Original Article

Association of Single Nucleotide Polymorphisms in the *PYGO2* and *PRDM9 G*enes with Idiopathic Azoospermia in Iranian Infertile Male Patients

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What's Known

- Infertility due to azoospermia is a global problem. Among the various factors, genetic factors are associated with non-obstructive azoospermia (NOA). However, the main cause of azoospermia has not been elucidated despite tremendous research efforts.
- *PYGO2* and *PRDM9* are the main two genes involved in the spermatogenesis process.

What's New

- Single nucleotide polymorphism (SNP) in the *Pygo2* gene (rs61758740) is significantly associated with NOA.
- In the case of *PRDM9*, SNPs rs2973631, and rs1874165 are of clinical importance in the diagnostic prediction of azoospermia.

Abstract

Background: Azoospermia is a risk factor for infertility affecting approximately 1% of the male population. Genetic factors are associated with non-obstructive azoospermia (NOA). *Pygo2* and *PRDM9* genes are involved in the spermatogenesis process. The present study aimed to assess the association of single nucleotide polymorphism (SNP) in the *Pygo2* (rs61758740 and rs61758741) and *PRDM9* (rs2973631 and rs1874165) genes with idiopathic azoospermia (IA).

Methods: A cross-sectional study was conducted from October 2018 to August 2019 at Rooya Infertility Centre (Qom, Iran). A total of 100 infertile patients with NOA and 100 men with normal fertility were enrolled in the study. Tetra-primer amplification refractory mutation system-PCR method was used to detect SNPs rs61758740, rs61758741, and rs2973631. The restriction fragment length polymorphism method was used for SNP rs1874165. In addition, luteinizing, follicle-stimulating, and testosterone hormone levels were measured.

Results: The results showed a significant increase in luteinizing and follicle-stimulating hormone levels in the patient group (P<0.001), but a non-significant difference in testosterone levels in both groups. SNP rs61758740 (T>C) was associated with the increased risk of azoospermia (OR: 2.359, 95% Cl: 1.192-4.666, P=0.012). SNP rs2973631 showed a significant difference in genotype frequency between the patient and control groups in the dominant, recessive, and codominant models. However, in the case of SNP rs1874165, the difference was significant in the dominant, codominant, and overdominant models.

Conclusion: There is an association between azoospermia and SNPs in *Pygo2* and *PRDM9* genes in Iranian infertile male patients with IA. SNPs can be considered a risk factor for male infertility.
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Introduction

Approximately half of all infertility cases are due to male factors and suboptimal sperm parameters. Azoospermia is just one

of such factors caused by various reasons, including testicular failure, i.e., partial or complete sperm production disorder.[1, 2] Non-obstructive azoospermia (NOA) accounts for approximately 60% of azoospermia cases. However, the cause has not been elucidated despite tremendous research efforts.[3]

Several infertility factors for NOA have been identified, including the involvement of autosomal genes.[4] One such gene is Pygopus 2 (*Pygo2*), shown to play a prominent role in idiopathic azoospermia (IA). The *Pygo2* gene is located on chromosome 1q21.3 and has three exons and a 3180 bp RNA transcript. Pygopus (PYGO) is a member of the conserved C-terminal plant homeodomain (PHD) finger protein family with two mammalian homologs, namely *Pygo1* and *Pygo2*.[5] The *Pygo2* protein is located in the nucleus of human cells and has two domains, the C-terminal PHD zinc finger motif and the N-terminal homology domain (NHD). *Pygo2* is a transcriptional coactivator of the Wnt pathway through its PHD. Together with other transcription factors (BCL9, ß-catenin, LEF/TCF), they form a transcription complex that activates the expression of specific target genes. NHD is involved in histone methylation and transcriptional activation by conscripting histone modification factors.[6] It has been reported that *Pygo2* is expressed in the elongating spermatid during chromatin remodeling.[7, 8] An animal study has shown that reduced *Pygo2* mutations during sperm production could lead to a selective reduction in protamine and significantly reduce acetylated histone H3 levels, ultimately leading to reduced sperm count and thus male infertility.[6]

Mutation in the *PRDM9* gene is also considered to play an important role in IA. This gene is located on chromosome 5p14.2 and contains 11 exons encoding two types of RNA, and two different types of proteins. This gene encodes a zinc finger regulatory protein that activates histone H3 lysine 4 trimethylation (H3K4me3) during the meiotic prophase. *PRDM9* is responsible for positioning recombination hotspots during the first stage of meiotic cell division and functions through a DNA-binding sequence of the meiosis-specific histone methyltransferase.[9, 10] In the case of *PRDM9* mutation in some species, including humans, the site of recombination that deals with double-strand breaks (DSBs) in DNA cannot be determined by a specific DNA sequence motif. It has been suggested that *PRDM9* is highly expressed in human testis, resulting in complete meiotic arrest.[11]

Given the above, for the first time, the present study aimed to assess the association of single nucleotide polymorphism (SNP) in the *Pygo2* (rs61758740 and rs61758741) and *PRDM9* (rs2973631 and rs1874165) genes with IA in Iranian infertile male patients.

Patients and Methods

A cross-sectional study was conducted from October 2018 to August 2019 at Rooya Infertility Centre (Qom, Iran). The study was approved by the Research Ethics Committee of Islamic Azad University of Medical Sciences, Tehran Medical Branch, Tehran, Iran (IR.IAU.PS.REC.1398.316). Written informed consent was obtained from all participants.

Sample Collection

A total of 100 infertile patients with NOA and 100 men with normal fertility were enrolled in the study. The inclusion criterion for infertile patients was azoospermia evaluated by an *in vitro* fertilization (IVF) laboratory and confirmed by an andrologist. Patients with no sperm in the ejaculate due to reduced sperm production in the testes were considered azoospermic.[4] The exclusion criteria were patients with a history of radiation or chemotherapy, varicocele, Klinefelter syndrome, cystic fibrosis, and Y chromosome deletions. The inclusion criteria for men with normal fertility (control group) were having at least one child and no family history of infertility.

Peripheral blood samples (3 mL) of all participants were collected in ethylenediaminetetraacetic acid (EDTA) tubes and stored at -20 °C. Luteinizing hormone (LH), follicle-stimulating hormone (FSH), and testosterone levels were measured using an electrochemical luminescence (ECL) kit (Roche, Switzerland).

DNA Isolation

DNA was extracted from the peripheral blood leukocytes in accordance with the standard salt precipitation method.[12] DNA quantification was evaluated using a NanoDrop™ OneC spectrophotometer (Thermo Fisher Scientific Inc., USA) at the wavelengths of 260 nm and 280 nm. The quality of DNA was confirmed using 1% agarose gel electrophoresis (Javaherian company, Iran).

Primer Design

Tetra-primer amplification refractory mutation system-PCR (4P-ARMS-PCR) method was used for the detection of SNPs in the *Pygo2* (*r*s61758740 and rs61758741) and *RPDM9* (rs2973631) genes. In a single PCR reaction, two inner primers and two outer primers were

combined. For SNP rs1874165, due to the lack of a suitable restriction enzyme, the restriction fragment length polymorphism (RFLP) method was used. The desired primers were designed using the web service for 4P-ARMS-PCR (http://primer1.soton.ac.uk/primer1.html). The specificity of the primers was checked using the basic local alignment search tool (BLAST) web service (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The primer sequences are shown in tables 1 and 2.

PCR Amplification

Each PCR contained 4 µL of genomic DNA, 10 µL of Taq DNA Polymerase 2× Master Mix RED (Amplicon, Denmark), and optimized concentrations of each primer in a final volume of 25 µL. Amplification was performed on a thermocycler (Kyratec, Australia) with the following sequence: 95 °C for five min for primary denaturation, denaturation at 93 °C for 30 s, annealing at the optimum temperature for each primer for 50 s (table 1), and extension at 72 °C for 45 s for a total of 35 cycles, with a final extension at 72 °C for 10 min. Then, 10 µL of PCR products were separated by running 1% agarose gel with a 100 bp DNA size marker, followed by ethidium bromide staining. DNA bands were visualized and analyzed using an ultraviolet transilluminator (Upland, USA). To confirm the accuracy of the genotypes, 10% of the samples were sequenced.

PCR-RFLP Method

The PCR-RFLP method was used for the amplification of SNP rs1874165 (C>T) in the *RPDM9* gene. The restriction enzyme MboII (Takara, Japan) was selected using NEBcutter software version 2.0 (BioLabs, USA). DNA was run on 2% agarose gel, stained, and visualized as described above. In order to check the accuracy of digestion, 10% of samples were sequenced. Primer sequences, annealing temperature, and fragment length are shown in table 2.

Statistical Analysis

The Kolmogorov-Smirnov test was used to evaluate normal levels of FSH and LH. The independent *t* test and Mann-Whitney U test

Table 1: Sequences for *Pygo2* gene primers, product size, and tetra-primer amplification refractory mutation system-PCR annealing temperature

Pygo2 gene	Primer sequence	Product size (bp)	Annealing temperature (°C)
rs61758740	Forward outer primer: TGGGTGAGATCATGGGACCAAATCCCCC	Control fragment: 353	58
	Reverse outer primer: AGATGACTTCGGAGCCCCCAAAGTGGGG	T allele: 214	
	Forward inner primer: TGGGGGGGCATGTTGAAAGCAGGGACT	C allele: 193	
	Reverse inner primer: CCACCCCCCTTCCCTCCCAATCCTCTG		
rs61758741	Forward outer primer: GTGCCAAGCTGTTGGCATCTGGAGTGC	Control fragment: 310	57
	Reverse outer primer: GGCCCACCTTCTCTGTCCCAACGATTTG	T allele: 174	
	Forward inner primer: GCAGTAGAAGCAGGTGGATTCAAGGGATC	C allele: 192	
	Reverse inner primer: CTGGCCCTGGTGGTGAGGATGGGGTGA		

Table 2: Sequences for *PRDM9* gene primers, product size, and tetra-primer amplification refractory mutation system-PCR annealing temperature

PRDM9 gene	Primer sequence	Product size (bp)	Annealing temperature (°C)
rs2973631	Forward outer primer: GTCCATTAGGAGAAGAAGCCACACTTCC	Control fragment: 422	57
	Reverse outer primer: AATCCGCCACTTTCATGACTAGAGATGA	A allele: 217	
	Forward inner primer: TGGACTCTTCTTGCCTTCAGTGATAATGA	G allele: 260	
	Reverse inner primer: AACCCCAAGTGTGTACCTCATTCGTC		
rs1874165	Forward primer: GTCCATTAGGAGAAGAAGCCACACTTCC	CC: 214, 210	50
	Reverse primer: TGAGGGAGGCATAATGATAA	TT: 424	
		CT: 424, 214, 210	

were used to assess the normal and non-normal distribution of data, respectively. Hardy-Weinberg equilibrium (HWE) was estimated using the Chi square test to compare the frequency of the observed and expected genotypes in the patient and control groups. To assess the effect of SNPs on the risk of azoospermia, the odds ratio (OR) and 95% confidence interval (CI) were determined using unconditional logistic regression analysis. Data were analyzed using SPSS software, version 24.0 (SPSS, Chicago, IL, USA) and SNPstat web service (https://www.snpstats.net; Gencat, Spain). P<0.05 was considered statistically significant.

Results

The mean age of the participants in the patient (n=100) and control (n=100) groups was 29.37±1.54 and 29.31±1.53 years, respectively. There was no significant difference in the mean age between the groups (P=0.783). FSH and LH levels were significantly higher in the patient group than the control group (P<0.001). Notably, there was no significant difference in the testosterone level between the groups (P=0.2) (figure 1).

Based on the HWE, the *Pygo2* SNP rs61758740 of the participants within and between the patient group and control group were in equilibrium (P=0.059). There was a significant difference in the observed frequency in the patient group compared to the control group (P=0.012). The calculated OR indicated that individuals with CT genotype were 2.359 times more likely to develop azoospermia. The genotype and allele frequencies of SNPs are listed in table 3. In terms of *Pygo2* SNP rs61758741, the study population in the control group was in equilibrium (P=0.118). However, they were not in equilibrium in the patient group and combined group (P=0.012 and P=0.048, respectively). The observed difference in genotype frequency between the participants in the patient and control groups was similar (P=0.355). The calculated OR indicated that individuals with CT genotype were 1.332 times more likely to develop azoospermia (table 4). In terms of *PRDM9* SNPs rs2973631 and rs1874165, the study population in the patient and control groups was not in equilibrium.

Four different genetic models (dominant, recessive, codominant, and overdominant) were analyzed using the unconditional logistic regression test. The results for *PRDM9* SNP rs2973631 showed a significant difference in genotype frequency in the dominant, recessive, and codominant models between the patient and
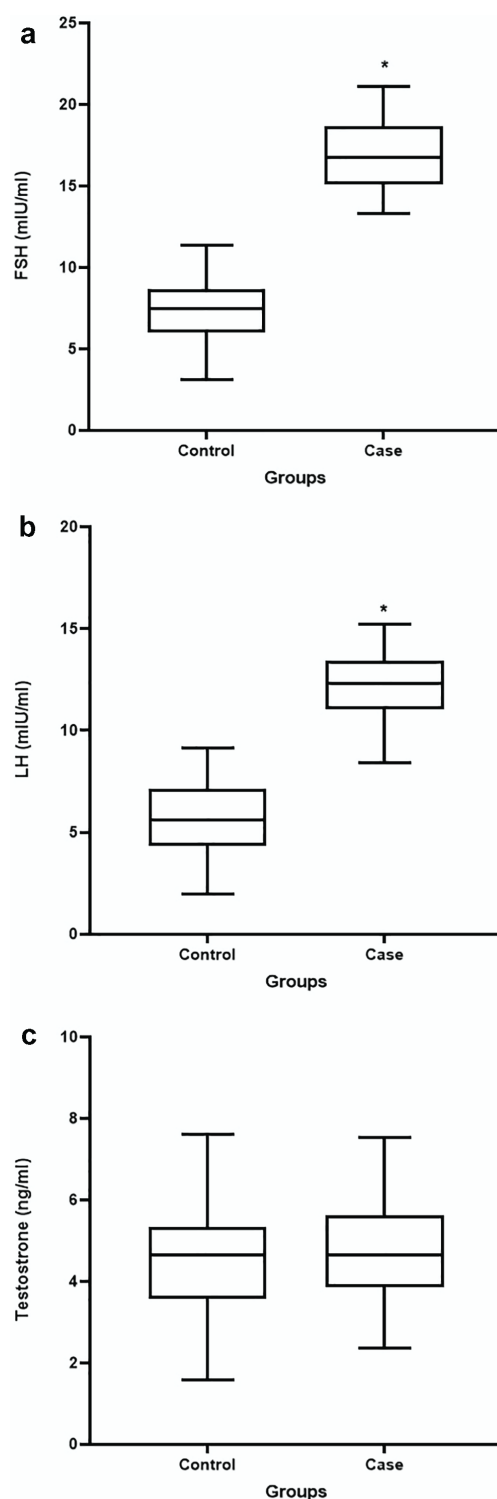
Figure 1: These figures show (a) FSH, (b) LH, and (c) testosterone levels in the control and patient groups. FSH and LH were analyzed using the Mann-Whitney U test and testosterone with an independent *t* test. Data are expressed as mean±SD. *P<0.001.

control groups (table 5). However, for *PRDM9* SNP rs1874165, the results showed a significant difference in the dominant, codominant, and overdominant models between the groups (tables 6 and 7).

Minor allele frequencies for the SNPs in the

PYGO2 and *PRDM9* genes were rs61758740: C>T (T=0.0018/9; 1,000 genomes), rs61758741: T>C (C=0.0004/2; 1,000 genomes), rs2973631: C>T (T=0.1116/559; 1,000 genomes), and rs1874165: C>T (C=0.2041/1022; 1,000 genomes) (table 7).

Table 3: Allele and genotype frequencies of rs61758740 (C>T) and association with the risk of azoospermia

Pygo2 rs61758740 (C>T)		Patient group (n, %)	Control group (n, %)	OR (95% CI)	P value
Genotype	CC	69 (69%)	84 (84%)	2.359 (1.192-4.666)	0.012
	CT	31 (31%)	16 (16%)		
Allele	C	169 (84.5%)	184 (92%)	2.109 (1.114-3.994)	0.02
	T	31 (15.5%)	16 (8%)		

Data were analyzed using an unconditional logistic regression test. OR: Odds ratio, CI: Confidence interval

Table 4: Allele and genotype frequencies of rs61758741 (C>T) and association with the risk of azoospermia

Pygo2 rs61758741 (C>T)		Patient group (n, %)	Control group (n, %)	OR (95% CI)	P value
Genotype	TT	67 (67%)	73 (73%)	1.332 (0.726-2.444)	0.355
	TC	33 (33%)	27 (27%)		
Allele	T	167 (83.5%)	173 (86.5%)	1.266 (0.73-2.197)	0.401
	C	33 (16.5%)	27 (13.5%)		

Data were analyzed using an unconditional logistic regression test. OR: Odds ratio, CI: Confidence interval

Table 5: The genotype relative risk of azoospermia for rs2973631 in *the PRDM9* gene

Model	Genotype	Patient group (n)	Control group (n)	OR (95%CI)	P value
Codominant	AA	57	40	1.00	0.014
	AG	30	32	0.658 (0.346-1.25)	
	GG	13	28	0.326 (0.151-0.705)	
Dominant	AA	57	40	1.00	0.016
	AG-GG	43	60	0.503 (0.286-0.883)	
Recessive	GG	13	28	2.603 (1.257-5.391)	0.009
	AG-AA	87	72	1.00	
Overdominant	AA-GG	70	68	1.00	0.76
	AG	30	32	0.911 (0.5-1.695)	
Allele	A	144	112	0.495 (0.326-0.75)	0.001
	G	56	88		

Data were analyzed using an unconditional logistic regression test. OR: Odds ratio, CI: Confidence interval

Table 6: The relative risk of genotypes for azoospermia of rs1874165 in *the PRDM9* gene

Model	Genotype	Patient group (n)	Control group (n)	OR (95%CI)	P value
Codominant	CC	65	44	1.00	0.005
	CT	20	40	0.338 (0.175-0.654)	
	TT	15	16	0.635 (0.285-1.415)	
Dominant	CC	65	44	1.00	0.003
	CT-TT	35	56	0.423 (0.239-0.748)	
Recessive	TT	15	16	1.079 (0.502-2.322)	0.845
	CT-CC	85	84	1.00	
Overdominant	CC-TT	80	60	1.00	0.002
	CT	20	40	0.375 (0.199-0.706)	
Allele	C	150	128	0.593 (0.385-0.912)	0.017
	T	50	72		

Data were analyzed using an unconditional logistic regression test. OR: Odds ratio, CI: Confidence interval

Table 7: Minor allele frequency and Hardy-Weinberg equilibrium of the study population

SNP	MAF	HWEP
rs61758740	0.0018	0.059
rs61758741	0.0004	0.012
rs2973631	0.1116	0.0001
rs1874165	0.2041	0.0001

SNP: Single nucleotide polymorphism; MAF: Minor allele frequency; HWEP: Hardy-Weinberg equilibrium P value; HWE was determined using the $\chi 2$ test.

Discussion

For the first time, we investigated the association of SNPs in the *PYGO2* and *PRDM9* genes with azoospermia in Iranian infertile male patients with IA. The results showed a significant increase in the FSH and LH levels in the patient group compared to the control group. However, the difference in testosterone levels between the groups was not significant. These results confirm that the cause of azoospermia in our patients was not pre-testicular.

Male fertility is a very complex process, and about 2,000 genes act via multiple signaling pathways to regulate spermatogenesis and support testicular development.[13] In humans, meiosis is controlled by many genes that are expressed throughout or at specific stages of spermatogenesis.[14] Previous studies investigated the role of genetics in spermatogenesis and male infertility, reporting that genetic factors account for 15%-30% of male infertility cases.[1, 15] One of the causes of infertility is azoospermia, where the IA type is a complex condition of unknown etiology. IA is a multifactorial disease with a genetic and epigenetic background. Epigenetic dysfunction refers to abnormal histones and protamine modification or abnormal DNA methylation, hypermethylation, and chromatin remodeling.[16] The *Pygo2* gene is expressed during chromatin remodeling in sperm cells.[5, 17] Our results on SNPs in the *pygo2* gene showed that individuals with CT genotype of SNP rs61758740 (M141I, missense variant) and SNP rs61758741 (K261E, missense variant) are significantly more likely to develop azoospermia. However, the results of the Chi square test for SNP rs61758740 showed a difference in the frequency of the observed and expected genotypes between the patient and control groups. In line with our findings, Ge and colleagues also identified these two non-synonymous SNPs in men with azoospermia and severe oligospermia. SNP rs61758740 (located at nucleotide position 2206) causes the replacement of a hydrophobic by a hydrophilic amino acid. Whereas, SNP rs61758741 (located at nucleotide position 2564) causes the replacement of basic amino acid with an acidic one.[7]

Previous animal studies have reported that *Pygo2* mutations during sperm production could lead to a selective reduction in protamine. It ultimately reduces the expression of H1fnt, PRM1, and PRM2 genes after meiosis, resulting in lower sperm count and male infertility by disrupting chromatin remodeling.[5, 18] In most mammals, DSBs and recombination in germ cell meiosis occur at preferred locations called hotspots, whose sites become marked by H3K4me3. The protein that determines these hotspots is histone methyltransferase *PRDM9*.[19] Chen and colleagues highlighted the important role played by PRDM9-mediated H3K4me3 in directing DSB fate and control of crossover homeostasis.[10] Zhang and colleagues showed that the dependence of normal synapses on recombination is a function of *PRDM9* in spermatocytes.[20] Therefore, *PRDM9* plays an important role in male infertility and testicular dysfunction.

The findings of the current study indicated that there is a significant difference in the *PRDM9* SNPs rs1874165 and rs2973631 between patients with azoospermia and healthy controls. The results of logistic regression analysis showed a significant difference in both SNPs in the *PRDM9* gene between the control and patient groups, at least in the codominant and dominant models. This is indicative of different variants of these SNPs in the Iranian male population with NOA, which could be associated with defective spermatogenesis. In contrast, He and colleagues reported no association between these SNPs and azoospermia.[9] The discrepancy could be due to several factors such as sample size, ethnicity, and study method. In an animal study, Mihola and colleagues showed that histone methyltransferase *PRDM9* is not essential for meiotic recombination in male mice. They stated that it can occur at sites of non-PRDM9-mediated H3k4me3, such as gene promoters and other functional sites.[21] However, in humans, the exact role of the *PRDM9* gene during meiosis is not clear. Nonetheless, some researchers confirmed the important role of this gene in determining DNA recombination initiated by DSBs.[22] Based on the position of both SNPs in the non-coding regions (rs2973631 at 3'UTR and rs1874165 at intron), the expression of the *PRDM9* gene may be affected by the spatial pattern of variation.

It is recommended to conduct further studies on *PYGO2* and *PRDM9* genetic variants and coding sequences in a large population of patients with azoospermia to establish a possible association between direct mutations and defective spermatogenesis.

Conclusion

There is an association between azoospermia and the SNPs in the *Pygo2* and *PRDM9* genes in Iranian infertile patients with IA. Considering a significant difference in the genotype frequency

between the patient and control groups, *Pygo2* SNP rs61758740 is a suitable prognostic criterion for azoospermia in infertile men. *PRDM9* SNPs rs2973631 and rs1874165 are also of clinical importance in diagnostic prediction.

Authors' Contribution

S.S.M, K.K.S, M.R, Z.P: All the authors had a substantial role in designing and conducting the work as well as analyzing and interpreting of the data. Drafting and revising of the article were also conducted by participating of them. All authors have read and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflict of Interest: None declared.

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