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ASSOCIATION STUDY OF *NAT2* rs1799931 POLYMORPHISM WITH MALE INFERTILITY

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SUMMARY

Infertility is a complex disease that is characterized by the failure to achieve pregnancy after 24 months of regular, unprotected sexual intercourse. Various factors result in male sterility, including genetic and non-genetic factors. Recently, scientists have drawn attention to the role of metabolic genes in contributing to the risk of male infertility by inducing reactive oxygen species (ROS). However, such studies about N-acetyltransferase 2 (*NAT2*), an enzyme that participates in phase II metabolism, remain limited. Thus, this study investigated whether the *NAT2* rs1799931 variant was associated with idiopathic male sterility in Vietnam. A total of 306 DNA samples (148 cases and 158 controls) were genotyped using the polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) methods. About 20% of randomly selected samples were confirmed using an allele-specific polymerase chain reaction (AS-PCR). The results showed that genotype frequencies were consistent with Hardy-Weinberg Equilibrium. Notably, there was an association between *NAT2* rs1799931 and male infertility in two genetic models: the additive model (GA genotype, OR = 0.606, 95% CI: 0.369–0.989, *p*-value = 0.044) and the dominant model (GA+AA genotypes, OR = 0.614, 95% CI: 0.382–0.981, *p*-value = 0.040). This study has enriched our knowledge about the roles of genetic factors in contributing to male infertility in the Vietnamese population.

Keywords: Idiopathic male infertility, *NAT2*, PCR-RFLP, polymorphism

INTRODUCTION

Infertility is a complex disease that is characterized by the failure to achieve pregnancy after 24 months of regular, unprotected sexual intercourse or due to abnormalities in the reproductive system of either male or female, or both (Zegers-Hochschild *et al.*, 2017). Infertility has affected more than 48 million couples worldwide. Impairment in male fertility accounts for 50% of infertile cases and contributes to 7% of the incidence in the world population (Krausz *et al.*, 2015). Both

genetic and non-genetic factors could result in male sterility. There are more than 2000 genes participating in spermatogenesis and genes implicated in apoptosis, DNA repair, xenobiotic metabolism or genes that response to reactive oxygen species (ROS) have been proposed to have an association with male infertility (Aydos *et al.*, 2009; Krausz *et al.*, 2015; Rubes *et al.*, 2005; Yarosh *et al.*, 2013). Among those, N-acetyltransferase 2 (*NAT2*) is active in xenobiotic metabolism and is one of the promising candidates for investigating the association between male infertility and genes related to the detoxification of foreign chemicals.

NAT2 is a highly polymorphic gene that is located on chromosome 8 (8p22) with 60 alleles and is responsible for encoding the N-acetyltransferase 2 (NAT2) enzyme. This enzyme functions in the detoxification of phase II metabolism by adding acetyl groups to various xenobiotic compounds such as arylamines, hydrazine, aromatic amines, and ROS (Summerscales, Josephy, 2004). Expression of *NAT2* in humans occurs within the male reproductive system, encompassing tissues in the testes, prostate, genital ducts, and exocrine glands. In these organs, the enzyme might play a protective function against substances that contribute to male urogenital disorders (Husain *et al.*, 2007). Differences in the metabolic capacity of the NAT2 enzyme among individuals are attributed to variations in the alleles of the *NAT2* gene. These allelic variations are determined by acetylator phenotypes which are categorized as slow (individuals with low-activity alleles in homozygosity), intermediate, or rapid (individuals carrying at least one high activity allele) (Summerscales, Josephy, 2004). Several slow acetylator variants of *NAT2* have been reported to be associated with idiopathic male infertility and other diseases in the male reproductive system (García-Closas *et al.*, 2005; Gong *et al.*, 2011; Hein *et al.*, 2002; Trang *et al.*, 2018). Previously, *NAT2* polymorphisms with a slow acetylation phenotype, including rs1799929 (c.481C>T) and rs1799930 (c.590G>A), were widely recognized and reported to be associated with an increased risk of male infertility (Trang *et al.*, 2018; Yarosh *et al.*, 2014). Besides, *NAT2* rs1799931 is also a slow acetylator, in which glutamine is replaced with glycine at position 286 of the amino acid sequence (NP_000006.2:p.Gly286Glu) (Zang *et al.*, 2007). However, only a few research efforts have investigated the connections between this *NAT2* polymorphism and the risk of having idiopathic infertility.

Here, we explored whether there is an association between *NAT2* rs1799931 and male infertility in a cohort of 306 Vietnamese male individuals. Genotyping was performed by the PCR-RFLP method, with subsequence statistical analysis to assess this variant. Our study is the first to examine rs1799931 in the Vietnamese population and contribute to expanding knowledge regarding male infertility.

SUBJECT AND METHODS

Study subjects and sample collection

Peripheral blood samples obtained from 306 male participants (including 148 infertile patients and 158 healthy men having at least one child without assisted reproductive technology intervention) were used for this research. Selected infertile patients were diagnosed with abnormal spermiograms, including non-obstructive azoospermia and oligospermia (lower than

15 million sperm/mL of semen). In addition, the medical history of all patients was checked to exclude those with a chromosomal abnormality, AZF deletion, reproductive disease (orchitis due to sexually transmitted diseases, testicular tumor, or genital trauma). All study participants volunteered and gave their written consent forms. Sample collection and research protocol were approved by the Human Research Ethics Committee of the Institute of Genome Research, Vietnam Academy of Science and Technology. Two mL of blood samples collected from the participants above were stored in an EDTA tube at ‑20 ºC.

SNP genotyping

Whole genomic DNA was extracted from 306 whole blood samples by using the GeneJET Whole Blood Genomic DNA Purification Kit of Thermo Fisher (USA), following manufacturer instructions. Genomic DNA was measured by spectrometer and electrophoresed on a 1% agarose gel for quality control. All qualified DNA samples were then diluted from the initial concentration to about 2.5 ng/µL and stored at –20 ºC.

The whole genomic DNA of 306 samples was used to amplify the region containing the single nucleotide polymorphism *NAT2* rs1799931 by PCR reaction with a specific pair of primers (Table 1). After that, PCR products were digested with the restriction enzyme *Bam*HI (Thermo Fisher, USA) to identify the genotypes of *NAT2* rs1799931. The restriction cut reaction was incubated at 37 ºC in a water bath for 5 hours, and then the digested products were viewed on a 1.5% agarose gel. Genotypes were determined based on the number of DNA bands and band size on electrophoresis gel (Table 1).

Table 1. Primers used for polymerase chain reaction - restriction fragment length polymorphism.

SNP/Gene	Primer sequence (5’-3’)	PCR product (bp)	PCR-RFLP	
			Genotype	Fragment size (bp)
rs1799931/ *NAT2*	F: 5’-AGGGGTTTACTGTTTGGTGG-3’	448 bp	AA	448
	R: 5’-GCATGAATCACTCTGCTTCCC-3’		GA	170, 278, 448
			GG	170, 278

Note: SNP: Single nucleotide polymorphism

Subsequently, the allele-specific PCR (AS-PCR) method was conducted to confirm the digestion results for 20% of the samples (60/306 samples). In AS-PCR experiments, there are two outer primers (F: 5’-AGGGGTTTACTGTTTGGTGG-3’; and R: 5’-CTAGCATGAATCACTCTGCTTCC-3’) designed to amplify the DNA fragment that contains *NAT2* rs1799931. Besides, two inner primers (Fg: 5’-TGCCCAAACCTGGTGATAG-3’; and Ra: 5’-GTTCCTTATTCTAAATAGTAAG

GGACT-3') are responsible for specifically genotyping the allele. All four primers were mixed with other necessary PCR components, and the AS-PCR reaction cycle was 95 ºC/4 minutes for initial denaturation, 35 cycles of 95 ºC/30 seconds, 63 ºC/25 seconds, 72 ºC/25 seconds, and 72 ºC in 5 minutes. The products of AS-PCR were electrophorized on a 2% agarose gel. The genotyping confirmation results were based on the number of DNA bands and band size on the gel (Table 2).

Table 2. Size and number of DNA bands of 3 genotypes of *NAT2* rs17999031 in AS-PCR method.

Genotypes	Number of DNA bands	Size of DNA bands (bp)
GG	2	451, 299
GA	3	451, 299, 197
AA	2	451, 197

Statistical analysis

Genotyping data generated from the PCR-RFLP method was analyzed by Microsoft Excel (Microsoft Corp., Washington, DC, USA) and R programming software version 4.2.1 (https://www.r-project.org) (R Core Team, 2018). The distribution of genotype of SNP *NAT2* rs1799931 was examined by a Chi-squared test to check whether the sample set was consistent with Hardy-Weinberg Equilibrium (HWE) (Graffelman, 2015). In order to assess the association between genotypes/allele frequencies of rs1799931 polymorphism and male infertility, three genetic models, including additive, dominant, and recessive, were performed using the R package "epitools" (Aragon, 2020). The magnitude of the association between polymorphism and male infertility was estimated by using the function "oddsratio" in the package to calculate 95% confidence interval (95% CI) and Odds ratio (OR). The estimation was considered significant if the *p*-value was < 0.05. All performed tests were two-sided.

RESULT AND DISCUSSION

Genotype distribution of *NAT2* rs1799931

PCR products of the DNA region containing *NAT2* rs1799931 were digested using the restriction enzyme *Bam*HI. Electrophoresis results of 8 samples on a 1.5% agarose gel showed that wells 2, 3, 5, 6, and 8 had two bands (278 bp and 170 bp), which indicate the GG genotype; well 1 showed one band of 448 bp corresponding to the AA genotype; wells 4 and 7 had three bands, which indicated the GA heterozygous genotype (Figure 1).

The genotype distribution and allele frequencies of the *NAT2* rs1799931 polymorphism are summarized in Table 3. The frequencies of the A allele (minor allele) in the case, control group, and whole study population were 0.18, 0.24, and 0.21, respectively. A similar pattern was also observed in the major G allele, with the frequencies being 0.82, 0.76, and 0.79,

respectively (Table 3). Furthermore, the result of the Chi-squared test revealed that the allele distribution of *NAT2* rs1799931 followed Hardy-Weinberg Equilibrium in all groups of the study population with a *p*-value > 0.05 (Table 3).

The confirmation outcomes of 60 random samples using AS-PCR were in concordance with the results of the PCR-RFLP method (Figure 2).

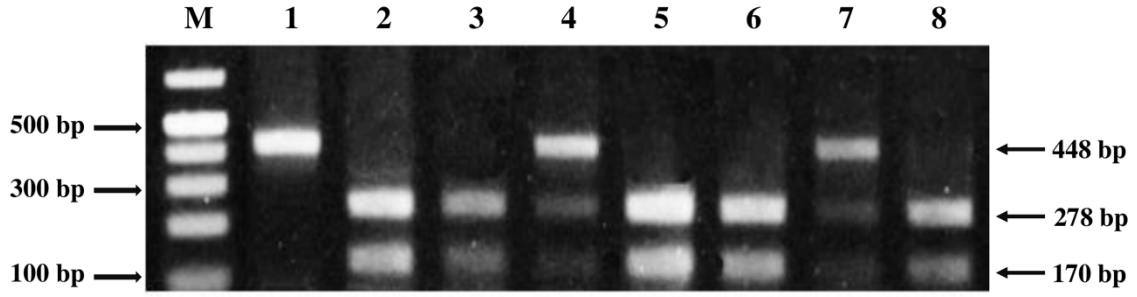

Figure 1. *Bam*HI-digested PCR products of *NAT2* rs17999031 on agarose gel 1.5%. M: Marker 100 bp (Thermo Fisher Inc.). 1: Homozygous AA (1 band of 448 bp); 4, 7: Heterozygous GA (3 bands of 448 bp, 278 bp, and 170 bp); 2, 3, 5, 6, 8: Wild-type GG (2 bands of 278 bp and 170 bp).

Table 3. Allele and genotype distribution of *NAT2* rs1799931 among infertile and healthy men.

	Genotype			Allele frequency		HWE (*p*-value)
	GG	GA	AA	G	A	
Case (n=148)	102	40	6	0.82	0.18	0.42
Control (n=158)	91	59	8	0.76	0.24	0.69
Total (n=306)	193 (0.63)	99 (0.32)	14 (0.05)	0.79	0.21	0.77

Note: n: Number of participants, HWE: Hardy-Weinberg Equilibrium.

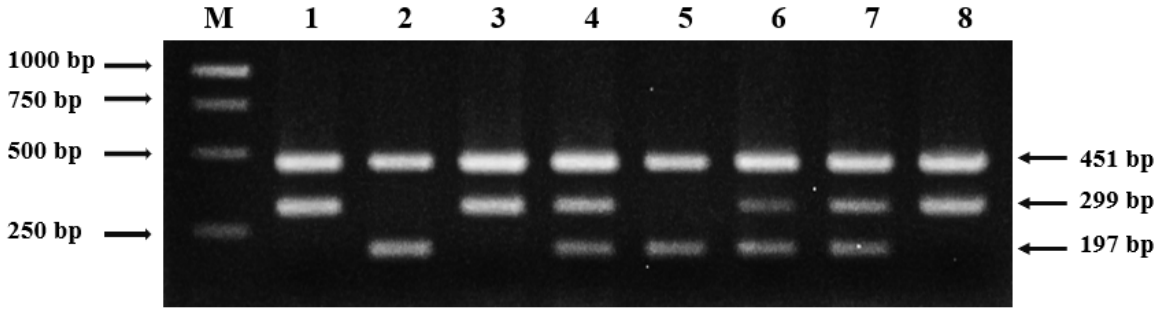

Figure 2. AS-PCR products of *NAT2* rs17999031 on agarose gel 2%. M: Marker 1kb (Thermo Fisher Inc.). 1, 3, 8: Wild-type GG (2 bands of 451 bp and 299 bp); 2, 5: Homozygous AA (2 bands of 451 bp and 197 bp); 4, 6, 7: Heterozygous GA (3 bands of 451 bp, 299 bp, and 197 bp).

Association analysis of SNP *NAT2* rs17999031

In order to understand the association between polymorphism and male infertility, four genetic models (additive, dominant, recessive, and alleles) were statistically performed (Table 4). The results obtained from the analysis showed significant associations in additive and dominant models, with *p*-values smaller than 0.05. Moreover, additive and dominant models in this study revealed a protective pattern against male infertility of the GA genotype (OR = 0.606, 95% CI: 0.369–0.989, *p*-value = 0.044) and the combination of GA + AA genotype group (OR = 0.614,95% CI: 0.382–0.981, *p*-value = 0.040), respectively.

Table 4. Association of *NAT2* rs1799931 and male infertility.

	Model	Case (n=148)	Control (n=158)	OR	95% CI	*p*-value
NAT2 rs1799931	**Additive**					**0.120**
	GG	102 (68.92%)	91 (57.60%)	1.000		
	GA	40 (27.03%)	59 (37.34%)	0.606	0.369 - 0.989	0.044
	AA	6 (4.05%)	8 (5.06%)	0.675	0.210 - 2.050	0.470
	Dominant					
	GG	102 (68.92%)	91 (57.60%)	1.000		
	GA+AA	67 (42.40%)	46 (31.08%)	0.614	0.382 - 0.981	0.040
	Recessive					
	GG+GA	150 (94.94%)	142 (95.95%)	1.000		
	AA	8 (5.06%)	6 (4.05%)	0.798	0.251 - 2.394	0.673
	Allele					
	G	241 (76.27%)	244 (82.43%)	1.000		
	A	75 (23.73%)	52 (12.57%)	0.686	0.460 - 1. 017	0.060

Note: n: Number; OR: Odds ratio; 95% CI: 95% confidence intervals; p-value was measured using Chi-squared test.

NAT2 is a highly polymorphic gene that is responsible for the detoxification of phase II xenobiotic metabolism. Several *NAT2* variants have impacted the enzyme activity of NAT2, resulting in the disruption of xenobiotic metabolism and increasing the level of reactive oxidative stress (ROS) (Hein, 2002). This defection in redox balance can impair sperm motility and damage sperm DNA (Gharagozloo, Aitken, 2011); thus, a substantial body of evidence has revealed a link between excessed production of ROS and male infertility (Agarwal, Said, 2005; Alahmar, 2019). Simultaneously, certain types of prescribed medication, such as isoniazid, sulfasalazine, and pyrazinamide, which have impacts on the male productive system by promoting high levels of oxidative stress, are metabolized by the NAT2 enzyme (Zentner *et al.*, 2020). In addition, oxidative stress-induced factors, such as alcohol addiction

and smoking habits, combined with the *NAT2* slow acetylator variant (rs1799930), could also elevate the risk of infertility (Yarosh *et al.*, 2014). Moreover, *NAT2* polymorphisms with slow acetylation phenotypes, rs1799929 (c.481C>T) and rs1799930 (c.590G>A), were associated with an increased risk of male infertility in the Vietnamese male population (Trang *et al.*, 2018). Therefore, *NAT2* variants could potentially contribute to chemically-induced anomalies in spermatogenesis.

NAT2 rs1799931 exchanges G for A at position 857 (NM_000015.3: c.857G>A), leading to the replacement of glycine at position 286 for glutamine in the amino acid sequence (NP_000006.2: p.Gly286Glu). The amino acid p.Gly286 is highly evolutionarily conservative in human NAT2 and other primate species such as chimpanzees, gorilla, and rhesus (https://genome.ucsc.edu). Hence, this amino acid change may affect the function of *NAT2*. A case-control study involving 636 cases and 442 healthy Chinese men yielded the result (OR = 0.94, 95% CI: 0.74–1.20, *p*-value = 0.840), indicating no significant association between *NAT2* rs17999031 and male infertility (Jiang *et al.*, 2016). However, in this study, there was an association between *NAT2* rs1799931 and male infertility in two genetic models (additive and dominant) (*p*-value < 0.05). Moreover, the odds ratio (OR) values (additive model: OR = 0.606, 95% CI: 0.369–0.989, *p*-value = 0.044; the dominant model: OR = 0.614, 95% CI: 0.382–0.981, *p*-value = 0.040) suggested that heterogeneous and homozygous genotypes lower the risk of male infertility. The inconsistency in results implies that methods of sample collection and some environmental elements such as lifestyle, ethnic diversity, and sample size may affect the outcomes. Therefore, more studies should be replicated in other populations to clearly define the association of *NAT2* rs1799931 with male infertility. Additionally, the roles of *NAT2* and its variants in spermatogenesis are not yet clearly established; hence, further research should be conducted to explore the variant functions of the NAT2 enzyme in sperm cells and different animal models to define the pathogenesis pathways of this enzyme in male infertility.

CONCLUSION

In conclusion, our study has reported the association between a *NAT2* polymorphism, rs1799931, and the susceptibility of male infertility in the Vietnamese population. In this pilot study, our statistical analysis has revealed that Vietnamese male subjects with heterozygous (GA) and homozygous variants (AA) of *NAT2* rs1799931 are protected against male infertility risk. Further studies should be performed on different populations to confirm our results, and functional studies should be carried out to inspect the effect of *NAT2* on male infertility.

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