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DMRT1, *RBMY*, and *AZFb* genes polymorphism and expression role in azoospermia susceptibility

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ABSTRACT

Male infertility can occur due to spermatogenesis defects. The most common reasons for male infertility are azoospermia and oligospermia, which have several underlying factors, one of which is genetic. This study was aimed to investigate the association of azoospermia with the *DMRT1* and *RBMY1A1* genes polymorphisms and *AZFb* region microdeletions in Iranian men. Expression of these genes was assessed by RT-PCR. A total of 100 Iranian men with azoospermia, oligozoospermia, or severe oligozoospermia and 100 fertile controls were included in this case-control study. Subjects were genotyped for *DMRT1* rs755383 and *RBMY1A1* rs1481942953 polymorphisms using Tetra-ARMS PCR. The existence of two sequence-tagged sites (STSs) on the *AZFb* region was also investigated by multiplex PCR. RT-PCR was used to analyze the expression in the testis of azoospermia cases. With a p-value of 0.038, rs755383 in the *DMRT1* gene was related to an elevated risk of azoospermia. However, no significant difference was found in genotype distribution in the *RBMY1A1* (rs1481942953) gene polymorphism. Four cases demonstrated Y chromosome microdeletions with sY127 and sY134 markers. Infertile males' cDNA analysis revealed low expression levels for DMRT1 and *PRY* (one of the main genes in the *AZFb* region) with a p-value<0.0001. In contrast, RBMY1A1 expression level did not differ between patients and control groups with a p-value of 0.112. ROC curve analysis was done to detect genes with biomarker potential. With AUCs of 83% and 77%, DMRT1 and PRY had diagnostic marker potential in azoospermia detection.

INTRODUCTION

Infertility affects one in six couples, and 50% of all contributing factors are male. According to prevalence research, azoospermia is detected in 10%–20% of male infertility cases (MI), affecting men's health globally [1]. One of the main reasons for male infertility is a lack of sperm, and semen quality is utilized as a respect for MI [2]. Semen assay is essential for both categorizing and identifying MI. The three types of male infertility are azoospermia (AS), severe oligozoospermia (OS), and infertility with normozoospermia, which can be categorized based on the quality of the semen. Male infertility is brought on by the accumulation of mistakes by both environmental and genetic variables that affect spermatogenesis, whereas genetic factors are a more frequent variable. Infertile men are about ten times more likely to have genetic disorders than the general population [3]. Spermatogenic issues connected to genetic defects cause more than 30% of all MI cases [2]. Azoospermia, or the absence of sperm in an ejaculation, has varied etiologies and therapies depending on whether it is clinically nonobstructive (NOA) or obstructive azoospermia (OA). Contrary to the OA phenotype, which exhibits normal spermatogenesis, NOA men's spermatogenesis is disrupted due to exposure to hazardous substances or defective testicular evolution [4]. The most severe type of male infertility is NOA, which is defined by three pathological phenotypes of the testicles: maturation arrest (MA), hypospermatogenesis, and Sertoli cell-only syndrome (SCOS) [1]. The newly discovered intracytoplasmic sperm injection

(ICSI) techniques may help a small percentage of NOA patients have biological children. This is a dangerous genetic risk, however, this may be passed down to future generations of infertile males [5]. The condition known as oligozoospermia mainly refers to sperm concentrations under the lower reference range of 15 million sperm/mL of ejaculate. Oligozoospermia can also be divided into moderate, severe, and mild groups [6].

Sertoli and germ cells express a conserved autosomal gene known as *DMRT1* in the developing mammalian testis. The regulation of sex determination in the vertebrate gonad and the transition of germ cells from mitosis to meiosis have been linked to *DMRT1* [7]. *DMRT1*, with the coordinate of 9p24.3, is a transcription factor that regulates the testis' development and proliferation of male germ cells, contributing to the determination of male sex and male sex differentiation [7, 8]. People with *DMRT1* mutations have a variety of aberrant testicular pathological characteristics, including spermatogonial arrest, MA, SCOS, and spermatocyte arrest [5]. It is well known that the 9p deletion syndrome and XY gonadal dysgenesis are associated with deletions of the short arm of chromosome 9 encompassing *DMRT1* [9].

Human chromosome Y, which contains many genes essential for male spermatogenesis, is vital for male fertility [10]. Infertility is primarily caused by genetic problems [11]. Structural alterations to loss or microdeletions of the area that differs on a short or long arm of the Y chromosome can fail spermatogenesis [12]. The area of Yq11 is where spermatogenesis occurs in the Y chromosomal organization. Azoospermic Factor (AZF) refers to these locations that are linked to the incidence of male infertility [13]. Infertility may result from disruption of spermatogenesis caused by deleting some portions of this AZF region. Although the Y chromosome's involvement in spermatogenesis is not fully understood, over 30 genes have been found there. The main symptoms caused by a loss in the AZF region range considerably, from the spermatogenic beginning at the stage of secondary spermatocyte development to Sertoli syndrome type 1 and type 2 [14].

Three AZF areas of Yq (*AZFa*, *AZFb*, and *AZFc*) were identified, and microdeletions in those parts may cause azoospermia [15, 16]. According to research, *AZF* microdeletions are present in 5–10% of males with severe oligospermia or azoospermia. The AZFb area is in the middle of subintervals 5M and 6B. This region is 3.2 Mb and contains multiple genes necessary for healthy spermatogenesis. The two main potential genes are the *RBMY* and *PRY* genes. The growth mechanism at the spermatocyte stage is stopped in patients with *AZFb* deletion [17].

RBMY1 is one of the potential genes located in the vicinity of AZF [14]. The two major RBMY1 gene types (*RBMY1F* and *RBMY1J*) found in *AZFb*'s nearby P3 palindrome and the four major *RBMY1* gene copies (*RBMY1B*, *RBMY1A1*, *RBMY1D*, and *RBMY1E*) found in distal u1 [18]. Ma and his colleagues [19] discovered partial *RBMY1* gene deletions on the Y chromosome of men with oligozoospermia in 1993, the first evidence of a possible unique influence of some *RBMY1* gene variants on men's testicular disease [20]. Only male germ cells, most likely premeiotic germ cells, produce proteins encoded by *RBMY1*. Postmeiotic germ cells, such as sperms, also expressed RBMY1 proteins, likely enhancing their motility [18].

Spermatids and spermatozoa included *PRY*-encoded proteins, and sperm samples with higher levels of apoptotic DNA damage showed a rise in the percentage of PRY proteins that stained spermatozoa positively from 1.5 to 51.2%. Furthermore, it is suggested that this protein phosphatase is related to the apoptotic destruction of non-functioning spermatozoa. The hypothesized effect of *PRY* gene deletions on human

spermatogenesis is most likely limited to the formation of postmeiotic sperm that regulates their apoptosis rate. Its role in testicular pathology is associated with meiotic arrest, which was observed in the majority of males with "classical" *AZFb* deletions [20]. According to evidence, male infertility, like other diseases, may be caused by mutations or single-nucleotide polymorphisms (SNPs) in various genes [21, 22]. In this study, we investigated *DMRT1*, and *RBMY* genes polymorphisms, and *AZFb* sub-region (PRY) microdeletion along with these gene expression levels in azoospermic men to detect any connection between these polymorphisms and their expression profile with different types of azoospermia.

MATERIALS AND METHODS

Study subjects

A total of 100 sterile Iranian men with ages ranging from 28 to 40 joined the study. For polymorphism detection, the cases were subjected to an andrological checkup, including medical history and physical checkup, semen evaluation, scrotal ultrasound, hormone assay, and karyotype analysis. Subjects underwent bilateral testicular tissue microdissection (to obtain spermatozoa for ICSI) for gene expression analysis. Preoperative tests comprised karyotyping, Y chromosome microdeletion assay and measuring the levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone. Normal ranges for hormones were 1 to 12 mIU/ml for FSH, 2 to 12 mIU/ml for LH, and 3 to 10 ng/mL for testosterone. The mean ± SD serum levels of testosterone, FSH, and LH in patient subgroups and fertile males are summarized in Table 1.

Table 1. Levels of serum LH, FSH, and testosterone in healthy individuals and clinical subtypes.

Groups	LH (mIU/ml)	FSH (mIU/ml)	Testosterone (ng/ml)
Azoospermia	14.01±1.19	23.27±2.17	2.76±0.38
Oligozoospermia	10.90±1.00	16.93±2.55	2.78±0.45
Severe oligozoospermia	10.91±1.07	17.59±2.26	2.85±0.39
Controls	8.06±0.58	9.47±0.71	4.47±0.29

Patients showed primary infertility and were not treated with hormones. The patients do not have a history of TESE, cryptorchidism, chromosomal abnormalities, cystic fibrosis, and Y chromosome microdeletion.

All controls were fertile with average sperm concentrations (more than 2.0 x 10^6 spz/mL). Men with proven fertility were the best control samples but were never referred to mTESE surgery. The research was accepted by the local ethics committee (NO. 52/422373/1).

Nucleic acid extraction and cDNA synthesis

Genomic DNA was obtained from the peripheral blood cells of infertile and fertile males using the salting-out DNA extraction technique.

Gene expression analysis was conducted on homogenized frozen testis tissues obtained via testicular biopsy under local anesthesia. Total RNAs were obtained using the extraction Kit (the RNeasy Plus Universal Mini, Qiagen) and then transferred to −80 ˚c. DNase treatment was done to remove DNA. The concentration and purification of RNA were determined by a Nanodrop spectrophotometer and completed by agarose

gel electrophoresis. cDNAs were created using the Revert Aid First Strand cDNA Synthesis Kit.

Tetra- ARMS PCR

Tetra-ARMS was applied for the amplification of rs755383 in the *DMRT1* gene. The outer primers amplify the 185bp DNA target; the forward-inner primer, which detects the "T" allele, and the reverse-inner primer, which detects the "C" allele, generate 112bp and 126bp amplicons, respectively (Table 2).

The PCR-amplification was carried out in a total volume of 20µl including 3.13µl buffer (10x), 1.2 µl MgCl2 (50 µM), 1 µl of each outer primer (1 µM), 0.3 µl of each inner primer, 0.62 µl dNTPs, 12.22 µl ddH2O, 0.23 µl Taq polymerase and 1 µl DNA (20–25 ng). The PCR was performed in a thermal cycler with the following cycling program: a denaturation step of 10 minutes at 95˚c, 30 seconds at 95˚c for denaturation, 30 seconds at 58˚c for primer annealing, 35 seconds at 72˚c for extension, and a final extension of 10 min at 72˚c, for a total of 40 cycles in the PCR program.

Furthermore, tetra-ARMS PCR was applied to amplify rs1481942953 in the *RBMY1A1* gene. The outer primers amplify the 303bp DNA target; the forward inner primer, which detects the "G" allele, and the reverse inner primer, which detects the "T" allele, generate 206bp and 153bp amplicons, respectively (Table 2).

20µl reaction mixture, including 3.13µl PCR buffer (10x), 1.2 µl MgCl2, 1 µl of each outer primer, 0.3 µl of each inner primer, 0.62 µl dNTPs, 12.22 µl sterile distilled H2O, 0.23 µl Taq DNA polymerase and 1 µl DNA was used to amplification. PCR was done in a thermal cycler as follows: a denaturation phase for 10 min at 95˚c, 30 seconds at 95˚c for denaturation, 30 seconds at 60˚c for primer annealing, 35 seconds at 72˚c for extension, with the number 40 cycles in the PCR program. Lastly, the amplified products were loaded on a 2% agarose gel.

Table 2. Polymorphism and microdeletion detection with specific primers.

Gene	Primer name	Primer sequence (5′-3′)	Amplicon size (bp)
DMRT1	Forward outer	CCTACTAACACACAGGGAGCTGAATTCTG	
	Reverse outer	CACCAGAAATTTAGTGGCGTAAATGACAC	185
	Forward inner (C allele)	CTGCTAGAGGCTCCAGAAAGAAATGC	126
	Reverse inner (T allele)	GTCTCTGTCAAGGTGTCTGTGGGGCTA	112
RBMY	Forward outer	CCTGTCAACAAAGGCGGAGGAAAGCAG	
	Reverse outer	GGCGGATTCCTTTGGTCTTTTCTGCAAA	303
	Reverse inner (T allele)	CCTCTTGCAGGTGGTGCACCATGAGA	153
	Forward inner (G allele)	CATATTATTTTACCAACCCTGCAGGGACCT	206
AZFb	sY127F	GGCTCACAAACGAAAAGAAA	
	sY127R	CTGCAGGCAGTAATAAGGGA	274
AZFb	sY127F	GTCTGCCTCACCATAAAACG	
	sY127R	ACCACTGCCAAAACTTTCAA	301

STS PCR

The PCR reaction was performed to analyze the *AZF* region of the Y-chromosome. The *AZFb* sub-region was evaluated, where STS primers were used. The European Academy of Andrology recommended the sY127 and sY134 primers and can distinguish most of the deletions in the *AZF* loci (Table 2).

The PCR comprised a total volume of 25µL, including 100–200 ng of human genomic DNA, 2.5mM dNTPs, oligonucleotide primers (0.1–2.0 µmol/L), 10XTaq DNA polymerase assay buffer, and 3U of Taq DNA polymerase.

The settings for thermocycling were standardized for the *AZFb* sub-regions, utilizing a TC-512 gradient thermocycler. Specimens were amplified using 35 cycles at 94˚c for 30 sec, 53˚c for 45 sec, and 72˚c for 60 sec.

The PCR products were transferred to electrophoresis on 2% agarose gels and marked with safe staining. In the event of detecting microdeletion at STS sites with a primer, the PCR assay was repeated three times for confirmation.

Real-time PCR

The expression of genes was normalized with β-actin. *DRMT1, RBMY1, PRY*, and *β-actin* specific primers and product lengths are shown in Table 3.

Primary denaturation was done at 95 ˚c for 8 min, continued by annealing at 60 ˚c for 30 s, and extension at 72 ˚c for 30 s. qPCR was performed in Step-One-Plus RT-PCR by 1.0 µl of cDNA, 10 µl of the SYBR Green, and 7.0 µl of DNase/ RNase-free water, 1 µl of primers. The average CT was used for further analysis. Relative expression analysis was done regarding the comparative CT method 2-ΔΔCT.

Table 3. Primer sequences, lengths, and amplicon sizes for Real-time PCR.

Gene	Primer name	Primer sequence (5′-3′)	Primer length	Ampliconsize
DRMT1	Forward	TGCTGATTCTGCTTCTGGGG	20 bp	
	Reverse	TGGCGGTTCTCCATGTTCTT	20 bp	138 bp
RBMY1A1	Forward	GAAGCCGTGGAGGAACAAGA	20 bp	179 bp
	Reverse	TCTTGCCACAGCAGAAGGAG	20 bp	
PRY	Forward	AGCTTCTGAGAGACCCAGGA	20 bp	120 bp
	Reverse	TTGGGATTGTTGAGGCCCAT	20 bp	
B-actin	Forward	AGAGCTSCGAGCTGCCTGAC	20 bp	184 bp
	Reverse	AGCACTGTGTTGGCGTACAG	20 bp	

Statistical analysis

Statistical tests of significance and χ2 analysis were done using R programming language version 4.0.5. The differences in allelic and genotypic frequencies of rs755383 and rs1481942953 between case and control groups were evaluated by a chi-square test with an odds ratio (OR). In the case of observing an allelic count less than 5, the statistical test was carried out by Fisher's exact test. Data were analyzed via GraphPad Prism 6, and P-values under 0.05 were estimated to be significant.

RESULTS

Characteristics of patients

This study comprised 100 men, which were categorized as follows: azoospermic (0 spermatozoa), severe oligozoospermic (less than 5x), and oligozoospermic (less than 20x). Their age was in the range of 28 to 40 years old, with a 34.07±2.95 mean age. Gonadotropin hormones and testosterone hormones were elevated. Patients' LH levels were normal; however, their FSH levels were elevated, and their testosterone levels were significantly lower (Table 1).

Association of polymorphism with male infertility

In our study, the associations of rs755383 of *DMRT1* (9p24.3) and rs1481942953 of *RBMY1A1* (Yq11.223) with male infertility were analyzed. Overall, the TC genotype of

rs755383 was associated with an increased risk of azoospermia (p = 0.014). In contrast, no significant differences were observed between allele frequencies in patients and the control group (p > 0.05). No significant association was observed in the genotype and allelic frequency of rs1481942953 in the *RBMY1A1* gene (Table 4 and Figure 1).

Table 4. Rs755383 and rs1481942953 genotypes and allelic frequencies.

Gene	Case (n=100)	Control (n=100)	P-value	OR (95% CI)
DMRT1 (rs755383)				
TT	14	29	0.038	1
TC	69	57	0.014	0.39 (0.19 – 0.82)
CC	17	14	0.062	0.39 (0.15 – 1.0)
C allele (Additive)	51.5%	42.5%	0.08	1.4 (0.96 – 2.1)
(TC/CC) Dominant	83	71		1.2 (0.81 – 1.9)
T allele	97	115	-	1
Recessive	17	14		0.79 (0.36 – 1.7)
RBMY1A1 (rs1481942953)				
TT	50	44	0.692	1
TG	37	42		0.747 (0.361 – 1.54)
GG	13	14		0.662 (0.271 – 1.61)
G allele (Additive)	31.5%	35%	0.483	1.17 (0.792 - 1.73)
(TG/GG) Dominant	50	56		1.37 (0.680 - 2.78)
T allele	68.5%	65%	-	1
Recessive	13	14		1.21 (0.600 - 2.44)

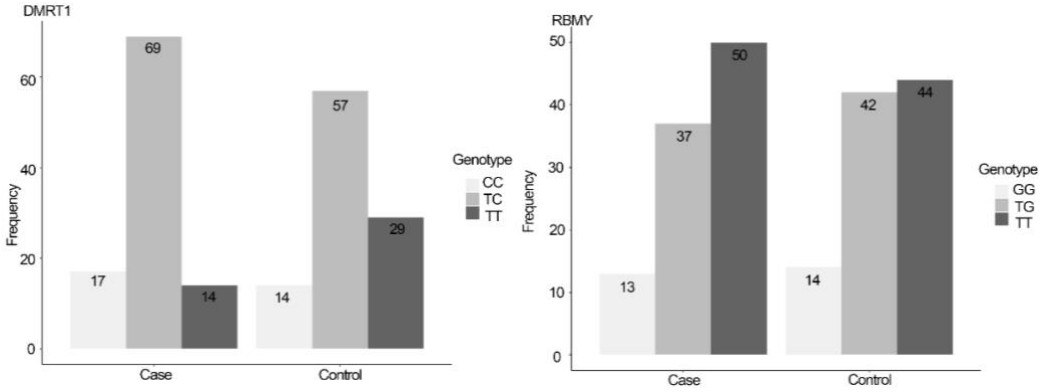

Figure 1. Genotype frequency of *DMRT1* and *PBMY1A1* in 100 infertile males.

Microdeletion detection in the *AZFb* region

Deletions in the *AZFb* region are the most severe among infertile males. sY127 and sY134 markers were used to detect microdeletions in this region. Only 4% of the patients had deletions within the *AZFb* region. Three cases of azoospermia and a case of severe oligozoospermia were detected with microdeletions in the *AZFb* region. Although most infertile males were affected by oligozoospermia, no deletion mutation was observed, as illustrated in Table 5.

Table 5. STS markers and AZFb microdeletions.

Groups	Total number	Micro-deletion in AZFb (sY127)	Micro-deletion in AZFb (sY134)
Azoospermia	33	1	2
Severe oligozoospermia	19	1	0
Oligozoospermia	48	0	0

Gene expression analysis

Statistical analyses showed significantly decreased *DMRT1* and *PRY* (one of the main genes of the *AZFb* region) expression levels in infertile males with p-values of less than 0.0001. However, RBMY1A1 expression did not statistically differ in patients and control males (p-value = 0.112) (Figure 2).

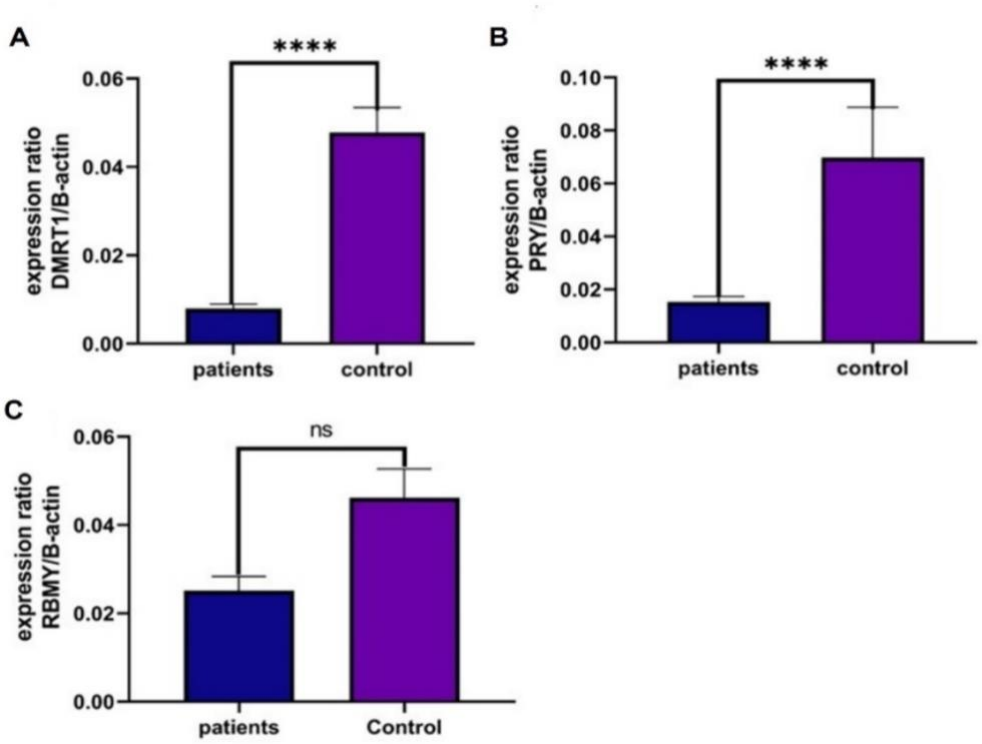

Figure 2. Decreased gene expression levels of A) the *DMRT1* gene (P-value<0.0001, 95% confidence interval (95% CI) 0.777 to 0.889) and B) the *PRY* gene (P-value<0.0001, 95% CI 0.706 to 0.835) are statistically considerable. However, C) *RBMY1A1* gene expression level did not show significant alteration in infertile males and healthy controls (P-value=0.112, 95% CI 0.484 to 0.646).

ROC-curve analysis

The ROC curve illustrates the diagnostic ability of the genes. The results demonstrate that *DMRT1* and *PRY*, with 83% and 77% area under the ROC curve (AUC), have the potential as diagnostic biomarkers. *RBMY1A1* with 56% AUC did not have a potential biomarker role in the case of azoospermia (Figure 3).

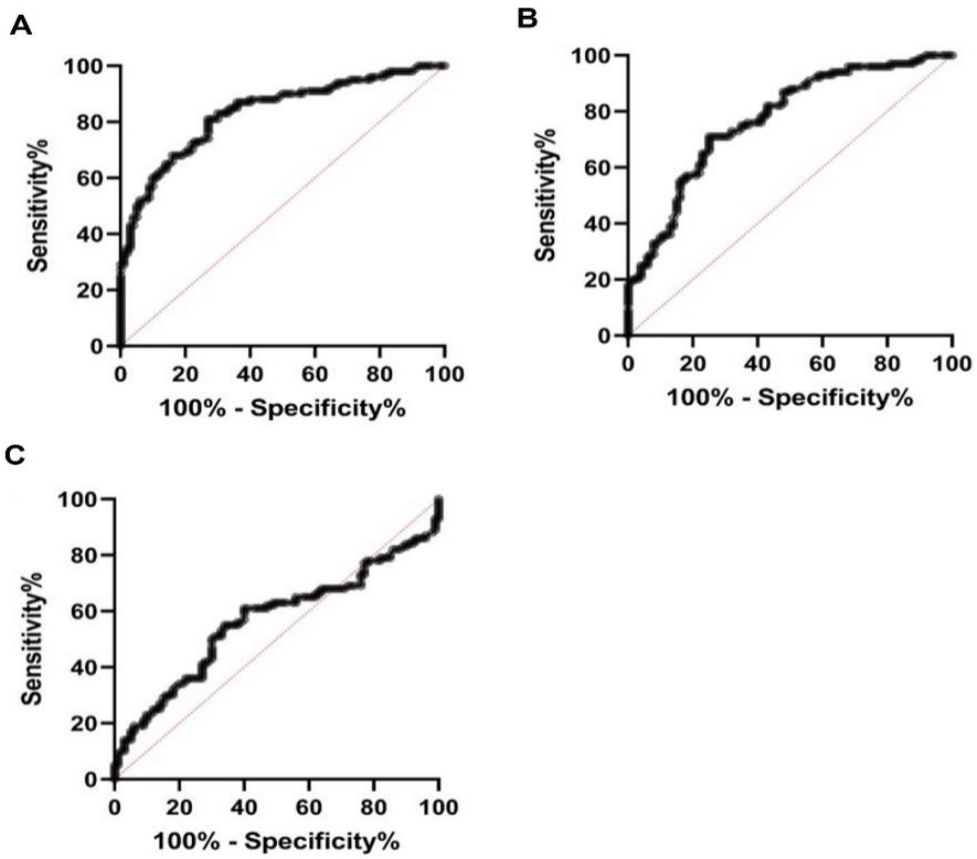

Figure 3. By analyzing A) *DMRT1* and B) *PRY* ROC curves, with respectively AUC= 83%, sensitivity=81%, specificity =73%, and AUC=77%, sensitivity =71%, and specificity =75%; these genes may have a potential biomarker role in infertility diagnosis. In contrast, C) *RBMY1A1* did not compose any diagnostic potential (AUC=56%, sensitivity =61%, and specificity =60%).

Levels of serum testosterone and gonadotropin in patients

Although testosterone levels are significantly higher in patients compared to fertile ($p<0.001$), the data of the hormonal analysis demonstrate a substantial drop in both LH and FSH levels with a P-value <0.001 (Figure 4).

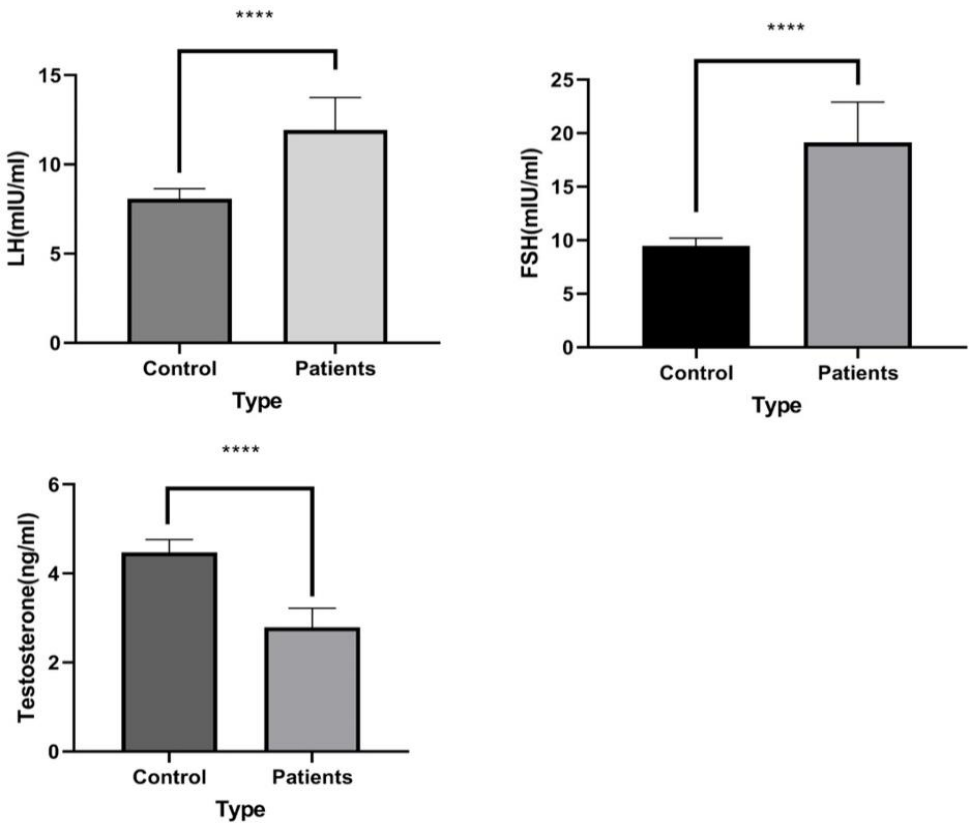

Figure 4. Subjects exhibited significant ($p<0.001$) variations in ratios of LH, FSH, and testosterone serum levels compared with the proven fathers' group.

DISCUSSION

One of the primary causes of male infertility is azoospermia, or lack of sperm in the semen. Klinefelter syndrome, microdeletions of the Y chromosome's azoospermia component, and *CFTR* gene mutations for OA are some genetic reasons for spermatogenic failure (SPGF). However, because monogenic factors are not examined, most instances remain unidentified [23].

Since DMRT1 prevents Sertoli cells in the human testis from transdifferentiating into a more granulosa-like cell type, Macdonald et al. have demonstrated that DMRT1 suppression alters the expression of essential genes associated with gonadal development. Additionally, they have shown that the fetal human testis experiences focal dysgenesis due to DMRT1 suppression [7].

In the human embryonic testis, suppressing the expression of the *DMRT1* gene can alter the expression of crucial genes required for gonadal development, resulting in a change from a male phenotype to one that is more ovarian-like [7]. Furthermore, disorders of sex development (DSD) or gender reversal have been linked to *DMRT1* gene deletions or mutations [24].

The postnatal XY gonad's robust control system that protects male fate is linked to *DMRT1*. It converts female granulosa cells to male ones when overexpressed in the pubertal or adult ovary [25]. The paired deletion of *DMRT1* and *SOX8/9* in the testis results in much more significant sexual trans differentiation than the removal of either DMRT1 or SOX8/9 alone, suggesting that DMRT1 maintains the male sexual cell destiny in cooperation with SOX8/9 [26]. According to research by Lindeman et al. in 2021, *SOX9* and *DMRT1* shares many chromatin locations accessible only to one sex in

Sertoli cells. DMRT1 can also make postnatal granulosa cell chromatin more receptive to SOX9 binding. Both transcription factors must completely regulate several sex-biased mRNAs [27]. It was acknowledged by Araujo *et al.* that the *DMRT1* gene mutation c.671A>G has already been linked to azoospermia [28]. According to a study, a Chinese patient with complete gonadal dysgenesis (CGD) had a homozygous *DMRT1* mutation, c.967G>A [28].

Human *RBMY1* genes are found on the Y chromosome, are expressed in the germ cells of men, and may be related to sperm motility [14], as the *RBMY1J* is linked to lower sperm counts in clinics [29]. The prevalence of the *AZF* microdeletion area on the Y chromosome was 7.7%, according to an investigation of *AZF* microdeletions on the Y chromosome of male fertility problems in Palembang utilizing the *RBMY1* STS *RBM1* gene [15].

Only SCOC syndrome was successfully associated with RBMY1 among the several azoospermia phenotypes, according to Javadirad *et al.*, indicating a substantial positive relationship between this gene and effective sperm retrieval (SR) [30]. RBMY1 has reportedly been linked to hepatocellular carcinoma, prostate cancer, and heart function [31, 32]. PRY is only present in the testis, and its product controls apoptosis and eliminates defective sperm [32]. According to a study, PRY may be a helpful indicator for abnormal spermatogenesis [33].

Particularly in distal *AZFb* close to the *PRY* and *RBMY1* gene variants in the highly variable MSY1 locus, hot spot areas for "polymorphic" Y chromosomal disruptions and rearrangements were discovered. Numerous total *PRY* deletions and substantial *RBMY1* gene copy deletions were found as a consequence of the investigation, and these findings appear to be associated with typical male fertility [20]. Based on a study, all *AZFb* genes, such as *RBMY* and *PRY*, were discovered to be deleted in a French man who was infertile but did not have meiotic arrest [34]. It is possible that the mentioned polymorphisms are influenced by the genetics of ethnicities [35]; therefore, additional research into different ethnic groups is recommended to support the current findings. In addition, other polymorphism sites in *DMRT1*, *RBMY1A1*, and *PRY* genes must be included in future studies.

CONCLUSIONS

This study was conducted to discover a potential correlation between male infertility and *DMRT1, RBMY1A1* polymorphisms, and *AZFb* microdeletions. Furthermore, DMRT1, RBMY1A1, and PRY expression levels were evaluated in testis samples. Our findings have shown that the TC genotype frequency of rs755383 in the *DMRT1* gene has a significant statistical relationship with male infertility. However, rs1481942953 in the RBMY1A1 gene had no statistical association with male infertility. With the help of sY127 and sY134 STS markers, four microdeletions in the AZFb region were discovered in azoospermia and severe oligozoospermia cases. In this study, patients had dysregulated *DMRT1* and *PRY* gene expression; however, *RBMY1A1* gene expression showed no significant dysregulation in infertile individuals. The ROC curve results indicate that DMRT1 and PRY could be useful biomarkers for detecting male infertility.

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AUTHOR CONTRIBUTIONS

RS and AHS were involved in the conception and design of the experiments. PB and AHS contributed to performing the experiments. ND analyzed data. AHS contributed to drafting the article. RS and AHS contributed to revising it critically for important intellectual content. RS made the final approval of the version to be published.

CONFLICTS OF INTEREST

There is no conflict of interest among the authors.

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