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Investigation of the Relationship between *HIWI3* rs11703684 (C>T) Polymorphism and Idiopathic Azoospermia/Oligozoospermia in the Kurdish Population of Kermanshah

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Abstract

Background & Objective: Genetic factors play a major role in the development of male idiopathic infertility. Because of the multi-genetic base of this disease, not all genetic factors have been investigated. *PIWI* genes have been reported to be involved in the regulation of piRNAs in spermatogenesis. Our study assessed the association between *HIWI3* rs11703684 (C>T) gene polymorphism with the risk of male idiopathic azoospermia/oligozoospermia in the Kurdish population of Kermanshah.
Materials & Methods: In this case-control investigation, we included two hundred individuals consisting of 100 men with idiopathic azoospermia/oligozoospermia and 100 fertile men as the control group. To determine genotypes of *HIWI3* C>T polymorphism, we used the Tetra Arms-PCR technique and significant values were considered as $p<0.05$.
Results: Our findings did not show a statistically significant difference in the genotype frequency of the recessive model (P = 0.118; OR = 0.158; CI, 0.019–1.339), dominant (P = 0.169; OR = 0.625; CI, 0.341–1.144) and co-dominant (P = 0.527; OR = 0.778; CI, 0.417–1.450). In addition, the results described a negative difference in allelic frequency of HIWI3 (rs11703684 C>T) in men with idiopathic azoospermia/oligozoospermia and control group (P = 0.288; OR = 0.749; CI, 0.463–1.212).
Conclusion: The current study does not indicate the probability effect of *HIWI3* rs11703684 (C>T) gene polymorphism on the male idiopathic azoospermia/oligozoospermia in the Kurdish population of Kermanshah. The critical role of *PIWI* genes in spermatogenesis and as a candidate risk factor for male infertility remained unknown.

Keywords: Azoospermia, Oligozoospermia, rs11703684, PIWI, *HIWI3* gene, Polymorphism

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Introduction

WHO states that infertility occurs when sexual inability does not allow a couple to achieve a spontaneous pregnancy within a year (1). Some abnormalities in the urinary tract, including congenital or acquired defects, such as malignancy or infections are involved in male infertility. The high temperature of the scrotal and disturbances of the endocrine also are known reasons for male infertility as well as immunologic factors and some known genetic causes (2). Some reasons such as Reactive Oxygen Species (ROS), genetic and epigenetic causes, and environmental pollution that lead to endocrine disturbance are proposed for idiopathic male infertility. Azoospermia occurs in the absence of spermatozoa and a very low amount of sperm

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leads to oligozoospermia (lower than 15 million/ mL). Teratozoospermia is caused by sperms with abnormal morphology and asthenozoospermia shows abnormal mobility sperm (3-5). Azoospermia is responsible for 11% of male infertility and derives into two groups: obstructive (40%) and non-obstructive (60%). The increase in FSH and LH concentration and the small size of the testis seem to be reasons for azoospermia (6). From 74% of all proteins expressed in the human testis, the amount of 1980 proteins is highly expressed and may involve in spermatogenesis. Mutations in mentioned genes may lead to the disturbance of spermatogenic processes and result in male infertility (7-9). These mutations are de novo and do not transfer to the next generation (3). Investigating these genes can clarify male infertility with unknown causes (idiopathic male infertility), which play a major role in infertility. *PIWI* gene (originally P-element-induced wimpy testis in *Drosophila*), encoded by *PIWIL1/HIWI*, *PIWIL2/HILI*, *PIWIL3/HIWI3*, and *PIWIL4/ HIWI2* genes, is a subfamily of argonaute proteins that are combined with some small RNAs in RNA-induced silencing complexes and have a role in piRNA production in germline cells (10-12). While there is no complete knowledge of how the *HIWI3* gene acts in spermatogenesis, some studies have revealed the association of this gene with idiopathic male infertility (13, 14).

Mutations alter the reproductive cells genome and are responsible for most cases of idiopathic infertility. Although single nucleotide polymorphism (SNP) studying of some genes that are involved in spermatogenesis gives us primary data about genetic causes of idiopathic infertility (15) and genome-wide association studies (GWAS) and comparative genomic hybridization-assay (CGH) help us to solve this complication, the genetic base of this kind of infertility remains unknown (16, 17). *TEX11* (X-chromosome meiotic genes) was the first validated gene whose loss of function is associated with non-obstructive azoospermia (18). Also, *ZMYND15*, *TAF4B*, *SYCE1*, *MCM8*, and *TEX15* are the other genes whose mutations are reported in non-obstructive azoospermia. These mutations are rare and it is hard to prove that mutations in these genes are monogenic causes of non-obstructive azoospermia (19-22). Mutations in the genes that cooperate in the synthesis of the cytoskeleton (23) and some of the other genes that are involved in energy metabolism and protein fold and degradation (24), may lead to idiopathic infertility. Post-translational modifications (25), oxidative stress, and DNA fragmentation may also be involved in infertility (26). Gu et al, revealed that individuals with *HIWI3* non-synonymous rs11703684 variant genotypes exhibited a significantly reduced oligozoospermia risk (13). According to the previous studies which have reported the potentially significant effects of nucleotide variation in gene encoding of *HIWI3* in spermatogenesis, a few investigations assessed the association between these SNPs with the risk of idiopathic male infertility (13).

In this study, based on previous information, we considered the *HIWI3* gene to investigate the effect of nucleotide change on idiopathic male infertility of Kurdish men population in Kermanshah city.

Materials and Methods

Samples

The case-control investigation was performed on the two hundred peripheral blood samples of individuals consisting of 100 idiopathic azoospermia/severe oligozoospermia infertile men and 100 healthy fertile men samples, who were referred to Motazedi Hospital in Kermanshah during 2015-2017. The case group, consisting of infertile patients with azoospermia and oligozoospermia with semen less than 5 million/ mL sperm, without detection of routine cytogenetic abnormalities by G-Banding method, without DAZ gene deletions and microdeletion of Y(AZF) chromosome. The patients are diagnosed by medical urology and genetics specialists as idiopathic azoospermia/severe oligozoospermia. The control group was selected from blood samples of men with normal fertility and no infertile family history. The protocols were

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confirmed by the Ahar Branch Islamic Azad University and signed informed consent and questionnaires were received from each case. If the cause of infertility was recognized, the sample was excluded.

DNA extraction and primer design

2mL of peripheral blood samples were taken from all cases and controls after confirmation of their abnormalities by the relevant specialists. First, genomic DNA was extracted by the salting out method and then the quality and quantity of extracted DNA were determined using two methods such as UV absorption analysis by spectrophotometry and agarose gel electrophoresis. To determine the frequency of *HIWI3* C> T gene polymorphism, we used the Tetra ARMS-PCR method. Forward and reverse primers are shown in (Table 1).

Table 1. The primers used in the *PIWIL3* gene polymorphism determination

Primers	Primer sequences	PCR size (bp)
PIWIL3-FI	5`-ACCAATTTTTTGTCCGTCCCGGGAATAG-3`	246
PIWIL3-RI	5`-CTTGCCCGATGTTTGCGTTTTTCAACAT-3`	183
PIWIL3-FO	5`-AGTTTTAGGGAAGGAAGGTACTGAAGAATCT-3`	
PIWIL3-RO	5`-TGAGCAGTCTGAAAAAAGAACAGCAGTCG-3`	374

PCR

In order to do DNA proliferation by Tetra ARMS-PCR, we used 12.5µL of Ampliqon Master Mix 2x (Denmark), 0.5µL of each inner primer (10 pmol/µL), 0.25µL of each outer primer, 100ng of extracted DNA, and 10µL of distillation water in the final volume to 25 µL. PCR included the first denaturation stage for 5min at 95˚C, then 35 cycles by denaturation phase at 95˚C for 30s, annealing phase at 61.5˚ C for 30s, and extension phase at 72˚C for 40s, and final extension phase at 72˚ C for 5min. Agarose 2% gel was used to analyze PCR products. Finally, to validate genotypes, we frequently sequenced 10% of the samples through random selection. PCR steps and material are summarized in (Table 2).

Table 2. PCR condition for gene polymorphism frequency analysis

Number of cycles	Stage	Temperature	Time
1	Initial denaturation	95	5 min
35	Denaturation	95	30 sec
	Annealing	61.5	30 sec
	Extension	72	40 sec
1	Final extension	72	5 min

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Results

Frequency of genotypes and allelic *HIWI3*

In this case-control investigation, the association between *HIWI3* (rs11703684 C>T) polymorphism with the risk of azoospermia/oligozoospermia idiopathic male infertility was assessed. The case group included 100 men with azoospermia/oligozoospermia idiopathic (mean age, 41 ± 2.27 (range 23 to 78), and the control group, 100 healthy men (mean age, 44 ± 2.25 (range 21 to 71). No significant difference in the mean age of groups was observed (P>0.05). The chi-square test was applied to show the difference in genotype of *HIWI3* polymorphism between healthy and patient groups. No significant difference was revealed (P=0.093) between groups (Table 3).

Table 3. The genotype frequencies of HIWI3 rs11703684 gene polymorphism between groups

Polymorphism (rs11703684HIWI3 C>T)	**Group**		**total**	***P***
	Numbers (percent)	Numbers (percent)		
	Healthy (control)	Patient (case)		
healthy homozygote (CC)	64 (46%)	74 (54%)	138	0.093
heterozygote (CT)	30 (55%)	25 (45%)	55	
Mutated homozygote (TT)	6 (86%)	1 (14%)	07	

The finding revealed that *HIWI3* (rs11703684 C>T) in models (CT vs CC+TT OR=0.778, CI 95%=0.417-1.450, p=0.527) and (CC vs CT+TT OR=0.625, CI 95%=0.341-1.144, P=0.169) and (TT vs TC+CC OR=0.158, CI 95%=0.019-1.339, p=0.118) are not associated with the risk of azoospermia/oligozoospermia idiopathic male infertility. In addition, our findings showed that the T allele of the *HIWI3* gene (rs11703684C>T) was negatively associated with increased susceptibility to male infertility (OR = 0.749; CI, 0.463–1.212, P = 0.288) (Table 4).

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Table 4. The frequency of wild and mutated alleles of *HIWI3* gene (rs11703684 C>T) in case and control groups

P	OR		CI (95%)	Total	Group		C>T(rs11703684*HIWI3*) polymorphism
	down	up			Numbers (percent) Healthy (control)	Numbers (percent) Patient (case)	
0.527	0.417	1.450	0.778				Co-dominantpattern
				55	30 (55%)	25 (45%)	CT
				145	70 (48%)	75 (52%)	CC+TT
0.169	0.341	1.144	0.625				Dominant pattern
				138	64 (46%)	74 (54%)	CC
				62	36 (58%)	26 (42%)	CT+TT
0.118	0.019	1.339	0.158				Recessive pattern
				7	6 (86%)	1 (14%)	TT
				193	94 (49%)	99 (51%)	CC+CT
0.288	0.463	1.212	0.749	69	42 (61%)	27 (39%)	Frequency of mutated T allele
				331	158 (48%)	173 (52%)	Frequency of wild C allele

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Discussion

Male infertility is not a simple and monogenic disease. There are many known and unknown causes that are involved in this multifactorial disorder (3, 27, 28). Morphological factors causing the disease have almost been diagnosed but the molecular mechanisms underlying the disease, which are the major causes of idiopathic male infertility are still unknown (29, 30). So, the study of these molecular mechanisms and interpretation of how the disease is made is useful for finding the disorder marker as well as personal treatment of idiopathic infertile men (31, 32). Previous studies state that many genes are responsible for coding proteins which are involved in spermatogenesis (33, 34). Also, other studies emphasized the role of non-coding RNAs in spermatogenesis. PIWI and TDRD proteins are associated with the evaluation and function that are most important non-codding mRNAs which have a critical role in spermatogenesis (35, 36). Therefore, mutation and polymorphism in these non-coding genes can play an important role in incomplete spermatogenesis and consequently male infertility. Because of the crucial role of PIWI proteins in the expression of piRNA molecules, special attention should be paid to the relationship between genetic alterations and the overall expression of mature and processed piRNA molecules in human testicular tissue (37). The results of the current study revealed that rs11703684 for the *PIWI3* gene is significantly associated with the idiopathic male infertility of the Kermanshah Kurdish population. AihuaGu et al. announced that a single nucleotide polymorphism (SNP) in the *PIWI* gene is more relevant to oligozoospermia rather than azoospermia. These SNPs operate with other genetic factors that eventually lead to azoospermia (13). Numerous shreds of evidence have shown that the presence of polymorphism will have only minor effects, while the presence of multiple genetic alterations in a single gene will have a far greater impact. Besides, based on previous findings, spermatogenesis is a multi-gene (polygenic) process and in the future individuals at high risk of infertility can identify it by analyzing and combining multiple factors (13). There are not many studies analyzing the effect of single nucleotide changes on idiopathic male infertility. Also, these few numbers of results have not provided accurate and explicit information to the scientific community because conflicting results have been reported, which may be due to genetic differences between different populations. AihuaGu et al. assessed nine SNPs of four *PIWI* genes, through the Taqman method, to analyze the effects of these SNPs on 490 azoospermia/oligozoospermia idiopathic infertile men and 468 fertile controls from China population. The results revealed that rs508485 from the *HIWI2* gene and the non-synonymous rs11703684 variant of *HIWI3* are associated with increased and decreased risk of azoospermia/ oligozoospermia idiopathic infertility, respectively (13). Our achievements, in a different study, conflicted with their findings in the case of the *HIWI2* gene and it may be due to differences in the population genome or volume of samples (38). But, our results in this study confirmed their findings in the case of the *HIWI3* gene. Zeebakamaliyan et al, in a case-control comparison, studied 226 non-obstructive azoospermia patients and 200 fertile males, from the Iranian population of Tehran to investigate the relationship between *HIWI2* rs508485 (T>C) and *HIWI3* rs11703684 (C>T) polymorphisms and idiopathic non-obstructive azoospermia. They revealed that *HIWI2* rs508485 (T>C) is associated with the non-obstructive azoospermia and a significant difference was observed in the dominant model but

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they do not show a significant difference between case and control groups for *HIWI3* rs11703684 (C>T) polymorphism (39). Our observations were also compatible with their findings and stated that *HIWI3* rs11703684 (C>T) polymorphism is significantly associated with azoospermia/oligozoospermia idiopathic infertility. We also exhibited that no significant difference is obvious in the three genetic models. The present study is the first study on the Kurdish population of Kermanshah, and the most important reason for consistency with past findings may be reflected in the non-significant effects of genetic variation for male infertility of these populations. On the other hand, Kamaliyan et al study was performed only on patients with non-obstructive azoospermia, and our study was on patients with idiopathic azoospermia/oligozoospermia. As previously mentioned, the association of single-gene mutations with oligozoospermia is not correlated with idiopathic azoospermia/oligozoospermia. Based on our findings, it can be concluded that *HIWI3* rs11703684 (C>T) polymorphism does not have a significant correlation with idiopathic azoospermia/oligozoospermia. This polymorphism cannot be identified as the main cause of the disease but in combination with the other genetic factors it is assessed as a probability risk factor of disease. To gain a more comprehensive interpretation of the effect of polymorphisms on infertility, increasing the number of study samples, increasing the number of SNP studied, and study of different states of the disease are suggested.

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Conflict of Interest

None.

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Ethical Considerations

This study were confirmed by Ahar Branch Islamic Azad University.

Code of Ethics

Code of this article is 932012.

Author's Contributions

S.S performed the molecular lab work and drafted the manuscript. S.GH, R.A, and F.K reviewed the procedure, study's design, and assisted with the manuscript's drafting. S. G. provided critical revisions. S.GH, R.A, and F.K made significant editions to the manuscript. The manuscript's published version was read by all authors, and they approved it.

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