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Original article

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Gene Polymorphism, Microdeletion, and Gene Expression of *PRM1*, *PRM2*, *AZFc* in Infertile Males

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Abstract

Background: Background: Male infertility contributes to roughly 15% of all infertility cases in couples. The most common cause of male infertility is azoospermia, which is caused by genetic mutations. The connection between various single nucleotide polymorphisms in the *PRM* genes and AZF region microdeletions with male infertility has not been reported.

Methods: In this case-control study, 100 infertile males (33 with azoospermia, 48 with oligozoospermia, and 19 with severe oligozoospermia) were chosen as the study subjects, and 100 fertile males were selected. Total DNA from peripheral blood was used to amplify two sequence-tagged site markers through multiplex PCR to detect AZFc partial deletions, and SNPs in *PRM1* and *PRM2* were determined through PCR-RFLP. Furthermore, quantitative real-time PCR was conducted to evaluate *PRM1*, *PRM2*, and *DAZ1* (found in the AZFc region) expression levels in testis tissue.

Results: The frequency of the rs779337774 SNP in the *PRM2* gene in the study population had no significant differences. However, a significant association was observed between the rs737008CA genotype (P= 0.013) and the C allele (P= 0.025) as a risk factor for male infant mortality. The deletion of sY254 and sY255 was discovered in azoospermia and severe oligozoospermia patients. Furthermore, all of these genes showed considerably low expression levels. However, only *DAZ1* was identified with diagnostic biomarker potential (AUC=0.742).

Conclusions: When these genes expression levels are reduced, the likelihood of spermatozoa retrieval in azoospermic individuals is elevated. Furthermore, no significant association was observed between PRM2 polymorphism and azoospermia; however, the CA genotype of PRM1 polymorphism is significantly associated with azoospermia incidence.

Keywords: AZFc, Gene expression, Male infertility, Polymorphism, PRM1, PRM2.

Introduction

Infertility affects 15% of marriages worldwide, and reproductive loss can be distressing for patients (1). Male infertility based on problems in spermatogenesis is significantly more challenging to treat than female infertility, which is frequently managed with hormone therapy to increase oocyte development (2). Congenital causes, such as Y chromosome AZF region microdeletion (AZF Del), only account for a few cases (3). Azoospermia is defined as the absence of spermatozoa on microscopy in the post-centrifuged ejaculate, and it can be present in up to 20% of infertile men (4, 5). The two major classifications of azoospermia, are obstructive and nonobstructive (6). Contrary to the obstructive azoospermia (OA) phenotype, which exhibits normal spermatogenesis, nonobstructive azoospermia (NOA) men's spermatogenesis is disrupted as a result of exposure to harmful

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substances or defective testicular evolution (7). The generation of hormones and spermatogenesis are retained in obstructive OA with normal testicular function. Physical blockage of the post-testicular vaginal canal is the pathophysiological basis for OA. Normal spermatogenesis occurs in the seminiferous tubule; however, this obstruction may occur in any area of the sperm excurrent ductal system. The blockage could be caused by a vasectomy, epididymal inflammation, hereditary conditions, or prostatic cysts (8). 10% to 15% of male infertility is caused by NOA (9). Due to minimal or absent spermatogenesis, NOA is defined by the lack of viable ejaculated spermatozoa (10). Nonobstructive azoospermia (NOA) may have several etiologies, some of which may be congenital in the form of abnormalities in gonadotropin levels or testicular failure (4). obstructive azoospermia (OA) is a condition where sperm concentrations decrease underneath the minimum reference limit of 15 million sperm/ml of ejaculate. There are three levels of oligozoospermia: severe (less than 5 million sperm/ml), moderate (5–10 million sperm/ml), and mild (10–15 million sperm/ml) (11).

Most of nuclear sperm protein is composed of protamine (PRM) genes, which are only expressed in the testis (12). The transition protein 2 gene (*TNP2*) and the protamine 1 gene (*PRM1*) are protamine genes located on chromosome 16: 16p13.3 and form a multigenic cluster. The repeating alanine elements seen at methylation sites are present in the matrix attachment regions (MARs) surrounding this cluster. These MARs play a crucial role in the proper controlling of protamine gene expression, regardless of the methylation state. The *PRM1-PRM2-TNP2* gene loci have a TATA-box, which is critical for the beginning of transcription by making it easier for transcription factors to bind to the promoter (13).

Protamine 1, found in all vertebrate species, and protamine 2 (*PRM2*), which only existed in mice and humans, are two kinds of protamine identified in mammals (14). DNA packaging, chromosome condensation, spermatogenesis, and sperm maturation are only a few crucial processes in which protamine genes play a role (15). Therefore, any alteration in the protamine genes underlying the DNA sequence may affect the sperm parameter and result in male infertility (16). It has been demonstrated that sperm count, motility, and morphology were all negatively impacted by protamine deprivation, leading to significant spermatogenesis disturbances and male infertility (14).

The genes required for typical spermatogenesis are located in the region of Yq11, known as the azoospermia factor (AZF) locus. Three significant locations called AZFa, AZFb, and AZFc are included in this locus (17, 18). The AZFa area was linked to Sertoli-cell-only syndrome (SCOS), the AZFb region to meiotic arrest (MA), and the AZFc region to a variety of phenotypes, including severe oligozoospermia (SOZ) and secretory azoospermia (SAZ) (18, 19). The deleted azoospermia (*DAZ*) gene family, which has been identified with four loci (*DAZ1*, *DAZ2*, *DAZ3*, and *DAZ4*), is found inside the AZFc area (20). This gene family interacts with several proteins to govern the translation of particular mRNAs in pre-meiotic germ cells, which controls appropriate spermatogenesis (21).

It was shown that deletions of the *DAZ1/DAZ2* gene doublet caused SOZ, with the potential to evolve into SAZ with various testicular morphologies (22). However, it was eventually revealed that only *DAZ1* was responsible for this phenotype. Recent research looked at the effects of AZF microdeletions on infertility therapies, including testicular and ejaculated sperm in patients (23). This investigation aimed to demonstrate PRM1 and PRM2 gene polymorphism, AZFc microdeletion, and *PRM1*, *PRM2*, and *DAZ1* gene expression levels in 100 azoospermic patients.

Materials and Methods

Study group

This case-control study included 100 infertile males aged 28-40 years old (34.07±2.95 mean

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age). Patients were categorized as follows: 33 azoospermic males (0 spermatozoa spz/mL), 48 oligozoospermic males (less than 20 x 106 spz/mL), and 19 males with severe oligozoospermia (less than 5 x 10^6 spz/mL) according to the criteria of the World Health Organization (WHO-1999). As controls, males with proven fertility and normal sperm concentration (more than 20 x 106 spz/mL) were selected.

All subjects had bilateral testicular tissue microdissection (mTESE) procedures to obtain spermatozoa for intracytoplasmic sperm injection (ICSI). The local Ethics Committee (NO.52/422368/1) approved this study, and all subjects provided written informed consent.

Before surgery, karyotyping and Y chromosome microdeletion analysis were performed, as well as serum levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone. Table 1 shows the Mean±SD blood levels of LH, FSH, and testosterone in different patient subgroups and fertile controls. The average levels of testosterone is about 3 to 10 ng/mL, and the expected levels of both LH and FSH are around 2 to 12 mIU/mL. Patients were not receiving hormone therapy, and all had primary infertility. None of the participants had a history of TESE or cryptorchidism. The study excluded patients with cystic fibrosis, chromosomal abnormalities, and Y chromosome microdeletion. The control group consisted of subjects with normal spermatogenesis. Men with proven fertility provided the best control samples.

A comprehensive andrological examination was performed on all patients, which included a medical history, physical examination, sperm analysis, scrotal ultrasound, hormone analysis, and karyotype screening. The volunteered controls underwent a detailed medical history and physical examination, fertile with at least one normal child.

Table 1. LH, FSH, and testosterone serum levels in clinical subgroups and healthy men.

Groups	LH (mIU/ml)	FSH (mIU/ml)	Testosterone (ng/ml)
Azoospermia	13.83±1.87	23.03±2.17	2.79±0.38
Oligozoospermia	10.71±1.00	16.69±2.55	2.81±0.45
Severe oligozoospermia	10.73±1.07	17.35±2.26	2.88±0.39
Controls	8.06±0.58	9.47±0.71	4.47±0.29

DNA extraction and cDNA synthesis

Peripheral blood samples were collected in EDTA tubes to examine gene polymorphisms and detect microdeletions. Genomic DNA samples from patients and control groups were extracted using the salting-out DNA extraction protocol for further investigation.

Frozen testis tissue was homogenized, and total RNA was extracted using the RNeasy Plus Universal Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol and stored at -80 °C. To eliminate DNA contamination, in-solution DNase digestion was performed. RNA concentration and purity were determined using a Nanodrop2000 spectrophotometer (Thermo Scientific, Wilmington, USA) and confirmed using agarose gel electrophoresis. The Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, Vilnius, Lithuania) was used to synthesize template cDNA from 1g of total extracted RNA in an Eppendorf Mastercycler Gradient using oligo dT and random hexamer primers simultaneously for each reaction (Hamburg, Germany).

RFLP PCR

The rs737008 (on the PRM1 gene) and the rs2069859885 (on the PRM2 gene) single nucleotide polymorphisms (SNPs) were

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genotyped using restriction fragment length polymorphism (RFLP). Primer pairs (SinaClon, Iran) were used in PCR amplifications (Table-2). For PRM1 (Gene ID: 5619) amplification, 32 cycles of denaturation at 94 °C for 1 minute, annealing at 66 °C for 1 minute, and extension at 72 °C for 1 minute were programmed. Furthermore, the PRM2 (Gene ID: 5620) PCR program conditions (Sensoquest, GmbH, Germany) included 32 cycles of 95 °C denaturation for 45 seconds, 70 °C annealing for 45 seconds, and 72 °C extension for 30 seconds. The CGCG sequence in the PRM1 gene was recognized using the BstuI restriction endonuclease enzyme (Sigma Aldrich, Germany). At 65 °C for 2 hours, the enzyme digestion produced 599-bp PCR products with the wild type C allele in 400 bp and 199 bp fragments. DNA fragments were isolated using 1.5% agarose gel electrophoresis and safely stained on gel documentation systems.

Table 2. SNPs primers and STS primers sequences and their amplicon size.

Gene	Primer name	Primer sequence (5′-3′)	Amplicon size (bp)
PRM1	F	ACAAAACCCAGCGTGACAACT	510
	R	GCAGATATTACCGCCAGAGACA	
PRM2	F	CTCCAGGGCCCACTGCAGCCTCAG	599
	R	GAATTGCTATGGCCTCACTTGGTG	
AZFc (DAZ1)	sY254F	GGGTGTTACCAGAAGGCAAA	400
	sY254R	GAACCGTATCTACCAAAGCAGC	
AZFc (DAZ2)	sY254F	GTTACAGGATTCGGCGTGAT	126
	sY254R	CTCGTCATGTGCAGCCAC	

Sequence Tagged Site PCR

The goal of the microdeletion analysis was to find AZFc) Gene ID: 106144558)microdeletions on the long arm of the Y chromosome. Two sequence-tagged sites (STS) primers (SinaClon, Iran), namely sY254 and sY255, were used to detect microdeletions. The European Academy of Andrology recommended these STS primers because of their capacity to detect 90% of AZFc deletions (Table-2).

The PCR amplification consisted of an overall volume of 25 µL, which contained 100-200 ng of human genomic DNA as template, 2.5mM dNTP's (2.5 mM each of dTTP, dCTP, dGTP, and dATP), oligonucleotide primers (0.1-2.0 µmol/L each of the forward and reverse primers), 10XTaq DNA polymerase assay buffer (Tris with 15mM MgCl2), and 3U of Taq DNA polymerase. Thermocycling conditions for the AZFc sub-regions were standardized using a TC-512 gradient thermocycler. Polymerase chain reaction amplification was performed on the samples in 35 cycles at 94 °C for 30 seconds, 53 °C for 45 seconds, and 72 °C for 60 seconds. The initial denaturation lasted 5 minutes at 94 °C, followed by a final extension of 10 minutes at 72 °C. Furthermore, the PCR assay was repeated three times for confirmation. The PCR-amplified products were electrophoresed on 2% agarose gels and stained with safe staining. The samples were mixed in a 1:1 ratio with gel loading dye before being loaded into the agarose gel wells. The gel documentation system was used to make a visual record of the gels.

Quantitative Real-time PCR

Quantitative Real-time PCR (qRT-PCR) was performed to study the expression of PRM1, PRM2, DAZ1 (Gene ID: 1617), and the housekeeping gene beta-actin on cDNA libraries (Table 3).

qRT-PCR initial denaturation began at 95 °C for 8 minutes, followed by 40 cycles of denaturation at 95 °C for 10 seconds, annealing at 60 °C for 30 seconds, and extension at 72 °C

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for 30 seconds. To avoid genomic DNA or primer dimer amplification, the melting curve was shaped by raising the temperature from 72 to 95 °C. The SteP One-Plus RT-PCR System was used to perform qPCR in triplicate on 48-well plates with 1.0 µl of produced cDNA, 10 µl of the SYBR Green master mix (Applied Biosystems ABI/PE, Foster City, CA), 7.0 µl of DNase/RNase-free water, and 1.0 µl of primers for the gene expression profile (Applied Biosystems). The average CT was used for further investigation, and all RT-PCR runs included non-template (cDNA) controls to eliminate potential contamination.

Table 3. qRT-PCR primers sequences and their amplicon size.

Gene	Primer sequence (5′-3′)	Amplicon size (bp)
PRM1	F-GCACATCCACCAAACTCCTG R-AGGCGGCATTGTTCCTTAGC	117
PRM2	F-CATCGCAGAGGCTGCAGAA R-TCACTCGGTGTTTCTTGGGC	195
DAZ1	F-AAGCCGTGGAATGGTAGCAA R-AGCCATTGAAAGAAGGGCCA	121
B-actin	F-AGAGCTSCGAGCTGCCTGAC R-AGCACTGTGTTGGCGTACAG	184

Statistical analysis

The comparative CT method was used to analyze relative gene expression. The $2^{-\Delta CT}$ parameter represents the expression fold in relation to the housekeeping gene beta-actin. To compare allele and genotype frequencies between the case and control groups, the chi-square test was used. To perform statistical significance tests and $\chi 2$ analysis, the R programming language version 4.0.5 was used. This test with an odds ratio (OR) was used to compare the allelic and genotypic frequencies of rs737008 and rs779337774 between case and control groups. When observing an allelic count of less than 5, the statistical test was carried out by Fisher's exact test. The results are confirmed as mean±SEM. The Mann-Whitney U test was used for statistical analysis. $P< 0.05$ were considered statistically significant. GraphPad Prism 6 software was used for statistical analysis.

Results

Single nucleotide polymorphism

The association of rs737008 and rs7779337774 with male infertility was investigated in this study. The CA PRM1 gene genotype was linked to an increased risk of infertility (OR 2.2, 95% CI=1.1-4.3) (Fig. 1). Furthermore, the C allele was more common in normal controls than the case group, with a significant difference (Table 4).

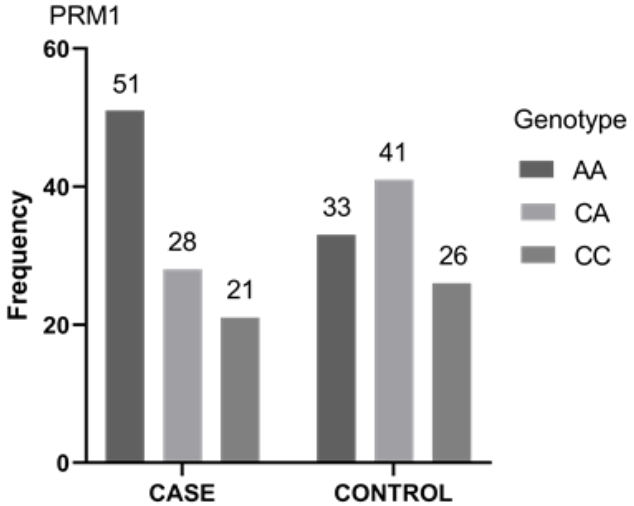

Fig. 1. Rs737008 *PRM1* gene genotype frequency. The CA *PRM1* gene genotype was linked to an increased risk of infertility.

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Table 4. Genotype and allelic frequency of *PRM1* rs737008 and *PRM2* rs7779337774.

Gene	Case (n=100)	Control (n=100)	P value	OR (95%CI)
PRM1(rs737008)				
AA	51	33	-	1
CA	28	41	0.013	2.2 (1.1 – 4.3)
CC	21	26	0.076	1.9 (0.92 – 3.9)
C allele (Additive)	35%	46.5%	0.025	0.61 (0.41 – 0.92)
A allele	79	74	-	1
PRM2(rs779337774)				
CC	78	67	0.214	1
CT	14	20		1.66 (0.780 – 3.54)
TT	8	13		1.89 (0.739 – 4.83)
T allele (Additive)	15%	23%	0.055	0.590 (0.355 - 0.982)
C allele	92	87		1

In contrast, no significant differences in genotyping and allelic frequencies were found between PRM2 rs7779337774 patients and the control group with a P> 0.05 (Fig. 2 and Table 4).

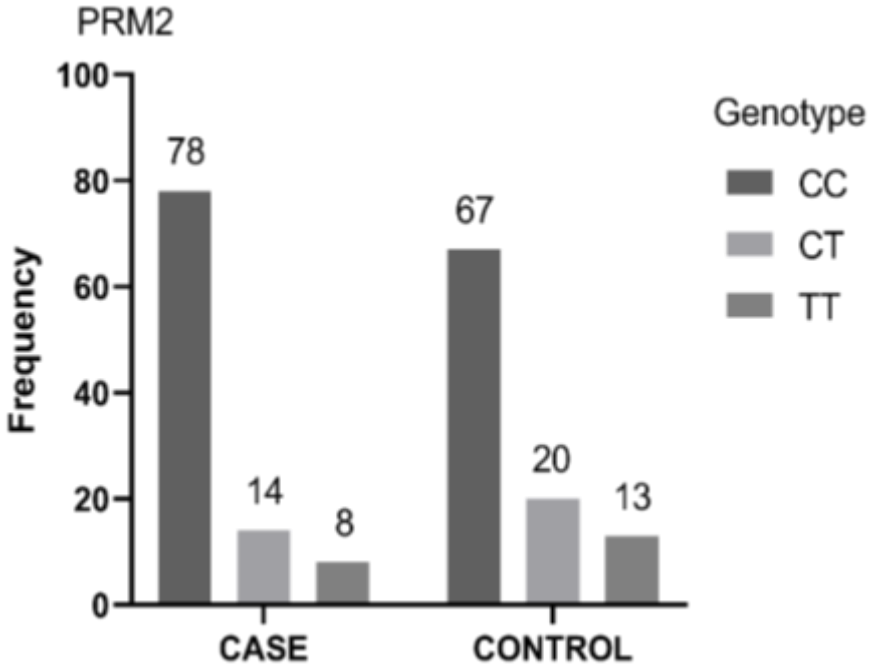

Fig. 2. *PRM2* rs7779337774 genotype frequency. No significant differences in genotyping and allelic frequencies were found between *PRM2* rs7779337774 case and the control group with a P> 0.05.

Microdeletion in AZFc region

AZFc microdeletions of the Yq chromosome were detected among the infertile males participating in this study. In total, 6% of infertile men had deletions within the AZFc region. Although the majority of infertile males were affected by oligozoospermia, no deletion mutation was observed among them.

Gene expression and ROC curve analysis in infertile males

As demonstrated by statistical analyses, *RPM1*, *RPM2*, and *DAZ1* (located in the AZFc region) had significantly decreased expression levels in sterile males compared to volunteered controls (Fig. 3)

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Furthermore, the receiver operating characteristic curve, or ROC curve, illustrated the diagnostic ability of these genes. The results show that only DAZ1 with a 95% confidence interval (CI), sensitivity= 69%, and specificity =65% has diagnostic value. In contrast, RPM1 and RPM2 did not have diagnostic values for azoospermia cases (Fig. 4).

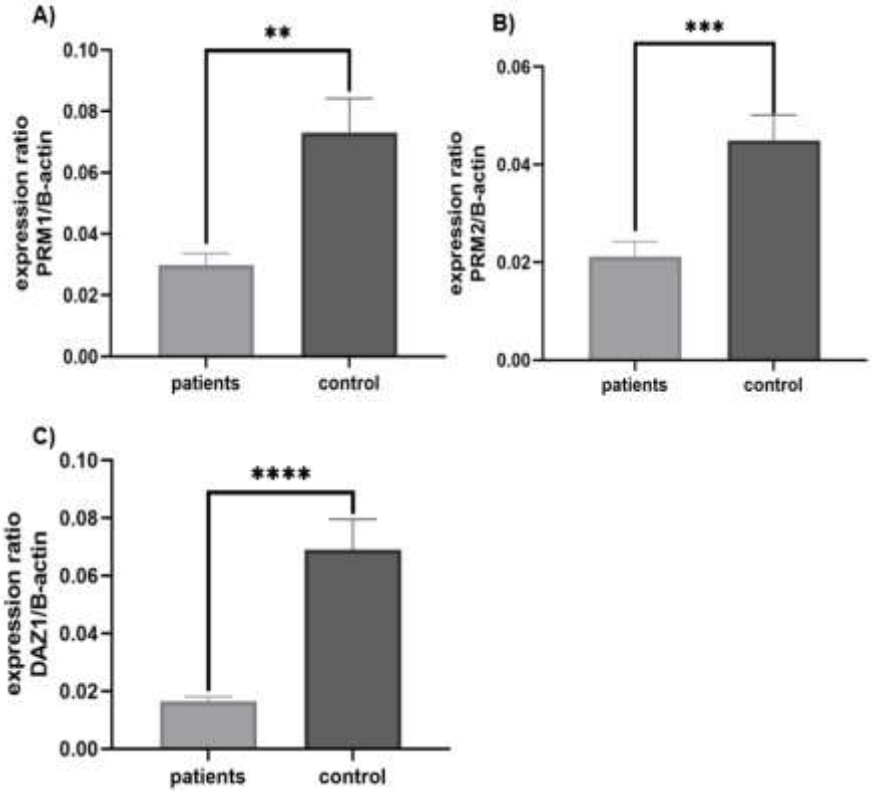

Fig. 3. Gene expression profile in infertile males compared to healthy controls A) *PRM1* with the P= 0.019; B) *PRM2* with the P= 0.0008; and C) *DAZ1* with a P< 0.0001, which all showed considerable low expression levels in the patients.

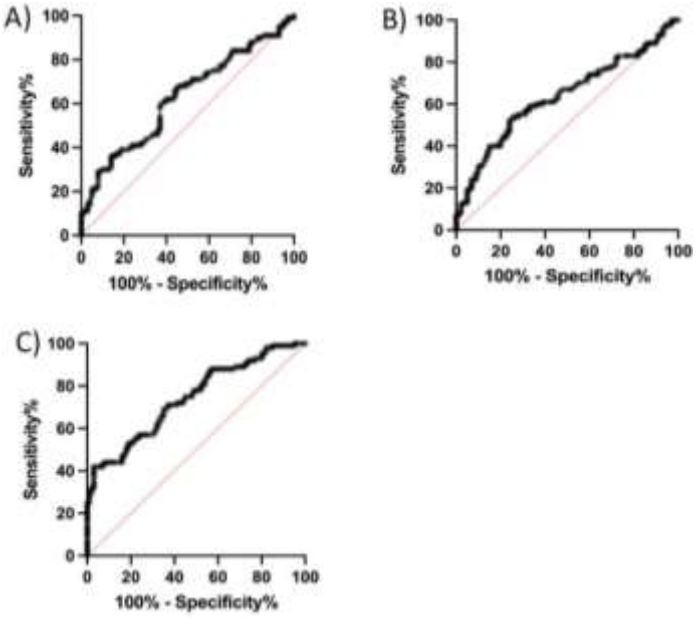

Fig. 4. Detecting biomarker potential with ROC curve analysis. A) From the lift side to the right side, respectively, *PRM1* and *PRM2* had a low biomarker value with an AUC of 0.636 and 0.626. B) With an AUC of 0.742, *DAZ1* might be a potential diagnostic biomarker to detect azoospermia.

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Serum gonadotropin and testosterone levels

The results of the hormonal analysis show a significant decrease in both LH and FSH levels, while testosterone levels are considerably high in patients compared to fertile healthy males (Fig. 5).

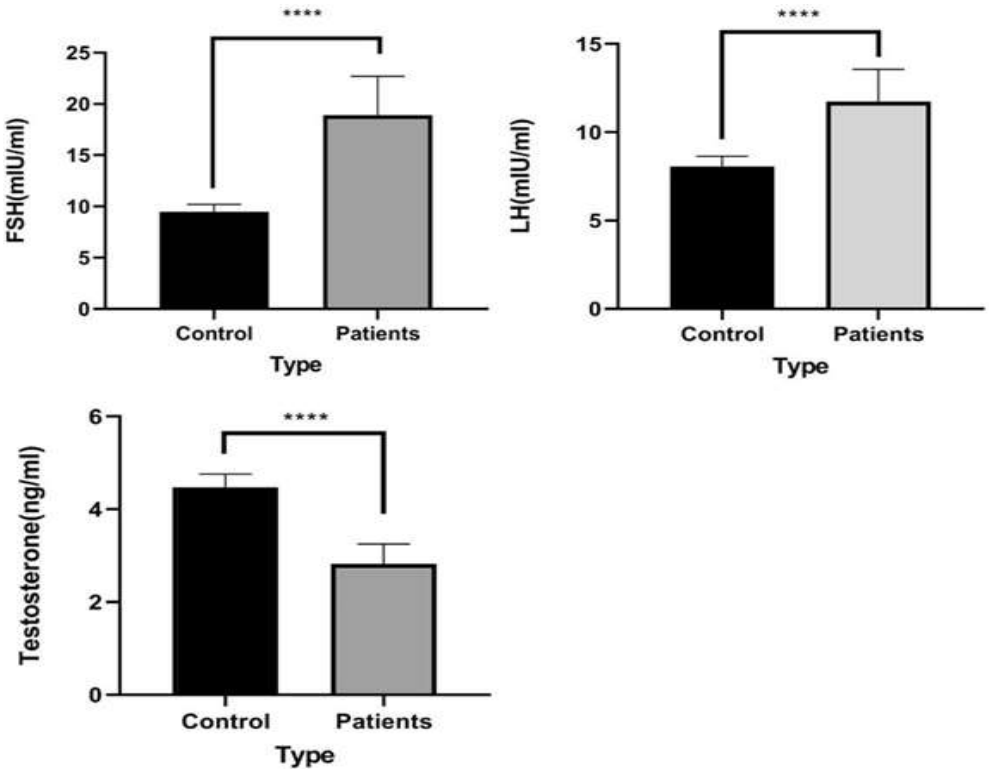

Fig. 5. The ratios of serum FSH, LH, and testosterone levels in patients were remarkably different in infertile subjects compared to fertile males, with a P< 0.001.

Discussion

This research investigated the gene polymorphism of *PRM* genes and microdeletions in the ZFc region. Additionally, the expression levels of *PRM1*, *PRM2*, and *DAZ1* and their biomarker potential were examined. As a result, no significant association was found between male infertility and the frequency of the rs779337774 polymorphism. In contrast, rs737008 *PRM1* showed a statistically significant association between CA genotype frequency and C allele frequency with male infertility. However, a comprehensive investigation of these SNPs within the protamine gene will help us provide more accurate information.

Humans have two forms of protamine, *PRM1* and *PRM2*, which bind DNA strongly when it is negatively charged. In the *PRM1* and *PRM2* genes, more than 20 SNPs have been discovered (24). A correlation between *PRM2* and male infertility has been shown in a number of studies (25). According to research by Hashemi et al., semen from people with typical semen characteristics has much higher relative expression of *PRM1* than semen from people with NOA. Additionally, successful sperm retrieval with mTESE (microdissection-testicular sperm extraction) showed a relatively high expression of *PRM1* mRNA compared to the failed cases. Since *PRM1* is only produced by germ cells within the testis, the presence of *PRM1* in semen also suggests that these NOA's testis has undergone some degree of effective spermatogenesis or focused spermatogenesis. According to the relatively high AUC with high sensitivity and specificity, this marker may have the potential to be used

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as a determining factor of sperm retrieval effectiveness in NOA in further studies (26).

The relevance of *PRM1* and *PRM2* cell-free seminal markers in diagnosing, distinguishing between OA and NOA, and categorizing their subtypes were examined in a study. The expression rates for PRM1 and *PRM2* were extremely low; therefore, seminal cell-free indicators can be used as non-invasive diagnostics to identify and classify the causes of azoospermia, but their usefulness needs to be established by extensive studies using more advanced molecular methods (27).

Male sterility could evolve, and spermatogenesis could be tricked by *PRM1*, which is indirectly downregulated by hsa-miR-27a-3p overexpression in NOA men compared to OA-control individuals (7). Moreover, a study found that the hormonal parameters and *PRM2* mRNA expression profile are substantially related to the effective retrieval of spermatozoa in azoospermic men. It is suggested that raising the *PRM2* mRNA expression profile will increase the frequency of spermatozoa retrieval in azoospermic individuals. By creating such a reliable marker, unwanted ICSI (intracytoplasmic sperm injection) operations and issues related to controlling azoospermia can be avoided (28).

The NOA infertile male with SCOS has an enhanced *PRM1/PRM2* mRNA ratio. Therefore, we may recommend protamine detection and assessment of its mRNA ratio (*PRM1/PRM2*) as an accurate and more dependable marker to evaluate sperm quality, which may affect the development capacity (29). In a study, *TXNDC2*, *PRM1*, and *PRM2* were introduced as reliable molecular indicators of sperm retrieval, with ROC analysis having a specificity of 95.5% and sensitivity of 96.8% (30).

Additionally, AZFc region microdeletions were detected in azoospermia and oligozoospermia patients by sY254 and sY255 STS markers (6%), but most of the patients did not have deletions in the AZFc region.

Microdeletions on the long arm of the human Y chromosome have been linked to abnormal spermatogenesis and a quantitative reduction in sperm in sperm samples. Microdeletions are estimated to affect 3.4% of oligozoospermic men, 12% of azoospermic men, and 1% to 7% of severely oligozoospermic men. Depending on the location of the microdeletion, the phenotypic presentation of spermatogenic failure may differ between patients. Infertile men with proximal deletions (the AZFa and AZFb regions) are more likely to have SCOS, whereas deletions of the distal AZFb and AZFc regions result in residual spermatogenesis (31). The discovery of YCMD in AZF a, b, c, and d sub-regions of azoospermic and severe oligozoospermic infertile populations by Dutta et al. confirms the critical role of genes located in these subregions in the control of spermatogenic function (31).

According to a meta-analysis study on the prevalence of Y chromosome microdeletions (YCMD) in the Indian population, 10.02% of azoospermic and oligozoospermic males had AZF deletions, which had the highest frequency of all deletions. A pooled investigation has found deletion frequency ranging from 0.59% to 32.62% (average = 13.48%) (32).

The partial AZFc deletions reduce the copy number of the genes inside the AZFc locus rather than completely removing the AZFc locus (33). Zhu et al. discovered 10 OMIM genes, including *CDY1*, *DAZ1*, *DAZ2*, *DAZ3*, and *BPY2*, in the AZFc area, with seven copy number variations (CNV) of roughly 3.6 Mb in size and four CNVs larger than 2.0 Mb. The fact that all of the patients with these CNVs had azoospermia or were severely oligozoospermic suggests that loss CNVs comprising all AZFc genes are linked to moderate oligozoospermia. Additionally, chromosome Yq11.223 q11.23 (24,889,425-28, 231, 736) was determined to be the most unstable location in the Y chromosome based on data from individuals with AZFc partial microdeletion (34).

Hashemi et al. have shown that OA people have a much higher relative expression of post-meiotic genes (such as *PRM1*) than NOA

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people. The relative expression of pre-meiotic genes, such as *DAZ*, is also reduced, but the difference between the OA and NOA groups was not statistically significant. According to the findings, sperm retrieval-negative people had much lower levels of post-meiotic indicators than sperm retrieval-positive people (21). More extensive research is needed to corroborate the findings, and more study is recommended to identify other polymorphisms in the RPM region, and AZFc sub-region microdeletion impacts on azoospermia.

Lastly, the expression profile of *PRM1*, *PRM2*, and *DAZ1* was observed to be considerably low in infertile patients. However, only *DAZ1* might be a diagnostic biomarker in azoospermia detection.

Conflicts of interest

None

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None

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