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ORIGINAL RESEARCH

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Investigation of genetic causes in non-obstructive azoospermic patients

Bulut Dural[1], Ilhan Gecit[2], Emre Aykanli[1,*], Cemal Ekici[2], Fatih Oguz[2], Ahmet Koc[3]

[1]Department of Urology, Faculty of Medicine, Erzincan Binali Yıldırım University, 24100 Erzincan, Turkey
[2]Department of Urology, Inonu University Faculty of Medicine, 44050 Malatya, Turkey
[3]Department of Medical Biology and Genetics, Inonu University Faculty of Medicine, 44050 Malatya, Turkey

***Correspondence**
emre.aykanli@sbu.edu.tr
(Emre Aykanli)

Abstract

Background: Male factor infertility is a health problem that affects millions of couples around the world. Male factor infertility is responsible for approximately more than half of all cases of infertility. About 15% of men and 10% of women with infertility may have genetic abnormalities, including chromosomal abnormalities and single gene mutations. In this study, results of genetic analys is of the infertile male patients who underwent testicular sperm extraction (TESE) with the diagnosis of non-obstructive azoospermia were evaluated in order to reveal genetic defects that impair or prevent spermatogenesis in male infertility. **Methods**: We compared the results of peripheral blood chromosome analysis, molecular karyotyping, male infertility genetic panel, and also testosterone, prolactin, follicular stimulating hormone and luteinizing hormone levels in non-obstructive azoospermic infertile patients aged 26–44 years, and investigated the relationship between these parameters and genetic mutations. **Results**: As a result of this research, among 26 patients, *INSL3* (insulin-like peptide 3) gene mutation, which is considered pathogenic according to the criteria published by the American College of Medical Genetics and Genomics (ACMG) was detected in 1, *FSHR* (follicle stimulating hormone receptor) gene polymorphism in 17, *CFTR* (cystic fibrosis transmembrane conductance regulator) mutations in 5, *CATSPER1* (cation channel sperm associated 1) and *TEX101* (testis expressed 101) in 1, *LHCGR* (luteinizing hormone/choriogonadotropin receptor) in 1, *ZMYND15* (zinc finger mynd-type containing 15) in 1, *DNAH5* (dynein axonemal heavy chain 5) in 2, and *DNAH11* (dynein axonemal heavy chain 11) changes in 1 patient. In the chromosome analysis, 47XXY Klinefelter syndrome was observed in 6 patients. **Conclusions**: The results have shown that non-obstructivea zoospermic patients with complaints of infertility may have other genetic abnormalities leading to infertility, despite the results of chromosomal analysis of the peripheral blood samples were within normal reference limits. Investigating these underlying genetic disorders helped us find the cause of infertility in ourpatient population.

Keywords

Azoospermia; Genetic; Infertility

1. Introduction

Infertility is defined as the failure to achieve a pregnancy by a sexually active couple after one year of regular unprotected sexual intercourse [1]. It is a public health problem that affects approximately 15% of all couples in the world [2]. In recent years, there has been increasing concern about a worldwide decline in male fertility, and the male factor is thought to be responsible for 40–50% of all cases of infertility in the world [3].

Abnormal semen parameters such as oligozoospermia, teratozoospermia or azoospermia are observed in only 50% of cases with male factor infertility [4]. Azoospermia is the most severe form of male factor infertility and is seen in approximately 10–20% of infertile couples [5]. Azoospermia, which means the absence of sperm in the seminal fluid, is caused by obstructive or non-obstructive etiologies [6]. Obstructive azoospermia (OA) is the absence of both spermatozoa and spermatogenetic cells in the semen and post ejaculate urine due to bilateral obstruction of the epididymis, seminal vesicles o rejaculatory ducts [4]. Non-obstructive azoospermia (NOA) is the absence of any sperm in these men analysis, without any evidence of obstruction in these pathways [7, 8].

Approximately 7% of infertile men have structural or numerical chromosomal abnormalities. The prevalence of karyotype anomalies in azoospermia is 10–15%, 5% in oligozoospermia and less than 1% in patients with normal sperm counts [9]. However, many other genetic abnormalities lead-

ing to infertility are still unknown.

Genetic research of human populations has made significant advances in recent years thanks to the development and availability of NGS (next generation sequencing) platforms. In contrast to the laborious process of single-gene mutation screening by exon-exon amplification and Sanger sequencing, NGS enables interrogation of large gene panels in a single experiment at a reasonable cost [10]. In recent years, genetic mutations in many diseases with established clinical diagnoses can be revealed thanks to the whole exon sequencing with NGS. At the same time, it has become possible to classify, and reveal the etiologies of different subtypes of the same disease.

In this study, we have aimed to evaluate the genetic analysis results of patients who applied with complaints of infertility and underwent micro-TESE operation with the diagnosis of NOA, using the NGS method. Our main aim in the study will be to evaluate the effect of genetic defects that impairor prevent spermatogenesis in male infertility based on micro-TESE results.

2. Material and methods

This study included 26 azoospermic NOA patients aged 26–44 who underwent TESE at İnönü University Faculty of Medicine, Turgut Özal Medical Center, Departmentof Urology, between 2014 and 2020. Detailed anamnesis of all participants was taken and physical examinations were performed. Patients with a history of pituitary and thyroid pathology, systemic disorders (such as diabetes, hypertension), those who had received chemotherapy or exogenous testosterone supplements were excluded.

Lymphocyte cell cultures were performed in all cases for karyotype analysis using heparinized peripheral venous blood samples. Chromosome analysis was performed on the metaphase plates stained using the high-resolution (450–550) G banding technique according to the standard protocol.

Semen analyses were performed with samples collected from male partners who masturbated after an ejaculatory abstinence period of at least 48 hours. Candidates with low semen volume (<1.5 mL) to be evaluated later for retrograde ejaculation and other obstructive pathologies were not included in the study. After the samples were kept in the incubator at 37 °C for 30 minutes, sperm concentrations were determined using a Makler counting chamber under a microscope at 200 times magnification. The results were evaluated based on WHO (World Health Organization) 2010 semen analysis reference values. When evaluating azoospermia and oligozoospermia, the results of at least 2 semen analyses performed 2–4 weeks apart were taken into account.

Venous blood samples collected from the participants early in the morning after an overnight fast were analyzed for testosterone, prolactin, follicle stimulating hormone (FSH) and luteinizing hormone (LH). Testosterone was analyzed using the chemiluminescent immunoassay method on a (Siemens Immulite 2000 immunoassay system, Siemens Healthineers, Erlangen, BY, Germany), while for the analyses of other hormonal parameters the Siemens Advia Centaur XPT immunoassay system, Siemens Healthineers, Erlangen, BY, Germany) was used.

DNA was extracted from the patients' peripheral venous blood samples using a commercial kit (11796828001, High Pure PCR Template Preparation Kit, Roche, NRW, Germany) in accordance with the protocol specified below.

1. 200 μL blood sample of each patient was placed in 1.5 mL Eppendorf tubes containing 200 μL binding buffer and 40 μL proteinase K.
2. Blood samples were incubated at 70 °C for 10 minutes.
3. 100 μL of isopropanol was added into each tube and the mixture was transferred to filtered tubes and centrifuged at 8000g for 1 minute.
4. After centrifugation, the bottom of the tube was gently emptied and 500 μL inhibitor removal buffer was added into each tube and incubated at 8000g for 1 minute.
5. Following this process, the remnants at the bottom of the tube were emptied and each tube containing 500 μL wash buffer was centrifuged at 8000g for 1 minute.
6. The procedure number 5 was repeated once more.
7. The bottom of the tube was evacuated and centrifuged at 13,000g for 10 seconds.
8. After the filtered section was transferred into new tubes, 200 μL elution buffer was added and DNA was extracted by centrifuging at 8000g for 1 minute.

The amount and purity of the isolated DNA samples were determined by measuring them on the NanoDrop ND-2000c spectrophotometer.

From the DNA samples extracted, 44 infertility-associated genes in Table 1 were examined using the next generation sequencing (NGS) method [11].

2.1 DNA quantification

Quantitative analysis of DNA samples was performed using a commercial kit (Q32851, Qubitds DNA HS Assay Kit, Waltham, MA, USA). The solubilizing solution to be used in the reaction was vortexed. Three working solutions to be used in the next steps were prepared for each sample including 1 μL of solubilizing solution and 199 μL of dilution solution. Then 198 μL working solution and 2 μL DNA sample were added into the Qubit Assay tubes, vortexed, and measurements were made. Finally, DNAs were diluted with nuclease-free water to 10 ng/μL.

2.2 Data processing and analysis

Sequencing was performed by the NGS method using primers covering the exons and exon-intron junctions of 44 genes examined. The results were analyzed using Qiagen Ingenuity Variant Analysis (Version4.0./QIAGEN, Redwood City, CA, USA) and Geneticist Assistant (Version1.8.1.0/SoftGenetics LLC, State College, PA, USA) analysis programs. The obtained data were evaluated with two different bioinformatics analysis methods in terms of base sequencing, filtering of low quality reads and artifacts, and annotation of variants.

In order to evaluate whether the detected variants were pathogenic or not, the guideline determined by the American College of Medical Genetics and Genomics (ACMG) in 2015 was used [53]. According to this guide:

Class 1: Pathogenic (pathogenicity: >99%): Changes whose disease-causing effect (pathogenicity) has been

TABLE 1. Infertility-associated genes examined in the study, and their functions.

Genes	Functions
AR	Repeat increases in CAG (cytosine, adenine, guanine) sequences cause low transcriptional activity in the AR gene which has been associated with male infertility [12].
AURKC	Spermatogenetic failure type 5 (SPGF5) Single nucleotide deletion on the Aurora kinase C gene has been found to be associated with infertility. In SPGF5, sperms with large-heads, variable number of tails and increased chromosomal content are detected [13].
CATSPER1	Encodes the protein responsible for the function of calcium channel gates on the plasma membrane in the sperm tail [14].
CATSPER2	Encodes the protein responsible for the function of calcium channel gates on the plasma membrane in the sperm tail [15].
CFTR	It has been associated with congenital bilateral vas deferens deficiency [16].
DDX25	Regulates production of gonadotropic hormones, which induce steroidogenesis in Leydig cells [17].
DMC1	It is responsible for homologous chromosome pairing in meiosis [18].
DNAH11	It is associated with primary ciliary dysgenesis and Kartagener syndrome [19].
DNAH5	It is associated with primary ciliary dysgenesis and Kartagener syndrome [20].
DNAI1	It is associated with primary ciliary dysgenesis and Kartagener syndrome [21].
DPY19L2	Spermatogenetic failure type 9 (SPGF9): is associated with globozoospermia [22].
ESR1	Deletion of this gene causes azoospermia, loss of sperm motility and fertility capacity [23].
ESR2	Deletion of this gene causes azoospermia, loss of sperm motility and fertility capacity [23].
ESX1	Induces the development of spermatocytes, spermatids and spermatozoa from sperm stem cells, respectively [24].
FSHB	Induces hypogonadotropic hypogonadism [25].
FSHR	Regulates the proliferation of Sertoli cells and the spermatogenic capacity of the testicles [26].
GNRHR	It has been associated with hypogonadotropic hypogonadism [27].
INSL3	Mutations in this gene have been detected in azoospermic cases with bilateral cryptorchidism [28].
KLHL10	It is characterized by germ cell loss and defective morphology of spermatids [29].
LHCGR	Inactivates human LH hormone receptor mutations [30].
NANOS1	Induces azoospermia and oligospermia [31].
NR5A1	Takes part in the regulation of genes involved in steroidogenesis and gender development [32].
PLCZ1	Controls cell cycle-dependent calcium oscillations in early embryogenesis. It is characterized by total fertilization failure [33].
PRM1	Takes part in the expression of protamine, which is involved in the packaging of sperm DNA [34].
PRM2	Takes part in the expression of protamine, which is involved in the packaging of sperm DNA [34].
PRM3	Takes part in the expression of protamine, which is involved in the packaging of sperm DNA [34].
RBMXL2	Defect in this gene product inhibits functional sperm production [35].
RXFP2	Mutations in this gene have been detected in azoospermic cases with bilateral cryptorchidism [36].
SEPT12	It has been associated with defective sperm annulus and spermatogenesis disorder [37].
SHBG	Ensures the delivery of sex hormones to target tissues and regulates androgen concentration [38].
SLC26A8	Mutations in this gene have been associated with severe asthenozoospermia and primary infertility [39].
SPATA16	SPATA16 expression is not observed in testicular samples of patients with globozoospermia, spermatogenic arrests or Sertoli cell-only syndrome [40].
SPO11	Low SPO11 expression further delays the activation of repair mechanism of meiotic double strand breaks [41].
STAG3	It is an important cohesin subunit and mutations in this gene can cause meiotic arrest and disorders in gametogenesis [42].
SYCE1	Encodes some proteins involved in meiosis [43].
SYCP3	This mutation has been observed in patients with azoospermia characterized by meiotic arrest [44].
TAF4B	Seminiferous tubules devoid of germ cells are observed in men with TAF4B mutation [45].

TABLE 1. Continued.

Genes	Functions
TAF7L	Affects spermatogenesis in the pre-meiotic phase [46].
TEX101	Stimulates the cumulus cells around the oocytes, increasing progesterone secretion and initiating the acrosome reaction [47].
TEX11	Induces elimination of spermatocytes in the anaphase 1 and pachytene stages [48].
USP26	It has been associated with Sertoli cell-only syndrome [49].
USP9Y	Its deletion causes azoospermia and severe oligospermia [50].
UTP14A	Its mutation prevents the differentiation of spermatogonia [51].
ZMYND15	Regulates the expression of haploid genes in spermatogenesis [52].

Abbreviations: AR: Androgen receptor; AURKC: Aurora Kinase C; CATSPER1: Cation Channel Sperm Associated 1; CATSPER2: Cation Channel Sperm Associated 2; CFTR: Cystic fibrosis transmembrane conductance regulator; DDX25: DEAD-box helicase 25; DMC1: DNA Meiotic Recombinase 1; DNAH11: Dynein Axonemal Heavy Chain 11; DNAH5: Dynein Axonemal Heavy Chain 5; DNAI1: Dynein Axonemal Intermediate Chain 1; DPY19L2: Dpy(dumpy)-19 Like 2; ESR1: Estrogen Receptor 1; ESR2: Estrogen Receptor 2; ESX1: ESX Homeobox 1; FSHB: Follicle Stimulating Hormone Subunit Beta; FSHR: Follicle Stimulating Hormone Receptor; GRHR: gonadotropin-releasing hormone receptor; INSL3: Insulin-like peptide 3; KLHL10: Kelch Like Family Member 10; LHCGR: Luteinizing Hormone/Choriogonadotropin Receptor; NANOS1: Nanos C2HC-Type Zinc Finger 1; NR5A1: Nuclear Receptor Subfamily 5 Group A Member 1; PLCZ1: Phospholipase C Zeta 1; PRM1: Protamine 1; PRM2: Protamine 2; PRM3: Protamine 3; RBMXL2: RNA Binding Motif Protein X-Linked Like 2; RXFP2: Relaxin Family Peptide Receptor 2; SEPTIN12: Septin 12; SHBG: Sex Hormone Binding Globulin; SLC26A8: Solute Carrier Family 26 Member 8; SPATA16: Spermatogenesis Associated 16; SPO11: SPO11 Initiator Of Meiotic Double Strand Breaks; STAG3: STAG3 Cohesin Complex Component; SYCE1: Synaptonemal Complex Central Element Protein 1; SYCP3: Synaptonemal Complex Protein 3; TAF4B: TATA-Box Binding Protein Associated Factor 4b; TAF7L: TATA-Box Binding Protein Associated Factor 7 Like; TEX101: Testis Expressed 101; TEX11: Testis Expressed 11; USP26: Ubiquitin Specific Peptidase 26; USP9Y: Ubiquitin Specific Peptidase 9 Y-Linked; UTP14A: UTP14A Small Subunit Processome Component; ZMYND15: Zinc Finger MYND-Type Containing 15.

demonstrated with sufficient data.

Class 2: Possibly Pathogenic (pathogenicity: 95–99%): Changes for which there is very strong data in favor of the presence of a disease-causing effect (pathogenicity).

Class 3: Variant with Unknown Pathogenic Effect (pathogenicity: 5–95%): Changes for which there is limited or controversial data on pathogenicity.

Class 4: Possibly Benign (pathogenicity: 1–5%): Changes for which there is very strong data in favor of not having a disease-causing effect (pathogenicity).

Class 5: Benign (pathogenicity: <1%): Changes that have been shown with sufficient data to have no disease-causing effect (pathogenicity).

3. Results

The patients participating in the study are numbered from 1 to 26 and the findings are stated in Table 2 to get her with the corresponding patient number. The INSL3 (insulin-like peptide 3) mutation in case number 14 was classified as Class 1, and all other mutations as Class 3 (Table 2).

Genetic analys is of all patients was performed and 47XXY Klinefelter syndrome was observed in 6. *AZF* (Azoospermia Factor) mutation in 2. *FSHR* variant change in 17, *CFTR* (Cystic fibrosis transmembrane conductance regulator) in 5, *CATSPER1* (Cation Channel Sperm Associated 1) and *TEX101* (Testis Expressed 101) in 1, *INSL3* mutation in 1, *LHCGR* (Luteinizing Hormone/Choriogonadotropin Receptor) in 1, *ZMYND15* (Zinc Finger MYND-Type Containing 15) in 1, *DNAH5* (Dynein Axonemal Heavy Chain 5) in 2 and *DNAH11* (Dynein Axonemal Heavy Chain 11) change in 1 patient, respectively.

4. Discussion

Among 44 genes we investigated, the *FSHR* (Follicle Stimulating Hormone Receptor) gene whose polymorphism was found in 17 (65.38%) patients, encodes the receptor that enables FSH to bind to the cell during initiation of spermatogenesis. It induces spermatogenesis in Sertoli cells in the testicles. In the absence of this receptor, FSH cannot bind to the cell and spermatogenesis cannot be initiated. The FSHR gene on chromosome 2p21 consists of 9 introns and 10 exons and a promoter region. Mutation screening has identified several single nucleotide polymorphisms (SNPs) in the FSHR gene [54]. Among these, linked SNPs at positions 307 and 680 in exon 10 of the FSHR gene have attracted wide attention. However, many studies investigating possible associations between Thr307Ala and Asn680Ser polymorphisms in the FSHR gene and male infertility, could not yield clear-cut results. Despite this uncertainty, FSHR polymorphisms presumably play an important role in male infertility. To further investigate the association between Thr307Ala and Asn680Ser polymorphisms in the FSHR gene and male infertility, a meta-analysis performed with a case-control study with 212 infertile and 164 fertile men from Northern China was published [55]. It has been shown that the FSHR gene polymorphisms found in this study have a higher frequency in infertile patients than in the control group [55].

TABLE 2. Genetic examination results of patients.

P.NO	Age	Hormone	Chromosome analysis	Molecular karyotyping	New generation sequencing infertility panel						
					Gene	Location	Variant	Zygosity	Class	Disease	Heredity
1	29	FSH: 28.23 mIU/mL LH: 10.18 mIU/mL T: 116 ng/mL	46XY	An increase of approximately 8.6 MB in the Yp11.32-p11.2 (236524-8875193) region; an increase of approximately 21.2 MB in the Yq11.21-q11.222 (14545-21311821) region; deletion of approximately 35.8 MB, in the Yq11.222-q11.223 (21353932-59311250 region; an increase of approximately 2.3 MB in the Xp22.33 (286524-2685674) region; and deletion of approximately 0.8 MB in the Xq28 (1849744667-155208244) region were detected.	-	-	-	-	-	-	-
2	34	FSH: 29.64 mIU/mL LH: 13.77 mIU/mL T: 126 ng/mL	46XY	Normal	CFTR	Exon 11	c.1516A>G (p.ıle506Val) rs1800091	heterozygous	Class 3	congenital bilateral absence of vas deferens	autosomal recessive
					FSHR	Exon 10	p.S680N	homozygous	Class 3	decreased FSH response	autosomal recessive
					FSHR	Exon 10	p.S307N	homozygous	Class 3	decreased FSH response	autosomal recessive

TABLE 2. Continued.

P.NO	Age	Hormone	Chromosome analysis	Molecular karyotyping	New generation sequencing infertility panel						
					Gene	Location	Variant	Zygosity	Class	Disease	Heredity
3	30	FSH: 10.29 mIU/mL LH: 5.81 mIU/mL T: 206 ng/mL	46XY	Normal	CFTR	Exon 14	c.2002C>T (p.Arg668Cys) rs1800100	heterozygous	Class 3	congenital bilateral absence of the vas deferens	autosomal recessive
					FSHR	Exon 10	p.S680N	homozygous	Class 3	decreased FSH response	autosomal recessive
					FSHR	Exon 10	p.S307N	homozygous	Class 3	decreased FSH response	autosomal recessive
4	30	FSH: 9.83 mIU/mL LH: 3.8 IU/mL T: 303 ng/mL	46XY	Normal	FSHR	Exon 10	p.S680N	heterozygous	Class 3	decreased FSH response	autosomal recessive
					FSHR	Exon 10	p.S307N	heterozygous	Class 3	decreased FSH response	autosomal recessive
5	32	FSH: 23.99 mIU/mL LH: 12.19 mIU/mL T: 188 ng/mL	46XY	A loss of approximately 1.1 MB was detected in the 22q11.21q11.22 (21444618-222580334) region.	-	-	-	-	-	-	-
6	37	FSH: 28.18 mIU/mL LH: 7.45 mIU/mL T: 51.9 ng/mL	46XY	Normal	DNAH11	Exon 15	c.2772G>A (p.Met924ıle) rs766050153	heterozygous	Class 3	primary ciliary dyskine-sia/Kartagener syndrome	autosomal recessive
7	39	FSH: 7.7 mIU/mL LH: 0.05 mIU/mL T: 417 ng/mL	46XY	Normal	DNAH5	Exon 43	c.7160C>A (p.Pro2387His) rs752767295	heterozygous	Class 3	primary ciliary dyskine-sia/Kartagener syndrome	autosomal recessive
					FSHR	Exon 10	p.S680N	homozygous	Class 3	decreased FSH response	autosomal recessive
					FSHR	Exon 10	p.A307T	homozygous	Class 3	decreased FSH response	autosomal recessive
8	38	FSH: 23.84 mIU/mL LH: 13.66 mIU/mL T: 608 ng/mL	46XY	Normal	-	-	-	-	-	-	-

TABLE 2. Continued.

P.NO	Age	Hormone	Chromosome analysis	Molecular karyotyping	New generation sequencing infertility panel						
					Gene	Location	Variant	Zygosity	Class	Disease	Heredity
9	44	FSH: 27.38 mIU/mL LH: 8.89 mIU/mL T: 205 ng/mL	46XY	Normal	DNAH5	Exon 68	c.11583C>A (p.Ser3861Arg) rs5760967758	heterozygous	Class 3	primary ciliary dyskine-sia/Kartagener syndrome	autosomal recessive
					FSHR	Exon 10	p.S680N	homozygous	Class 3	decreased FSH response	autosomal recessive
					FSHR	Exon 10	p.A307T	homozygous	Class 3	decreased FSH response	autosomal recessive
10	38	FSH: 6.39 mIU/mL LH: 2.44 mIU/mL T: 250 ng/mL	46XY	Normal	CFTR	Exon 22	c.3683A>G (p.Glu1228Gly) rs1190355027	heterozygous	Class 3	congenital bilateral absence of the vas deferens	autosomal recessive
					FSHR	Exon 10	p.S680N	heterozygous	Class 3	decreased FSH response	autosomal recessive
					FSHR	Exon 10	p.A307T	heterozygous	Class 3	decreased FSH response	autosomal recessive
11	33	FSH: 61.15 mIU/mL LH: 24.33 mIU/mL T: 400 ng/mL	47XXY	47XXY	ZMYND15	Exon 4	c.1027C>T (p.Arg343Trp) rs117858568	heterozygous	Class 3	spermatogenesis disorder type 15	autosomal recessive
					FSHR	Exon 10	p.S680N	heterozygous	Class 3	decreased FSH response	autosomal recessive
					FSHR	Exon 10	p.A307T	heterozygous	Class 3	decreased FSH response	autosomal recessive
12	36	FSH: 63.74 mIU/mL LH: 19.74 mIU/mL T: 19.74 ng/mL	46XY	Normal	LHCGR	Exon 11	c.1985A>G (p.Asn662Ser) rs200467316	homozygous	Class 3	LHCGR related diseases	autosomal domi-nant/autosomal recessive
13	38	FSH: 24.57 mIU/mL LH: 5.12 mIU/mL T: 323 ng/mL	46XY	Normal	-	-	-	-	-	-	-

TABLE 2. Continued.

P.NO	Age	Hormone	Chromosome analysis	Molecular karyotyping	New generation sequencing infertility panel						
					Gene	Location	Variant	Zygosity	Class	Disease	Heredity
14	34	FSH: 45.81 mIU/mL LH: 14.55 mIU/mL T: <20 ng/mL	46XY	Normal	INSL3	Exon 2	c.305G>Ap.-Arg102Hisrs121-9122556CM-036014	heterozygous	Class 1	cryptoorchidism	autosomal dominant
					FSHR	Exon 10	p.S680N	homozygous	Class 3	decreased FSH response	autosomal recessive
					FSHR	Exon 10	p.S307N	homozygous	Class 3	decreased FSH response	autosomal recessive
15	30	FSH: 13.01 mIU/mL LH: 5.37 mIU/mL T: 288 ng/mL	46XY	Normal	FSHR	Exon 10	p.S680N	heterozygous	Class 3	decreased FSH response	autosomal recessive
					FSHR	Exon 10	p.S307N	heterozygous	Class 3	decreased FSH response	autosomal recessive
16	39	FSH: 18.29 mIU/mL LH: 6.73 mIU/mL T: 368 ng/mL	46XY	AZFc Deletion	FSHR	Exon 10	p.S680N	heterozygous	Class 3	decreased FSH response	autosomal recessive
					FSHR	Exon 10	p.S307N	heterozygous	Class 3	decreased FSH response	autosomal recessive
17	30	FSH: 57.67 mIU/mL LH: 38 mIU/mL T: 66.8 ng/mL	47XXY	47XXY	FSHR	Exon 10	p.S680N	homozygous	Class 3	decreased FSH response	autosomal recessive
					FSHR	Exon 10	p.S307N	homozygous	Class 3	decreased FSH response	autosomal recessive
18	44	FSH: 33.3 mIU/mL LH: 31.73 mIU/mL T: 112 ng/mL	47XXY	47XXY	FSHR	Exon 10	p.S680N	heterozygous	Class 3	decreased FSH response	autosomal recessive
					FSHR	Exon 10	p.S307N	heterozygous	Class 3	decreased FSH response	autosomal recessive
19	33	FSH: 64.86 mIU/mL LH: 34.95 mIU/mL T: 83.6 ng/mL	47XXY	47XXY	CATSPER1	Exon 6	c.1834C>T (p.Arg12Cys) rs200131049	heterozygous	Class 3	total fertilization failure	autosomal recessive
					TEX101	Exon 5	c.38C>Trs759-736291 (p.Thr13ıle)	heterozygous	Class 3	TEX101-associated related diseases	autosomal domi-nant/autosomal recessive
20	40	FSH: 16.74 mIU/mL LH: 6.04 mIU/mL T: 148 ng/mL	46XY	Normal	FSHR	Exon 10	p.S680N	heterozygous	Class 3	decreased FSH response	autosomal recessive
					FSHR	Exon 10	p.S307N	heterozygous	Class 3	decreased FSH response	autosomal recessive

TABLE 2. Continued.

P.NO	Age	Hormone	Chromosome analysis	Molecular karyotyping	New generation sequencing infertility panel						
					Gene	Location	Variant	Zygosity	Class	Disease	Heredity
21	36	FSH: 26.94 mIU/mL LH: 13.23 mIU/mL T: 208 ng/mL	46XY	Normal	FSHR	Exon 10	p.S680N	heterozygous	Class 3	decreased FSH response	autosomal recessive
					FSHR	Exon 10	p.S307N	heterozygous	Class 3	decreased FSH response	autosomal recessive
22	34	FSH: 49.02 mIU/mL LH: 34.19 mIU/mL T: 131 ng/mL	47XXY	47XXY	FSHR	Exon 10	p.S680N	homozygous	Class 3	decreased FSH response	autosomal recessive
					FSHR	Exon 10	p.S307N	homozygous	Class 3	decreased FSH response	autosomal recessive
23	28	FSH: 33.98 mIU/mL LH: 15.16 mIU/mL T: 31.4 ng/mL	47XXY	47XXY	CFTR	Exon 15	c.2562_2563-delTGinsGA (p.Val855ıle) rs1584813846	heterozygous	Class 3	Congenital bilateral absence of the vas deferens	autosomal recessive
					FSHR	Exon 10	p.S680N	heterozygous	Class 3	decreased FSH response	autosomal recessive
					FSHR	Exon 10	p.S307N	heterozygous	Class 3	decreased FSH response	autosomal recessive
24	26	FSH: 14.38 mIU/mL LH: 3.88 mIU/mL T: 314 ng/mL	46, X, i(Y)(p10)	AZFa, AZFb and AZFc deletions were detected.	-	-	-	-	-	-	-
25	29	FSH: 33.82 mIU/mL LH: 17.76 mIU/mL T: 445 ng/mL	46XY	Normal	-	-	-	-	-	-	-
26	36	FSH: 50.96 mIU/mL LH: 27.29 mIU/mL T: 306 ng/mL	46XY	Normal	CFTR	Exon 18	c.2981T>G (p.Phe994Cys) rs397508469	heterozygous	Class 3	Congenital bilateral absence of the vas deferens	autosomal domi-nant/autosomal recessive
					FSHR	Exon 10	p.S680N	heterozygous	Class 3	decreased FSH response	autosomal recessive
					FSHR	Exon 10	p.A307T	heterozygous	Class 3	decreased FSH response	autosomal recessive

Abbreviations: AZFa: Azoospermia Factor a; AZFb: Azoospermia Factor b; AZFc: Azoospermia Factor c; CATSPER1: Cation Channel Sperm Associated 1; CFTR: Cystic fibrosis transmembrane conductance regulator; DNAH5: Dynein Axonemal Heavy Chain 5; DNAH11: Dynein Axonemal Heavy Chain 11; FSH: Follicle Stimulating Hormone; FSHR: Follicle Stimulating Hormone Receptor; INSL3: Insulin-like peptide 3; LH: Luteinizing Hormone; LHCGR: Luteinizing Hormone/Choriogonadotropin Receptor; T: Testosterone; TEX101: Testis Expressed 101; ZMYND15: Zinc Finger MYND-Type Containing 15.

In our study, FSHR polymorphism with "unclear pathologenic effect" was found in 17 patients. FSH levels were found to be higher than normal in 11 of these 17 patients (64.7%). In addition, *FSHR* polymorphisms were detected in 5 of our 6 patients with Klinefelter syndrome and FSH levels were observed to be higher than normal in 6 of them.

Mutations in the CFTR gene cause cystic fibrosis (CF) [56]. This disease is characterized by progressively worsening lung disease, pancreatic dysfunction, elevated sweat electrolytes, and male infertility. Given the fact that almost all male patients with CF are infertile due to congenital bilateral absence of the vas deferens (CBAVD), the question has arisen as to whether *CFTR* is also involved in infertility due to CBAVD alone. A small-scale French study reported that patients with CF had ongenital aplasia of the epididymis and vas deferens seven cases out of 17 azoospermic men (41%), while only 2.8% of individuals in the general population were reportedly heterozygous for the F508del mutation [57]. In another study, *CFTR* gene mutation was found in 9 of 750 unselected oligozoospermic patients [58]. Similar results have recently been described in a German population study. A heterozygous *CFTR* gene mutation was found in 5 (19.23%) of 26 nonobstructive azoospermic patients. Each of the mutations was located in different regions of genes.

The *CATSPER1* gene encodes a calcium channel that is involved in the acquisition of motility of mature sperm in the final stage of spermatogenesis. It is predicted that mutations in this gene will disrupt the motility of the sperm flagellum and cause infertility [59]. In our study, we found *CATSPER1* mutation in only one of our patients (number 19). This patient who had also TEX101 mutation underwent 47 XXY chromosome analysis.

TEX101 is a glycosylphosphatidyl inositol (GPI)-anchored glycoprotein that has been identified as a molecular marker of germ cells. Although there are indications that malfunction in TEX101 may affect male fertility, little is known about its exact physiological function and underlying molecular mechanisms. Recently, a study has showed that in the absence of the *TEX101* gene, sperms were unable to pass through the uterotubal junction or bind to the ZP (zona pellucida), leading to male infertility [60]. Additionally, a study found that a group of mice with the *TEX101* mutation had a uterotubal migration defect and that sperms had no ability to adhere to the surface of the female genital tract [61]. In our study, *TEX101* mutation was found in patient number 19 with a diagnosis of Klinefelter syndrome who also had a *CATSPER1* mutation.

It has been shown that INSL3 and its receptor LGR8 (leucine-rich repeat-containing G-protein-coupled receptor 8), the LH/-LHCGR system are effective in the descent of the testicles from the inguinal canal into the scrotal sac [62]. The *INSL3* gene localized on chromosome 19, close to the Janus kinase 3 (JAK3) gene contains two exons with an intron interrupting the C-peptide coding domain [63]. *INSL3*, produced by prenatal Leydig cells, is required for the transabdominal descent of testicles through stimulation of growth hormone and differentiation of the gubernaculum, as shown in rodents [64]. Since several mutations leading to amino acid substitution have been found incases with *INSL3* and *LGR8* mutations, it has been suggested that they may have a role in human cryptorchidism [65]. A review of the literature has shown that these mutations are prevalent in 4–5% of men with a past or present history of cryptorchidism [66]. In addition to its role in testicular descent and cryptorchidism, *INSL3* hormone has possible important but undefined endocrine and paracrine effects in adults. Deficiency of this hormone may represent an important manifestation of functional hypogonadism [66]. In a recent study, *LGR8* expression in germ cells showed that *INSL3* is linked to *LGR8*. Suppression of apoptosis by binding suggested that *INSL3* plays an active role in the survival of germ cells [67]. A study comparing serum *INSL3* levels in 135 fertile men and 85 male patients with testicular disorders showed that serum *INSL3* levels were within the normal range in fertile men, but decreased in patients with testicular disorders [68]. In our study, *INSL3* mutation and *FSHR* gene polymorphism were detected in patient number 14. The patient's bilateral testicles are located scrotally and their sizes are smaller than normal.

It is well known that LH and its receptor play a role in testicular descent. Patients with hypogonadotropic hypogonadism or inactivating mutations of the *LHCGR* gene have undescended testes due to androgen deficiency [69].

A study conducted on 278 patients with abnormally located testicles, 277 infertile men, and 271 men with normal spermatogenesis showed that patients with testicular disorders had a high LH level to maintain normal testosterone levels but this LH elevation was not associated with any *LHCGR* genotype.

It has been determined that the presence of *LHCGR* mutation is associated with spermatogenic damage rather than testicular dysfunction [70]. In our study, homozygous *LHCGR* mutation was observed in patient number 12.

ZMYND15 gene has been shown to act as a histone deacetylase-dependent transcriptional repressor which regulates the normal transient expression of haploid cell genes during spermatogenesis. Inactivation of *ZMYND15* results in premature activation of the transcription of several important haploid genes, including *Prm1* (Protamine 1), *Tnp1* (Transition Protein 1), *Spem1* (Spermatid Maturation 1) and Catpser3, causing late spermatid exhaustion and male infertility. ZMYND15 is the first transcriptional repressor gene identified as essential for sperm production and male fertility [51]. In a study, *ZMYND15* mutation was found in 3 patients in Sanger sequencing of 219 patients with severe oligozoospermia [71]. In our study, *ZMYND15* mutation and *FSHR* gene polymorphism were observed in patient number 11 with Klinefelter syndrome.

Kartagener syndrome, which is a type of Primary Ciliary Dyskinesia (PCD), has three components: chronic bronchitis and bronchiectasis, infertility with immotile sperm or low motility, and situs inversus. The diagnosis can be confirmed by electron microscopic examination of ciliary axonemal structures [72]. Most of the mutations identified to date have occurred in the *DNAH5* (dynein axonemal heavy chain 5) and *DNAI1* (dynein axonemal intermediate chain 1) genes, and a very small number of mutations involve other genes. A study conducted with 90 asthenozoospermic and 200 normospermic individuals, in which *DNAH5*, *DNAI1* and *DNAH11* mutations were investigated, reported that mutations were found in 7

patients in the asthenozoospermic group [73]. In our study, we found *DNAH11* variant in patient number 6 and *DNAH5* class 3 variant in patients number 7 and 9. Additionally, patients number 7 and 9 had *FSHR* gene polymorphisms.

The reason why pathogenic and possible pathogenic mutations were not detected in most of the patients in our study is that there may be other genes associated with infertility. In addition, many changes classified as "variants of uncertain clinical significance" were detected in our study according to the ACMG 2015 criteria and *in-silico* generated databases. However, these changes are not directly associated with infertility today; some of them are classified as polymorphisms and some as variants of unknown significance. These criteria may change as databases are constantly updated and new data is added.

Our study has some limitations. Most importantly our study population consisted of small number of patients. Therefore, mutations that might turn out to be pathogenic in a higher number of patients may have been evaluated as mutations with unknown pathogenic effects in this study. In addition, due to the small number of patients in our study population, rarely seen gene mutations might not be detected. Secondly, due to the lack of a control group in the study, the mutations detected could not be compared with those found in fertile male individuals. In fact, the high cost of commercial kits has restricted our ability to study infertility-associated mutations in greater number of patients. Nevertheless, we believe that our further study on the genetic evaluation of patients with non-obstructive azoospermia will contribute to the literature and lead to conduction of more comprehensive studies.

5. Conclusions

As a result, although many mutations and polymorphisms we found in our study were evaluated as "variants of uncertain clinical significance", they may be considered "pathogenic" variants in the future. We think that more research is needed to understand whether these changes cause infertility, and that next-generation sequencing can be used as a routine diagnostic test to determine mutations and polymorphisms related to *FSHR*, *DNAH5* and *DNAH11* genes in infertility cases of unknown etiology.

AVAILABILITY OF DATA AND MATERIALS

The data presented in this study are available on reasonable request from the corresponding author.

AUTHOR CONTRIBUTIONS

BD and IG—designed the research study. BD and AK—performed the research. CE and FO—provided help and advice on collection of samples. EA—analyzed the data. BD and EA—wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was conducted with the approval of the ethics committee of Inonu University Faculty of Medicine, Turgut Ozal Medical Center (ethics committee approval no: 2020/105). The patients provided informed consent and agreed to publication of the details of this research.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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