



The Assessment of 5 Polymorphisms in the *USP9Y* Gene in Infertile Patients with Non-obstructive Azoospermia

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Abstract

Background: Male factor infertility that is the cause of about half of the infertility cases, may occur due to azoospermia. Because spermatogenesis defects may lead to non-obstructive azoospermia (NOA), investigating the factors involved in spermatogenesis, including hormones and genes, is one of the most important aspects in understanding the mechanism of NOA in men.

Objectives: Male infertility is a complex disorder that affects a large proportion of men, yet many of its causes are unknown. Clarifying its genetic basis may help to identify the causes of infertility and provide effective treatment for patients.

Methods: In this case-control study, single nucleotide polymorphisms (SNPs) located in the *USP9Y* gene were investigated. Accordingly, 100 healthy men and 100 NOA patients were regarded as control and case groups, respectively. Testis tissue samples were taken during the testicular sperm extraction (TESE) procedure, DNA extraction was done, and ARMS PCR was designed and performed. The rs3212292, rs2032597, rs2032604, rs2032598, and rs717268 polymorphisms in the *USP9Y* gene were analyzed by Tetra-ARMS PCR. Furthermore, the serum levels of sex hormones were assessed by the ELISA technique. Finally, the obtained data were analyzed by SPSS software.

Results: According to the obtained results, there was no significant difference in case of genotype frequency of investigated SNPs between the case and control groups ($P \geq 0.05$). The mean FSH and LH levels in the control group were relatively lower than the case group ($P \leq 0.05$), whereas no significant difference in the mean testosterone level was observed between the two groups ($P \geq 0.05$).

Conclusions: Polymorphisms of the assessed SNPs showed no effect on the frequency of azoospermia; Thus, polymorphisms did not increase the risk of NOA and also did not have a protective effect against the disease. Also, the results showed that the serum levels of FSH and LH increased in NOA patients.

Keywords: Male Infertility, Non-obstructive Azoospermia, Single Nucleotide Polymorphisms, *USP9Y*, ARMS PCR

1. Background

Azoospermia is one of the most serious reproductive disorders that occurs in 1% of the male population. Various factors are involved in azoospermia; however, its precise etiology is almost unknown (1). Most of these factors are complex gene-environment interactions (2). This reproductive-related disorder is classified as obstructive azoospermia (OA), induced by ejaculatory pathway obstruction, and non-obstructive azoospermia (NOA) caused by a failure in spermatogenesis (3). NOA is a disorder of genetic origin that is represented as a failure of spermatogenesis within the testis, including various reasons, such as chromosomal abnormalities and Y chromosome microdeletions (4). This phenotype has different causes (affecting various functions, such as differentiation of gonads, the activity of the hypothalamic-pituitary axis,

and spermatogenesis), making it a complex and heterogeneous disorder (5). Essential genes involved in spermatogenesis are placed on the proximal area of the Y chromosome (Yp11). Azoospermia factor (AZF) is located in this region, which includes sub-regions of AZFa, AZFb, and AZFc (6). Ubiquitin-specific peptidase 9, Y-link (*USP9Y*), is a functional gene on the AZFa region with a DNA of 170 kb and at least 46 exons. *USP9Y* is responsible for encoding a protein (7) that acts as ubiquitin C-terminal hydrolase, which its elimination is related to azoospermia or violent oligospermia (8). *USP9Y* expression is limited to spermatids, and point mutations in this gene can result in various clinical phenotypes of male infertility, including spermatid maturation arrest, oligospermia, or asthenozoospermia, linked to decreased sperm cell mobility and concentration (9). Also, complete loss of the gene is associated with

NOA (10, 11). In many complex diseases, genetic susceptibilities have been identified through genome-wide association studies (GWAS) using single-nucleotide polymorphism (SNP) arrays (12, 13). There is not enough information about the relationship between the *USP9Y* gene polymorphisms and azoospermia; thus, this study aimed at detecting the relationship between 5 polymorphisms of *USP9Y* and azoospermia susceptibility in the target population.

2. Objectives

Because different genes are responsible for the process of spermatogenesis and the formation of azoospermia, it is important to evaluate the genetic basis of infertility. In this study, we focused on the probable communication between the polymorphisms in the *USP9Y* gene and NOA.

3. Methods

This case-control study was approved by the Academic Center for Education, Culture, and Research (ACECR), Qom province, and the Biomedical Research Ethics Committee (IR.ACECR.JDM.REC.1397.015).

Regarding the azoospermia frequency in the population (1%), and the earlier findings, the minimum sample size was calculated based on the following formula (14):

$$\frac{\left(Z_{1-\frac{\alpha}{2}}\right)^2 p(1-p)}{d^2}$$

Accordingly, 200 men attending the infertility treatment center from August 2016 to August 2017 were divided into the case (NOA) and control groups, and written informed consent was obtained before enrolling in the study. The inclusion criteria were as follows: Participants aged 25 - 35 years old and being physically and anatomically healthy with normal karyotype (Table 1). Healthy people for the control group had at least one healthy child with no more than two years passing since the birth of the last child. Semen from both cases and controls were analyzed according to WHO protocol, and urological examinations were performed for anatomical integrity of the genital system. Also, the NOA of the cases was approved by an urologist. Patients with anatomic disorders of genitalia, testis neoplasms, chromosomal numerical and structural abnormalities, or Y chromosome microdeletions were excluded.

In order to confirm NOA, testicular biopsies of patients who had no sperm according to their semen analysis were assessed by a pathologist. Furthermore, serum levels of LH, FSH, and testosterone were measured using an ELISA kit in the case and control groups.

Table 1. Inclusion Criteria for Non-obstructive Azoospermic Men

Karyotype	46, XY
Microdeletion chromosome Y	NO
Spermogram with centrifuge	No sperm
TESE	No sperm
Age (y)	25 - 35
Varicocele	No
Gonadotropins (chemotherapy/radiation)	No

Genomic DNA was extracted from blood samples using GeneAll® Exgene™ kit. We used the tetra-primer amplification refractory mutation system- polymerase chain (PCR) method, in which two pairs of primers are used to analyze *USP9Y* rs2032597 (A>C), rs2032604 (T>G), rs2032598 (T>C), rs717268 (G>A), and rs3212292 (T>A) genotypes. Primers were designed by Primer1 online software (<http://primer1.soton.ac.uk/primer1.html>). PCR primers are given in Table 2.

Statistical analysis for genotype and allele frequencies, azoospermia association, and hormonal evaluation were performed by the χ^2 and paired *t*-test using SPSS software. A P-value of less than 0.05 was considered to be statistically significant.

4. Results

The mean age of the control and case subjects was 31.12 ± 1.38 and 31 ± 1.688 years, respectively. Table 3 presents the frequencies of genotypes and alleles in 5 SNPs. The χ^2 test showed that there was no significant difference between the genotypic and allelic frequencies between the case and control groups, and these genotypes did not increase the chance of disease or showed no protective effect against NOA. The electrophoresis results of 5 SNPs are shown in Figure 1.

The mean FSH level in the control and case group was 7.43 ± 1.947 and 16.88 ± 2.188 mIU/mL, respectively. The Mann-Whitney U test showed that the observed mean difference was significant ($P < 0.001$), and the FSH hormone increased in cases. The mean LH level was 5.7 ± 1.828 and 12.18 ± 1.471 mIU/ml in the control and case groups, respectively, and the observed difference between the two groups was significant ($P = 0.002$). The mean testosterone level in the control and case group was 4.6 ± 1.262 and 4.83 ± 1.292 ng/dL, respectively. The independent *t*-test indicated that the difference in mean testosterone level observed between the two groups was not significant ($P = 0.849$) (Table 4).

Table 2. Nucleotide Sequences for Designed Primers

Rs	Nucleotide Sequence	PCR Product (bp)
rs3212292		FO-RO = 463; FO-RT = 296; RO-FA = 221
FA (A allele)	339 GCTTGGGATTTTCTCTGGACATCA 364	
RT (T allele)	391 ACCTTAAAGCAATCAAAAAGATGATGAA 364	
FO (5' - 3')	96 TGCCAGGTATTAATGACAGTTCTAAAGCA 125	
RO (5' - 3')	558 TGATTGGAAGAAACAATGACCAATT 531	
rs2032597		FO-RO = 441; FO-RC = 289; RO-FA = 208
FA (A allele)	496 TTTGTTAAATAATTCATGTTTGTC 522	
RC (C allele)	550 ACATAACTTAATAAGAGCTGCAATTACTG 522	
Forward outer primer (5' - 3')	262 TAAATTTTCCATTCTAGTAGTCTTAC 289	
Reverse outer primer (5' - 3')	702 AAAAGAACTAAACTTGCCAATTACTTTC 675	
rs2032598		FO-RO = 318; FO-RT = 174; RO-FC = 197
FC (C allele)	331 ATAATGGCCAGCAATTTAGTATTGACC 357	
RT (T allele)	382 AACAGCACATGCATTAGTAAAAGGCA 357	
Forward outer primer (5' - 3')	209 AAAGAAAAGGCTCTTACATTACAGGACC 236	
Reverse outer primer (5' - 3')	526 GAGCAAGATCCATCTAAAACAAAACA 499	
rs2032604		FO-RO = 505; FO-RG = 265; RO-FT = 296
FT (T allele)	493 TATCCCCAAACCCATTTTGATGCCTT 519	
RG (G allele)	547 GAAAATAATAATTGAAGACCTTTTAAATC 519	
Forward outer primer (5' - 3')	252 AAGATGTTAAAGAGGCCAGCTTTGTG 277	
Reverse outer primer (5' - 3')	756 GGTTACAAAGAATATATCAATGGCAA 729	
rs717268		FO-RO = 373; FO-RA = 204; RO-FG = 226
FG (G allele)	234 ATAGGATGATTATTTCTATGATTCTGAG 263	
RA (A allele)	289 TGTTCTTTACATATGAGATGATGTT 263	
Forward outer primer (5' - 3')	86 AACTTCTGAGTGAATTTCTAGTTGAG 113	
Reverse outer primer (5' - 3')	458 CATAGTACTCAGATGCTGATTATACAAA 431	

5. Discussion

Infertility is a multifactorial issue in which various factors, such as the environment and genetics, can be influential. Studies have shown that 50% of infertilities are related to men. The most important factors in male infertility are genetic factors that affect important physiological processes, such as hormonal homeostasis, spermatogenesis, and sperm quality (15). About 200 genes have been found so far that can regulate spermatogenesis, 30 of which are on the Y chromosome. Two major genetic factors in men's infertilities are Y chromosome microdeletions and chromosomal abnormalities (16, 17). Around 25% of NOA cases can be explained by single genetic abnormalities (including chromosome aberrations and point mutations), with the remaining patients considered idiopathic. It has been indicated that idiopathic NOA has a multifactorial etiology, in which both environmental and genetic factors may con-

tribute to the development of the disease. In these cases, wide variations in the human genome, mainly single nucleotide polymorphisms (SNPs) and copy number variants (CNVs), are likely to confer genetic predisposition, complicating the elucidation of the underlying pathological mechanisms (18, 19). According to the influential advances in infertility treatment using assisted reproductive techniques (ART), which increase the likelihood of transferring genetic abnormalities to the next generations, identification of genetic abnormalities sounds valuable (20). In fact, the frequency of male infertility varies among different geographical regions and ethnic groups. It was found that among azoospermia cases, 83.7% were due to NOA (21). Also, Fogle et al. showed that 93% of azoospermia cases were recorded as NOA (22). However, the most geographical and ethnic reports in male infertility are on Y chromosome microdeletions.

Malekasgar and Masoudi showed that 51.6% of

Table 3. Allele and Genotype Frequencies of rs508485 (T>C) Its Association with the Non-obstructive Azoospermia Risk

rs/Genotype & Allele	Non-obstructive Azoospermia, No. (%)	Control, No. (%)	P-Value	OR (95% CI)
rs3212292 (T>A)			0.346	1.568 (0.612 - 4.019)
A	12 (12)	8 (8)		
T	88 (88)	92 (92)		
rs2032597 (A>C)			0.744	0.899 (0.474 - 1.705)
A	76 (76)	74 (74)		
C	24 (24)	26 (26)		
rs2032598 (T>C)			0.313	1.405 (0.724 - 2.728)
C	26 (26)	20 (20)		
T	74 (74)	80 (80)		
rs2032604 (T>G)			0.192	1.784 (0.742 - 4.292)
G	15 (15)	9 (9)		
T	85 (85)	91 (91)		
rs717268 (G>A)			0.39	1.652 (0.521 - 5.236)
A	8 (8)	5 (5)		
G	92 (92)	95 (95)		

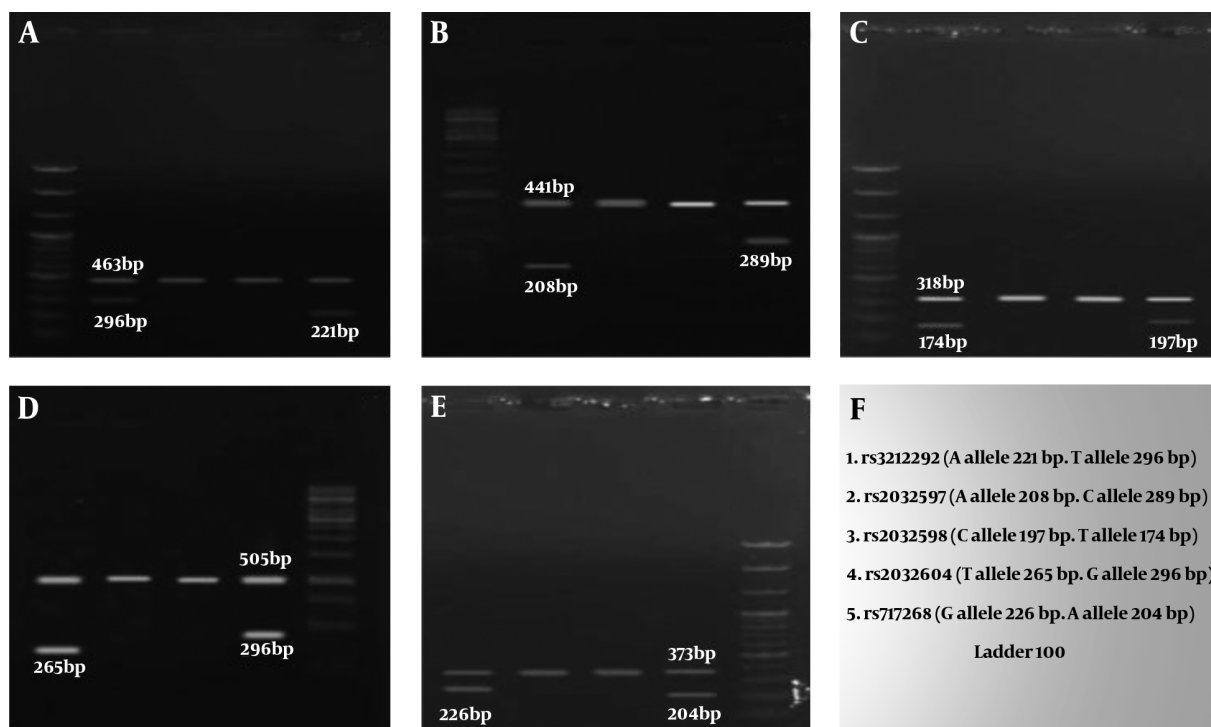
**Figure 1.** Gel electrophoresis

Table 4. Comparison of Hormonal Levels in the Control and Non-obstructive Azoospermic Men

Hormone	Control	Non-obstructive Azoospermia	P-Value
FSH	7.43 ± 1.95	16.88 ± 2.19	< 0.0001
LH	5.7 ± 1.82	12.18 ± 1.47	0.002
Testosterone	4.6 ± 1.26	4.83 ± 1.29	0.849

azoospermic infertile men and 52.6% of severe oligozoospermic patients from north Iran carry Y chromosome microdeletions (23, 24). In the Iranian population, the Y microdeletion frequency, according to the databases from 2003 - 2012, has shown variable ranges from 5 - 52%, which is within the worldwide range (15). This wide range of variability may be the consequence of different factors, such as the sample size, type of patient selection, and the variances among the populations (25). Also, Omrani et al. reported 24% microdeletions in infertile men from West Azarbayjan (26). It should be noticed that our study was performed on 200 participants referring to the infertility treatment center of ACECR in Qom province. One of the genes involved in the spermatogenesis process is the *USP9Y* gene, which was selected to examine in this study. Deletions affecting *USP9Y* were initially highly associated with mild to severe azoospermia or oligozoospermia (27). It was later documented that partial deletion of *USP9Y* in men is correlated with the milder infertility phenotype, indicating a minor effect for *USP9Y* in spermatogenesis (28). A study by Sun et al. on 576 infertile men and 96 healthy men showed that single-gene deletion associated with spermatogenic insufficiency occurred in *USP9Y* (29). Thus, *USP9Y* is not required for normal spermatogenesis, but its effects on fertility may be effective in combination with other genes in the AZFa region (30). In 2000, Foresta et al. analyzed 173 infertile men focusing on the AZFa region and found deletions in 9 patients, of whom one case showed deletions in the *USP9Y* gene (31). There is contradictory evidence about the impact of this gene on infertility and spermatogenesis, and the association between polymorphisms in the *USP9Y* gene and azoospermia has also not been investigated. We tested 5 polymorphisms of the *USP9Y* gene in this study. Some SNPs of this gene have been evaluated and shown to be associated with some other diseases. In a study, Wang and Park showed that the risk of schizophrenia, autism, and prostate cancer could be increased by rs2032598 and rs2032604 SNPs; however, no population-based study on azoospermia has yet been performed (32, 33).

It is of particular interest that the ubiquitin protein encoded by the *USP9Y* gene plays an important role in infertility and can affect the polymorphism of the *USP9Y* gene in male infertilities (34, 35). Studies have shown that the levels of sex hormones, such as LH, FSH, and testosterone were

significantly higher in infertile men in comparison with healthy men. These hormonal changes were much more seen in azoospermic men than in other infertile subgroups (36, 37). These results suggest that the FSH, LH, prolactin, and testosterone levels cannot predict microdeletions in AZF in infertile men. More studies with a larger sample size should be considered to assess these microdeletions (38). Zhao et al. stated that sperm density, total sperm count, number of motile sperm, and percentage of motile sperm were positively associated with the testicular volume (39). However, our results in this study showed a decrease in FSH and LH hormones in the case group compared with the control group. Also, testosterone hormone levels in the case and control groups did not show a significant difference.

5.1. Conclusion

In general, our results showed that in the studied population, the frequency of genotypes and alleles did not play a role in developing NOA. The results of this study showed that one of the important reasons for NOA can be hormonal disorders so that in NOA, the levels of FSH and LH hormones are increased, which indicates that in people with sperm disorders, hormone levels should be checked at first, and then other tests and more detailed investigations should be regarded.

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Footnotes

Authors' Contribution: Study concept and design: N.K., and F.D.; Analysis and interpretation of data: N.K. and F.D.; Drafting of the manuscript: H.F., and A.Sh.; Critical revision of the manuscript: F.D., A.Sh.; Statistical analysis: N.K.

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