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ASSOCIATION STUDY OF *NR5A1* rs1110061 WITH INFERTILE MALE IN 401 VIETNAMESE INDIVIDUALS

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SUMMARY

Male infertility is a reproductive issue involving defects in the quantity and quality of sperm. Besides the exogenous elements such as harmful habits, pollution, genetic factors including disorders in a single gene, group of genes, or chromosomes, are the etiology causing infertility in men. *NR5A1* is well-known as a candidate gene involved in male infertility, including 46, XY disorders of sex development (DSD), cryptorchidism, micropenis, spermatogenic failure, non-obstructive azoospermia, and oligozoospermia. This study aimed to identify the single nucleotide polymorphism (SNP) associated with male infertility in the *NR5A1* gene in a Vietnamese cohort of 202 infertile men and 199 healthy controls. By directly sequencing the coding region of the *NR5A1* gene in 56 cases and 21 controls, only one missense mutation c.437G>C (p.Gly146Ala; rs1110061) in exon 4 was found. The variant was detected in 34 patients, of which 27 were heterozygous (GC) and 7 were homozygous (CC). In the control group, 15 individuals carried this mutation, including 10 with heterozygous (GC) and 5 with homozygous (CC). To further investigate the association of the polymorphism *NR5A1* rs11100061 with male infertility disease, we performed PCR-RFLP in 202 infertile and 199 healthy men to assess the genotypes and alleles. The results indicated that the distribution of the genotypes of the polymorphism was in accordance with Hardy-Weinberg equilibrium (p-values > 0.05). However, no association was detected between the polymorphism and male infertility (p-values > 0.05).

Keywords: direct sequencing, male infertility, *NR5A1*, PCR-RFLP, rs1110061

INTRODUCTION

Infertility, defined as the inability of a sexually active couple to conceive after 1 year of regular intercourse without contraception (Zegers-Hochschild *et al.*, 2017), affects approximately 15% of couples, and male factor is the cause in 50% of the cases (Vander Borght *et al.*, 2018; Sudhakar *et al.*, 2018). Thousands of genes taking place in spermatogenesis, testicular development, and endocrine regulation of testicular function are considered as the etiology of the disease. At least 15% of the infertile men are presented with defects in such genes (Asero *et al.*, 2014). *NR5A1* is among the genes that proved to be associated with male infertility by biological and functional evidence and is replicated in numerous independent studies (Tüttelmann *et al.*, 2018).

NR5A1 (Nuclear receptor subfamily 5 group A member 1, NM_004959.5) is located on chromosome 9q33, spanning about 30 kb long, and consisting of 7 exons (1 non-coding exon followed by 6 coding exons) (Oba *et al.*, 1996). The steroidogenic factor 1 (SF1) protein, encoded by the *NR5A1* gene, plays a pivotal role in steroidogenesis, sexual and adrenal developments, and reproduction (Cannarella *et al.*, 2019). It is expressed in Sertoli and Leydig cells of the developing testis and Sertoli cells of the prepubertal and adult testis. Attempts to identify mutations of the *NR5A1* gene revealed several point mutations, which impair its function, leading to severe spermatogenic failure and male infertility. Currently, more than 188 different mutations in *NR5A1* were described, and they scattered throughout all the protein domains, with a predominance in DNA-binding domain (DBD, 35%) and ligand-binding

domain (LBD, 42.3%) (Fabbri-Scallet *et al.*, 2020). They are found in a wide range of infertile phenotypes, including 46 XY disorders of sex development (DSD) (Askari *et al.*, 2020; Bashamboo *et al.*, 2016; Brauner *et al.*, 2016; Domenice *et al.*, 2016; Tuhan *et al.*, 2017), cryptorchidism (Ferlin *et al.*, 2015), non-obstructive azoospermia, and oligospermia patients (An *et al.*, 2021; Werner *et al.*, 2017; Zare-Abdollahi *et al.*, 2015). Moreover, case-control association studies between polymorphisms and different types of male infertility have also been conducted in diverse populations, generating different outcomes (Röpke *et al.*, 2013; Sudhakar *et al.*, 2018; Wada *et al.*, 2005, 2006; Zare-Abdollahi *et al.*, 2015).

Although the effects of mutations in *NR5A1* on men with reproductive impairment have been widely studied, mutations in Vietnamese idiopathic infertile patients have not yet been investigated. Therefore, we conducted this study to identify candidate variants associated with male infertility in the *NR5A1* gene in the studied group of 202 infertile male patients and 199 healthy men of Vietnamese ethnics.

SUBJECTS AND METHODS

Sample collection

A total of 202 men who had idiopathic infertility fulfilled the following criteria were selected for this study: (1) inability to conceive after 12 months of intercourse without contraception; (2) normal karyotype and no deletion in the AZF region; (3) low sperm concentration (oligozoospermia) with 5 to 20 million sperm/ml ejaculate or no sperm in the ejaculate (azoospermia); (4) non-obstructive azoospermia; (5) no medical history of viral infections such as mumps, which causes testicular inflammation and other infectious diseases, which cause male infertility due to *Chlamydia trachomatis*, *Mycoplasma genitalium*, *Streptococcus faecalis*, hepatitis B virus. Group of 199 men with at least one biological child were selected as the control. All subjects were of Vietnamese group and gave written informed consent to donate their blood at Hanoi Medical University Hospital. The study was approved by the Institutional Review Board of the Institute of Genome Research, Vietnam Academy of Science and Technology.

Mutation analysis

Genomic DNA was extracted from 2 ml EDTA-preserved blood using GeneJET Whole Blood Genomic DNA Purification Mini Kit (ThermoFisher, catalog number: K0781). PCR was carried out with specific primers (Table 1) for all exons of *NR5A1* (exons 2–7; NM_004959.4). Exons 2 and 3 (exon 2/3) were amplified together. Each polymerase chain reaction (PCR) was carried out in a 20 µL using 0.2 µM of forward and reverse primers, 20 ng genomic DNA, 100 µM dNTPs, 3% glycerol, 0.1 unit Taq polymerase (ThermoFisher). PCR reaction conditions used for the amplification of all the exons were initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30s; 55°C - 59°C for 30s, 72°C for 1 min and a final extension at 72°C for 5 min (Table 1). For sequencing, the PCR products were treated with GeneJET PCR Purification Kit (ThermoFisher, catalog number: K0701) to remove unused nucleotides and excessive primers. Sanger sequencing of the amplicons was performed using an ABI 3500 automated DNA sequencer (Applied Biosystems Division, Foster City, CA, USA) and the Big Dye Terminator V1.1 cycle sequencing kit. DNA analysis and sequence assembly with the reference sequence were done using AutoAssembler software.

The mutation analysis was then extended in the cohort of 146 infertile patients and 178 healthy controls using restriction enzyme *Hha*I (Thermo Fisher) to determine the genotypes of *NR5A1* rs1110061. The total volume of digestion reaction was 5 µL, including 3 µL of PCR product; 0.5 µL of buffer Tango (10X); 0.1 µL of *Hha*I (10 U/µL); and H_2O. The mixture was incubated at 37°C in a water bath for 5 hours and then examined in agarose gel 1.5%. The genotypes of *NR5A1* rs1110061 were determined based on the number and size of DNA bands (Table 2).

Statistical analysis

Acquired data were statistically computed using Microsoft Excel (Microsoft Corp., Washington, DC, USA) and R version 4.0.3 (R Core Team, 2020). Chi-square test ($\chi 2$) of package "Hardy-Weinberg" was used to determined the Hardy-Weinberg equilibrium (HWE) of the population (Graffelman, 2015). The correlation between the polymorphism with male infertility in 3 test models: additive, dominant, and recessive and allele forms were evaluated by package "epitools" (Aragon, 2020). Odds ratio with a confidence interval of 95% was used to estimate the association. All the statistical tests were two-sided. The computing outcomes were considered to be statistically significant if *p*-value is less than 0.05.

Table 1. Specific sequence primers used for amplifying coding regions (exons 2 to 7) of *NR5A1.*

Exon	Forward primer (5'-3')	Reverse primer (5'-3')	Ampliconsize (bp)	Tm (ºC)
2 and 3	GGGCACAGAGAGGGGATTAC	GTTCTCTTGCAGCGACTGG	592	58
4	GTGTTGAGCAGGGGAGAGAG	AAGGATGGCCCTATCCAAAG	829	55
5	ATCTGGGTAGATGGGCACAG	GTGGGGAAAGGGCTGATAAT	395	55
6	TCTGACCTGCACCTCCAATC	CTCTGGCTGTCTCCACCTCT	301	55
7	GTGACCGAGAACCTCCCTTT	TGGGCATCAGAAAATGAACC	378	59

bp: base pair; Tm: annealing temperature.

Table 2. Number and size of DNA bands of *NR5A1* rs1110061 genotypes.

Genotypes	Number of DNA bands	Size of DNA bands (bp)
GG	2	117, 710
GC	4	117, 185, 525, 710
CC	3	117, 185, 525

RESULTS

Genetic findings in *NR5A1*

The entire coding regions of the *NR5A1* gene (exons 2 to 7; NM_004959.4), including the flanking boundary regions, were directly sequenced to identify variants associated with male infertility. Sequencing of coding regions and exon-intron boundary sites of 77 samples (56 cases and 21 controls) enabled us to detect only one variant, c.437G→C in exon 4 (Figure 1). This one-base change resulted in a Gly at position 146 to Ala missense variant, named rs1110061 in the dbSNP. No other variants were found in any other exon regions. The variant was detected in 34 patients, of which 27 with heterozygous (GC) and 7 with homozygous (CC). In the control group, 15 individuals carried this variant, including 10 with heterozygous (GC) and 5 with homozygous (CC).

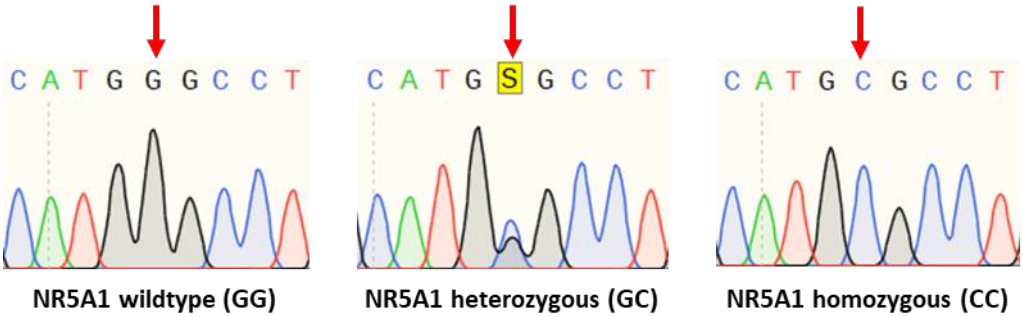

Figure 1. Direct sequencing of the PCR products of exon 4 (including the flanking boundary regions) reveals the variant c.437G>C (Gly146Ala). An altered base at the second (G→C) position of codon 146 (indicated by arrow), resulting in a substitution of Gly (GGG) for Ala (GCG).

Genotyping *NR5A1* rs1110061

To further analyze the correlation of the variant with male infertility, we amplified the exon 4 in 401 samples (202 cases and 199 controls) including 77 sequenced samples with the above-mentioned primers and digested them with restriction enzyme *Hha*I. Six representative *Hha*I-digested products (1-6) (Figure 2) indicated that: sample 1 and 2 were heterozygous (GC), sample 3 and 6 were homozygous (CC), and sample 4 and 5 were wild type (GG).

Genotypes and allele frequencies of the polymorphism *NR5A1* rs1110061 were described in

Table 3. The minor allele frequencies in the case, control, and the whole population were 0.376, 0.432, 0.404, respectively. Using Chi-square test, we showed that the distribution of the polymorphism rs1110061 was in accordance with Hardy-Weinberg equilibrium in case, control, and the studied population (*p*-value > 0.05).

Association analysis of *NR5A1* rs1110061 with male infertility

The correlation of polymorphism *NR5A1* rs1110061 with the male infertility was further assessed by statistical analysis of three models (additive, dominant, recessive) and allele forms (Table 4). The resulted *p*-values were much higher than the standard threshold, indicating the insignificant difference between the case and control groups. In conclusion, genotypes of *NR5A1* rs1110061 (GG/GC/CC) and alleles (G/C) were not associated with male infertility in any test models.

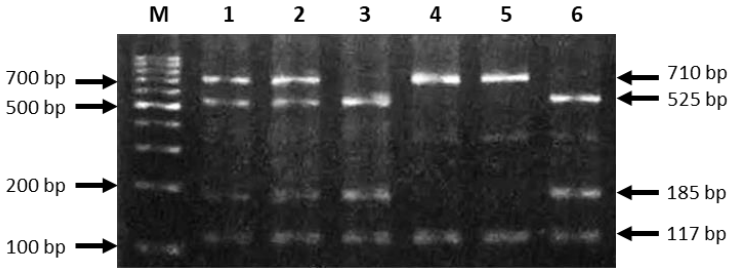

Figure 2. Restriction enzyme-digested PCR products on agarose gel 1.5%. M: 100bp marker; 1 and 2: heterozygous GC (4 bands of 710 bp, 525 bp, 185 bp and 117 bp); 3 and 6: homozygous CC (3 bands of 525 bp, 185 bp, and 117 bp); 4 and 5: wild type GG (2 bands of 710 bp and 117 bp).

Table 3. Summary of genotypes and allele frequencies of rs1110061.

	Genotypes/Frequency			Allele frequency		HWE *p*-value
	GG	GC	CC	G	C	
Case (n = 202)	80 (0.396)	92 (0.455)	30 (0.149)	0.624	0.376	0.673
Control (n = 199)	67 (0.337)	92 (0.462)	40 (0.201)	0.568	0.432	0.413
Total (n = 401)	147(0.366)	184 (0.459)	70 (0.175)	0.596	0.404	0.345

n: number; HWE: Hardy-Weinberg equilibrium.

Table 4. Association between *NR5A1* rs1110061 and male infertility.

Test model	Cases (n=202)	Control (n=199)	OR	95% CI	*p*-value
Additive					
GG	80 (39.60%)	67 (33.67%)	1.000		
GC	92 (45.54%)	92 (46.23%)	1.193	0.772 – 1.847	0.424
CC	30 (14.86%)	40 (20.10%)	1.587	0.894 – 2.840	0.111
Recessive					
GG+GC	172 (85.14%)	159 (79.90%)	1.000		
CC	30 (14.86%)	40 (20.10%)	1.439	0.856 – 2.442	0.166
Dominant					
GG	80 (39.60%)	67 (33.67%)	1.000		
GC+CC	122 (60.4%)	132 (66.33%)	1.291	0.859 – 1.945	0.217
Allele					
G	252 (62.38%)	226 (56.78%)	1.000		
C	152 (37.62%)	172 (43.22%)	1.261	0.951 – 1.675	0.107

n: number; OR: odd ratio; 95% CI: 95% confident intervals, p-value is measured using Chi-square test.

DISCUSSION

Infertility in men is a complex multifactorial disease, influencing approximately 7% of men in the general population (Krausz *et al.*, 2015). Genetic defects might cause 15% of the fertile men, and at least 300 SNPs distributed in more than 123 genes have been identified to relate to male infertility. Approximately 70% of the genes participate in common cell functions but are relevant to germ cells such as apoptotic process, DNA repair, detoxification, response to reactive oxygen species, etc. (Araujo *et al.*, 2020; Krausz *et al.*, 2015, 2018), while the remaining 30% involve in the male reproductive system.

NR5A1, also known as steroidogenic factor 1 (SF-1), encodes for a protein involved in the transcriptional regulation of multiple genes involved in steroidogenesis, reproduction, and male sexual differentiation. This gene product interacts with *SRY*, *SOX9*, *GATA4*, and other proteins during sex and adrenal development pathways (Karpova *et al.*, 2015; Lin *et al.*, 2008; Sekido *et al.*, 2008). It is demonstrated that *NR5A1* knock-out in mice could directly lead to male infertility (Jeyasuria *et al.*, 2004). Additionally, mutations in *NR5A1* are suspected of associating with azoospermia and oligozoospermia (Bashamboo *et al.*, 2010; Ferlin *et al.*, 2015; Röpke *et al.*, 2013). In an early *NR5A1* mutational analysis study, 7 heterozygous missense mutations were found in 315 idiopathic spermatogenic failure individuals of non-Caucasian ancestry. They were not observed in more than 4000 controls and might lead to reduced protein function *in vitro* (Bashamboo *et al.*, 2010).

During the course of finding the potential mutations in *NR5A1* from patients with idiopathic male infertility, rs1110061 appeared to present frequently. It is first reported to reduce the SF-1 trans-activation function for the adrenal-specific cyp11A promoter and the ovary-specific cyp19 promoter II by approximately 20% (WuQiang *et al.*, 2003). In 2013, a large comprehensive sequencing study in a cohort of 488 infertile patients (218 severe oligozoospermia and 270 non-obstructive azoospermia) and 237 controls of Caucasian origin was performed to detect candidate mutations (Röpke *et al.*, 2013). Three mutations were found, including rs1110061, which presented in 16 patients and 5 controls. No significant difference was detected between patient and control groups in terms of allele frequencies ($p = 0.41$) and genotype distribution ($p = 0.62$). Furthermore, the polymorphism was only found in patients without a medical history of cryptorchidism. However, in another research, such polymorphism is proposed to be associated with more severe forms of spermatogenic impairment related to cryptorchidism (Ferlin *et al.*, 2015). The highest prevalence of the polymorphism (7/61, 11.5%) was found in men with azoospermia and cryptozoospermia with a history of cryptorchidism ($p < 0.05$ vs. normozoospermic controls). In 2015, no association between male infertility and the polymorphism rs1110061 was demonstrated in a cohort of 90 Iranian azoospermic men and 112 fertile men (Zare-Abdollahi *et al.*, 2015). Three patients and one control were identified with this polymorphism, indicating the prevalence of 3.3% in the azoospermic patients. Recently, 7 mutations (6 intronic and 1 exonic), including rs1110061 are demonstrated not to be associated with non-obstructive azoospermia (NOA), oligozoospermia ($p = 0.76$) in a cohort of 916 Indian individuals (502 infertile men and 414 controls) (Sudhakar *et al.*, 2018). The study also stratified the data based on the linguistic affiliation (Indo-European and Dravidian) to analyze; however, the result remained the same. Similarly, we detected no association between azoo/oligozoospermia with rs1110061 ($p > 0.05$) in any test models and allele forms in the Vietnamese population.

CONCLUSION

In this study, we performed direct sequencing in 56 azoospermic/severe oligozoospermic patients and 21 control individuals of Vietnamese ethnics to identify candidate variants in the coding region (exon 2-7) of the *NR5A1* gene. Only one polymorphism c.437G>C (p.Gly146Ala; rs1110061) was found in exon 4. The polymorphism was subsequently analyzed in the 401 studied subjects to reveal the frequencies of genotypes GG/GC/CC are 0.366/0.459/0.175, respectively, and their distribution all followed Hardy-Weinberg equilibrium. However, no association was established between *NR5A1* rs1110061 and male infertility ($p > 0.05$). This study would help contribute more insights into *NR5A1* mutations in the Vietnamese population.

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PHÂN TÍCH SỰ LIÊN QUAN CỦA ĐA HÌNH *NR5A1* rs1110061 VỚI BỆNH VÔ SINH NAM Ở 401 CÁ THỂ NGƯỜI VIỆT

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TÓM TẮT

Vô sinh nam là vấn đề sinh sản ở nam giới có liên quan tới sự thiếu hụt về số lượng hoặc chất lượng của tinh trùng. Bên cạnh các yếu tố ngoại cảnh như các thói quen có hại cho sức khỏe, ô nhiễm; các yếu tố di truyền bao gồm rối loạn ở gen đơn, nhóm gen hoặc nhiễm sắc thể là nguyên nhân gây ra vô sinh ở nam giới. *NR5A1* được biết đến như là một gen liên quan tới bệnh vô sinh nam gồm các loại như rối loạn phát triển giới tính ở nam giới có bộ nhiễm sắc thể 46 XY, tinh hoàn ẩn, dương vật nhỏ, suy sinh tinh, vô sinh không bế tắc, và vô sinh ít tinh trùng. Nghiên cứu này nhằm mục đích phát hiện đa hình liên quan tới vô sinh nam ở trên gen *NR5A1* ở quần thể người Việt Nam bao gồm 202 bệnh nhân và 199 người khỏe mạnh. Trước tiên, chúng tôi đã sử dụng phương pháp giải trình tự trực tiếp vùng mã hóa của gen *NR5A1* ở 56 bệnh nhân và 21 người khỏe mạnh và xác định được một đa hình sai nghĩa c.437G>C (p.Gly146Ala; rs1110061) ở exon 4. Điểm đa hình này xuất hiện ở 34 bệnh nhân, trong đó 27 người mang kiểu gen dị hợp (GC) và 7 người mang kiểu gen đồng hợp (CC). Ở nhóm đối chứng, 15 người mang đa hình, bao gồm 10 dị hợp (GC) và 5 đồng hợp (CC). Để tiếp tục nghiên cứu *NR5A1* rs1110061, chúng tôi thực hiện phương pháp PCR-RFLP trên 202 người bệnh và 199 người khỏe mạnh để đánh giá kiểu gen của đa hình này. Kết quả cho thấy sự phân bố của đa hình này tuân theo định luật cân bằng Hardy-Weinberg ($p > 0,05$). Tuy nhiên, chúng tôi không tìm thấy sự liên quan nào giữa đa hình này và bệnh vô sinh nam ở quần thể người Việt Nam ($p > 0,05$).

Từ khoá: g*iải trình tự trực tiếp*, vô sinh nam, *NR5A1*, PCR-RFLP, rs1110061