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STUDY ON THE ROLE OF *TDRD1* VARIANTS IN MALE INFERTILITY AMONG 310 VIETNAMESE INDIVIDUALS

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ABSTRACT

Infertility is a global reproductive health burden affected by various genetic factors, including spermatogenic defects. The instability of the germ cells' genome caused by the unregulated replication of transposable elements is one of the causes of spermatogenic impairment. Tudor domain-containing 1 (*TDRD1*) expressed only in germ cells plays a significant role in the piRNA (PIWI-interacting RNA) pathway to maintain genome integrity via suppressing transposon elements during spermatogenesis. Despite the protein's role in male germline development, *TDRD1* has not been studied intensively. In this study, we established the relationship between male infertility and single nucleotide polymorphisms (SNPs) of *TDRD1* (rs541192490, rs77559927) among 310 Vietnamese men (160 infertile patients and 150 healthy controls). Genotypes of single nucleotide polymorphisms of the *TDRD1* gene (SNPs) were identified using the PCR-RFLP method. The results showed that *TDRD1* SNPs were not associated with male infertility in all three test models (additive, dominant, and recessive) (p-value > 0.05). Haplotype analysis of the two SNPs also showed similar findings. This study would contribute to the knowledge of *TDRD1*'s association with male infertility in the Vietnamese population.

Keywords: male infertility, PCR-RFLP, rs541192490, rs77559927, *TDRD1*, Vietnam.

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INTRODUCTION

Infertility is a global reproductive health burden affecting about 10–15% of couples that have regular unprotected intercourse (Sharlip et al., 2002) and male factors are responsible for approximately 40–50% of infertile cases (Hirsh, 2003). Among male sterility-related factors, the spermatogenic defect is a common cause of male infertility and idiopathic primary testicular dysfunction, which many genetic factors are considered to link with this complex disease (Anawalt, 2013; Krausz et al., 2015). A hundred candidate genes relating to spermatogenesis have been found in animal models, however, the relationship between these genes and human spermatogenesis impairment is not fully unraveled (Cooke & Saunders, 2002; O'Bryan et al., 2006; Yan, 2009; Gu et al., 2010).

Not only being regulated by protein-coding genes but the complex process of spermatogenesis is also influenced by non-coding RNAs (Yan, 2009; Kosova et al., 2012). PIWI-interacting RNAs (piRNAs), non-coding RNAs that are abundant in germ cells, play an important role in the integrity and stability of the germ cells' genome (Aravin et al., 2006; Lau et al., 2006). The piRNAs participate in a defence mechanism that occurs only in the germ cells to suppress transposons which are the main factors causing gene mutations (Slotkin & Martienssen, 2007). In mammals, transposons and their homologs make up about 46% of the human genome (Lander et al., 2001). In this suppression process, PIWI proteins play a key role in repressing transposons (Shoji et al., 2009). Interestingly, all function-impairing mutations of the piRNA pathway genes have been reported to lead to male infertility in mice, while the females remain reproductive. This phenomenon suggests that the piRNA pathway is indispensable in maintaining germline development in mammals (Pillai & Chuma, 2012). MILI, one of the three PIWI proteins, has been shown to play a central role in piRNA interaction and is expressed during spermatogenesis (Reuter et al., 2009). Interacting with MILI, Tudor domain-containing 1 (*TDRD1*) is a member of the Tudor family of proteins that occur only in germ cells. This is the first protein of the TDRD family found to interact with the mouse Piwi protein (Mili) (Chuma et al., 2006; Reuter et al., 2009). In embryonic germ cells, *TDRD1* acts as a scaffold for the correct assembly of the Piwi complex which is essential for male germline development. Indeed, loss-of-function mutations of *TDRD1* lead to male-specific sterility with meiosis defects in the same manner as *Mili* mutations (Pillai & Chuma, 2012). Furthermore, *TDRD1* protein was also found throughout all stages of spermatogenesis from fetal prospermatogonia to round spermatids, indicating the role of *TDRD1* in normal germline.

Research by Zhu et al. (2016) has demonstrated the association of polymorphism rs77559927 of *TDRD1* with impaired spermatogenesis in the Han Chinese population. The polymorphism rs77559927 is located at NC_000010.11:g.114179297, which changes the thymine nucleotide to cytosine at the 5'-UTR of exon 1 of the *TDRD1*. This polymorphism can lead to the increased or decreased expression of several different transcripts of *TDRD1* (Zhu et al., 2016). Changes in the expression level of *TDRD1* can interfere with the interaction of piRNAs, affecting the ability to repress transposable elements, thereby leading to male infertility (Pillai & Chuma, 2012). Thus, this study was conducted to determine the association of *TDRD1* rs77559927 polymorphism with male infertility in the Vietnamese population. Together with SNP rs77559927, a novel polymorphism of *TDRD1* (rs541192490) was also studied individually and combined for haplotype analysis of the two SNPs.

MATERIALS AND METHODS

Study participants and collection of blood samples

The case-control study recruited a total of 160 infertile males including idiopathic NOA

and oligospermia (< 15 million sperms/mL) men from several hospitals in northern Vietnam and 150 healthy Vietnamese men. The qualified infertile individuals must satisfy all following criteria: (1) inability to conceive after at least 12 months of consecutive attempts at fertilization; (2) being diagnosed with non-obstructive azoospermia, oligospermia, and/or azoospermia; (3) a normal 46, XY karyotype and absence of Y-chromosome microdeletions of AZF region; (4) no history recording of infection or other diseases that could affect fertility. The control group includes healthy men who had fathered at least one child and had normal semen concentration, motility, and morphology according to WHO guidelines. All participants that met the requirements above gave informed consent for blood collection. The study was approved by the Ethical Review Committee of the Institute of Genomics Research. The peripheral blood samples (2 mL) were collected and stored in EDTA tubes at -25 ^{o}C.

Methods

Total DNA extraction from blood samples

Genomic DNA was extracted from whole blood samples of participants using Gene JET Whole Blood Genomic DNA Purification Kit (Thermo Fisher, USA). DNA quality was assessed by measuring genomic DNA using both electrophoresis and spectrophotometry, then the samples were diluted to the final concentration of approximately 2.5 ng/μL and stored at -20 ^{o}C.

SNP genotyping

The primers were designed for the PCR-RFLP method (Kim et al., 2017) (Table 1) 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 enzyme *Bst*BI and *Psp*1460I to identify the genotypes of *TDRD1* rs77559927 and rs541192490, respectively (Table 1). Both variants presented all three genotype forms.

*Table 1***.** Primers used for PCR-RFLP

SNP	Primer sequence	PCR product length (bp)	PCR-RFLP	
			Genotype	Fragment (bp)
rs77559927	F: 5'-CCACGGTGACCCAAGTGACCTTC- 3' R: 5'-CCTGCACTTCCCTCGCTTCGA-3'	279	TT	258, 21
			TC	279, 258, 21
			CC	279
rs541192490	F: 5'-TTGAGTGCCTCAGAACAGGAG-3' R: 5'-GTTTCTCACCCTGGGCAGTAG-3'	435	GG	435
			GA	145, 297, 435
			AA	145, 297

Statistical analysis

All collected data were analyzed by using R statistics software version 4.1.2 (R Core Team, 2020), and Microsoft Excel (Microsoft Corp., Washington, DC, USA). Hardy-Weinberg equilibrium (HWE) of the population was tested by a goodness-of-fit Chi-square (χ2) test of package "Hardy Weinberg" (Graffelman, 2015). The correlation between polymorphisms and the risk of male infertility was assessed using the package "epitools" (Aragon, 2020) under three test models: additive, dominant, and recessive. Using the control group as a reference, we estimated the odds ratio with a confidence interval of 95% to estimate the association. All the statistical tests were two-sided, with a *p*-value < 0.05 considered the threshold of significance.

RESULTS

Genetic analysis of *TDRD1* polymorphisms

DNA regions containing *TDRD1* rs77559927 and rs541192490 were successfully amplified using specific primers. Electrophoresis on agarose gel 1% showed specific, sharp, and bright DNA bands with the appropriate molecular weight

(data not shown). After that, PCR products were digested with *Bst*BI and *Psp*1406I to determine the genotypes of *TDRD1* rs77559927 and rs541192490, respectively (Fig. 1). For rs77559927, the third band (21 bp) could not be seen likely because of its small size lead to its migration out of the gel.

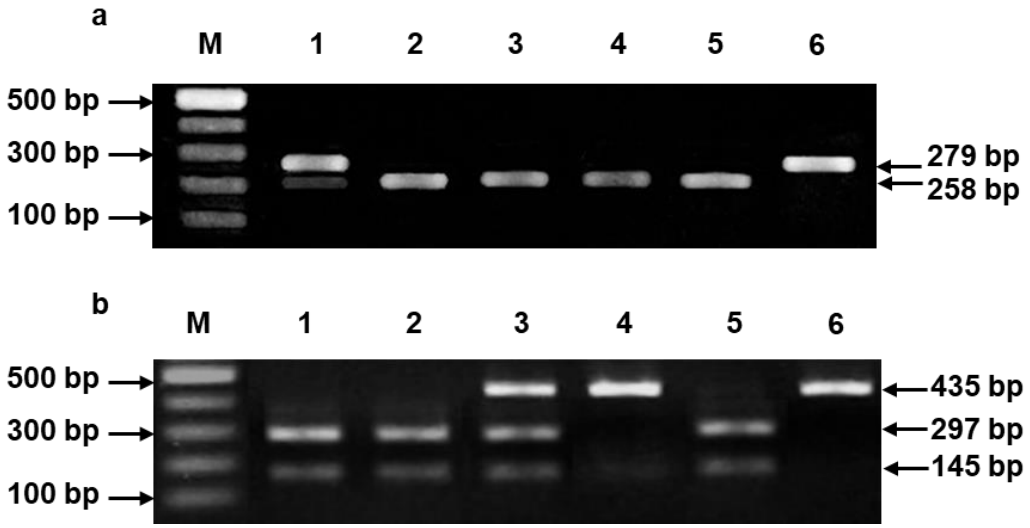

Figure 1. Restriction enzyme-digested PCR products on an agarose gel. **a**: *Bst*BI-digested PCR products of *TDRD1* rs77559927 on 3% agarose gel. M: Marker 100 bp; 2, 3, 4, 5: Wildtype TT (1 band of 258 bp); 1: Heterozygous TC (3 bands of 279 bp, 258 bp, and 21 bp); 6: Homozygous CC (1 band of 279 bp). **b**: *Psp*1406I-digested PCR products of *TDRD1* rs541192490 on 1.5% agarose gel. M: Marker 100 bp; 1, 2, 5: Wildtype GG (2 bands of 297 bp, 145 bp); 3: Heterozygous GA (3 bands of 435 bp, 297 bp, and 145 bp); 4, 6: Homozygous AA (1 band of 435 bp)

For rs77559927, the minor allele frequencies (MAFs) in the case, control, and the overall population are 0.094, 0.0625, and 0.08, respectively (Table 2). No significant difference between the MAFs of the studied groups was observed. The same pattern was found for rs541192490 in case (0.041), control (0.05), and overall population (0.045). The distribution of the two studied SNPs was confirmed to follow HWE using the Chi-square test in case, control, and the overall population (p-values > 0.05).

Table 2. General information of *TDRD1* rs77559927 and rs541192490

SNP	Alleles	MAF case	HWE case	MAF control	HWE control	MAF whole population	HWE whole population
rs77559927	T>C	0.094	0.224	0.0625	0.687	0.08	0.6683
rs541192490	G>A	0.041	0.135	0.05	0.283	0.045	0.072

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

Associations of *TDRD1* SNPs with male infertility

Associations of the single nucleotide polymorphisms of the *TDRD1* gene (rs77559927 and rs541192490) with male infertility were established on three test models (additive, dominant, and recessive) (Table 3). The p-values obtained from analysis of the correlation between the identified genotypes

with male infertility in three models and alleles were higher than 0.05, indicating no significant differences between the patient group and the control group. A combination analysis of the two SNPs was attempted but no significant results were found.

Table 3. Associations between *TDRD1* SNPs and male infertility

SNP of *TDRD1*	Test models	Cases (n = 160)	Controls (n = 150)	OR	95% CI	*p*-value
rs77559927	Additive					0.388
	TT	85 (53.13%)	80 (53.33%)	1.582	0.673 - 3.871	0.294
	TC	65 (40.63%)	55 (36.67%)	1.817	0.757 - 4.535	0.171
	CC	10 (6.24%)	15 (10.00%)	1.000		
	Dominant					
	CC + TC	75 (46.88%)	70 (46.67%)	1.000		
	TT	85 (53.12%)	80 (53.33%)	1.008	0.644–1.578	0.970
	Recessive					
	TT + TC	160 (94.11%)	145 (90.56%)	0.603	0.252–1.386	0.225
	CC	10 (5.89%)	15 (9.44%)	1.000		
rs541192490	Additive					0.839
	GG	148 (92.5%)	136 (90.7%)	1.000		
	GA	11 (6.9%)	13 (8.7%)	0.779	0.329–1.817	0.554
	AA	1 (0.6%)	1 (7.7%)	0.919	0.023–36.089	0.952
	Dominant					
	GG+GA	159 (99.4%)	149 (99.3%)	1.000		
	AA	1 (0.6%)	1 (7.7%)	1.067	0.027–41.866	0.963
	Recessive					
	GG	148 (92.5%)	136 (90.7%)	1.000		
	GA+AA	12 (7.5%)	14 (9.3%)	1.266	0.561–2.902	0.561

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

DISCUSSION

Spermatogenesis is a complex process involving a series of strictly regulated genes. Several studies have shown that genes involved in the piRNA biosynthesis pathway play an important role in spermatogenesis (Klattenhoff & Theurkauf, 2008; Pandey et al., 2013; Yadav & Kotaja, 2014). Thus, variations in these genes may play a role in the formation of defects in this biological process. Mutations in *TDRD1* have been shown to selectively halt piRNA formation, leading to an increase in the number of the most diverse family of retrotransposon LINE1 (L1) (Aravin et al., 2008; Reuter et al., 2009). If left unregulated, L1 insertions can result in gene variation, mRNA destabilization, non-allelic homologous recombination, and epigenetic changes (Kohlrausch et al., 2022).

Furthermore, sperm dysfunction can also be caused by the increased methylation of *TDRD1* (Chuma et al., 2006). In a recent study, Zhu et al. found that the *TDRD1* rs77559927 was significantly different between 342 individuals with nonobstructive azoospermia and 493 controls in a Han Chinese population (Zhu et al., 2016). The change from T>C of *TDRD1* rs7755992 was associated with a reduced risk of spermatogenesis defects, so individuals containing the C allele are likely to be protected against male infertility. Another study also claimed that *TDRD1* expression was significantly reduced in spermatogenic cessation compared with healthy controls

(Babakhanzadeh et al., 2020), thus further confirming the role of *TDRD1* in spermatogenesis. Other than *TDRD1*, proteins belonging to the TDRD family were extensively studied (Gu et al., 2010; Yabuta et al., 2011; Arafat et al., 2017; Gou et al., 2017; Kamaliyan et al., 2018). Despite playing a pivotal role in protecting germ cells against transposons, the role of *TDRD1* in human spermatogenesis was hardly discussed outside of the two aforementioned studies. Thus, a new study of *TDRD1* SNPs in a different cohort would be a beneficial addition to the knowledge of male-infertility-related genes. The rs541192490 replaces the guanine at position c.1009 to an adenine, resulting in an exchange of valine for isoleucine at codon 337 (p.V337I). The amino acid p.V337 is evolutionarily highly conservative among human TDRD and its ortholog in other species such as chimp, gorilla, mouse, and alpaca (https://genome.ucsc.edu). Thus, this amino acid change could potentially affect the function of *TDRD1*. However, we found no association between *TDRD1* polymorphisms rs77559927 and rs541192490 with the risk of male infertility in the Vietnamese cohort. Thus, the association of *TDRD1* rs77559927 with male infertility is heterogeneous across different populations. The cause of this variation may be alluded to variations of genetic characteristics between human populations, habitats, or other conditions.

CONCLUSION

In this study, we have determined the genotypes of *TDRD1* SNPs (rs77559927 and rs541192490) in 310 Vietnamese individuals. The distribution of genotypes as well as allele frequencies of these polymorphisms followed the Hardy-Weinberg equilibrium ($p > 0.05$). The results showed no association between genotypes of the studied polymorphisms with male infertility ($p > 0.05$). To reaffirm the association between *TDRD1* polymorphisms and male infertility, studies on other polymorphisms of the *TDRD1* gene need to be performed.

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