table>

*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**

<table>
<thead>
<tr>
<th>SNP/Gene</th>
<th>Test model</th>
<th>Cases (n = 175)</th>
<th>Controls (n = 201)</th>
<th>OR</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs4666689/FSIP2</td>
<td>Additive</td>
<td>TT 0 (0.00%)</td>
<td>4 (1.99%)</td>
<td>1.00</td>
<td></td>
<td>0.096</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TA 36 (20.57%)</td>
<td>32 (15.92%)</td>
<td>0.200</td>
<td>0.007 - 1.396</td>
<td>0.089</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AA 139 (79.43%)</td>
<td>165 (82.09%)</td>
<td>0.264</td>
<td>0.010 - 1.744</td>
<td>0.156</td>
</tr>
<tr>
<td></td>
<td>Recessive</td>
<td>TT 0 (0.00%)</td>
<td>4 (1.99%)</td>
<td>1.00</td>
<td></td>
<td>0.139</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TA + AA 175 (100.00%)</td>
<td>197 (98.01%)</td>
<td>0.250</td>
<td>0.009 - 1.649</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dominant</td>
<td>TT + TA 36 (20.57%)</td>
<td>36 (17.91%)</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AA 139 (79.43%)</td>
<td>165 (82.09%)</td>
<td>1.186</td>
<td>0.707 - 1.991</td>
<td>0.513</td>
</tr>
<tr>
<td></td>
<td>Allele</td>
<td>T 36 (10.28%)</td>
<td>40 (9.95%)</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>A 314 (89.72%)</td>
<td>362 (90.05%)</td>
<td>1.038</td>
<td>0.642 - 1.671</td>
<td>0.879</td>
</tr>
</tbody>
</table>

**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**

<table>
<thead>
<tr>
<th>SNP/Gene</th>
<th>Test model</th>
<th>Case (n = 80)</th>
<th>Control (n = 98)</th>
<th>OR</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs7493/PON2</td>
<td>Additive</td>
<td>GG 54 (67.50%)</td>
<td>68 (69.39%)</td>
<td>1.00</td>
<td></td>
<td>0.742</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GC 24 (30.00%)</td>
<td>29 (29.59%)</td>
<td>0.959</td>
<td>0.500 - 1.849</td>
<td>0.901</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CC 2 (2.50%)</td>
<td>1 (1.02%)</td>
<td>0.425</td>
<td>0.013 - 5.375</td>
<td>0.441</td>
</tr>
<tr>
<td></td>
<td>Dominant</td>
<td>GG 54 (67.50%)</td>
<td>68 (69.39%)</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GC + CC 26 (32.50%)</td>
<td>30 (30.61%)</td>
<td>0.916</td>
<td>0.484 - 1.741</td>
<td>0.787</td>
</tr>
<tr>
<td></td>
<td>Recessive</td>
<td>GG + GC 78 (97.50%)</td>
<td>97 (98.98%)</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CC 2 (2.50%)</td>
<td>1 (1.02%)</td>
<td>0.429</td>
<td>0.014 - 5.395</td>
<td>0.446</td>
</tr>
<tr>
<td></td>
<td>Allele</td>
<td>G 132 (82.5%)</td>
<td>165 (84.18%)</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>C 28 (17.5%)</td>
<td>31 (15.82%)</td>
<td>0.886</td>
<td>0.504 - 1.560</td>
<td>0.671</td>
</tr>
</tbody>
</table>

**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
Association of FSIP2 rs4666689 and PON2 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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