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RESEARCH ARTICLE

CONTRIBUTIONS TO RARE PHENOTYPES IN KLINEFELTER SYNDROME

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ABSTRACT

Purpose: Klinefelter Syndrome (KS; 47, XXY) and Y chromosome microdeletions are the most common genetic causes of male infertility. Our goal was to assess these factors contributing to male infertility in our region.
Material and Methods: In this current study, 58 patients diagnosed with azoospermia/oligozoospermia were invited to the polyclinic and 2 ml peripheral blood samples were collected. Genotyping by employing PCR based-fragment analysis was conducted after isolating genomic DNA from the peripheral blood samples of patients who consented to participate in our study. Patients' FSH, LH, and testosterone levels, as well as their physical examinations, were carefully evaluated.
Results: We found that high follicle stimulating hormone (FSH) value can be used as a predictive factor in azoospermia. We successfully revealed the potential of KS (3.2%) but no Y chromosome microdeletions are responsible for primary male infertility. A patient with KS was identified, exhibiting not only short stature but also a lack of breast enlargement.
Conclusion: Non-genetic factors such as varicocele (28%) and smoking (28%) may have greater potential to explain primary infertility in our region. Physicians should be aware that unexpected features such as short stature may accompany KS in adult patients who have not received growth hormone treatment.

Keywords: Klinefelter Syndrome, short stature, Y-chromosome microdeletion, delayed diagnosis, smoking.

INTRODUCTION

According to the definition of the World Health Organization (WHO), infertility; is the absence of pregnancy as a result of sexual intercourse 2-3 times a week regularly and unprotected during a year (1). About 15% of couples of reproductive age are affected by infertility (2). Male factors account for half of infertility cases, and recently, the rise in infertility rates has gained attention, particularly due to declining sperm quality. Azoospermia, or the absence of sperm in semen analysis, is observed in 11.2% of infertile men (3). The most common genetic cause of infertility is Klinefelter syndrome (47, XXY), which is a sex chromosome anomaly, and this syndrome is also the most common (1/650 newborn male) sex chromosome anomaly (4). In this syndrome, spermatogenesis and androgen hormone production are interrupted (5). High LH (luteinizing hormone) production affects estradiol production and causes gynecomastia, and insufficient Leydig cell number causes low testosterone levels (6). Klinefelter syndrome (KS) is observed in 3% of all infertile men, whereas KS is seen in 14% of non-obstructive azoospermic cases (7–9).

According to the data obtained from the studies carried out between 1984-2003; chromosomal anomaly is seen in 13.1% of azoospermic cases (67% KS), while a chromosomal anomaly is observed in 4.3% of oligozoospermic cases (12% KS) (10). Y-chromosome microdeletions are one of the most common genetic cause of male infertility (11). Y-chromosome hosts the AZF (AZoospermia Factor) region, which has important roles in spermatogenesis, and there are 4 different AZF

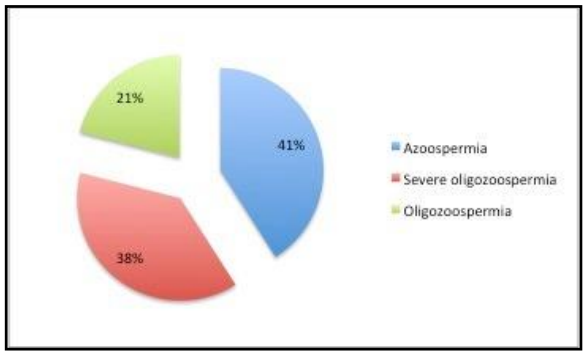

Figure 1. Classification of patients by sperm count

regions (AZFa, AZFb, AZFc and AZFd) (12). Three genes (*DDX3Y*, *USP9Y*, *UTY*) and 1 testis-specific transcription unit (*TTTY15*) located in the AZFa locus are present in a region of 1150 (kb), and single or combined deletions of these first 3 genes occur in Sertoli cell only (SCO) cases (9-55%) have been reported (13–15). The deleted AZFb locus, which is also associated with infertility, has 6 protein-coding genes and 9 TTTYs in a 6.2 Mb region, where the genes are *EIFA1Y*, *RPS4Y*, *SMCY*, *HSFY*, *PRY*, and *RBMY* genes (16). Mass deletions of AZFc are seen in 5-15% of infertile men and are responsible for oligozoospermia (17–20). In recent studies, the AZFd region is located between the AZFb and AZFc regions as a separate gene region, and patients with AZFd deletion may have mild oligozoospermia or normal sperm counting. However, it has been shown that they have a dysmorphic sperm structure (12).

We assessed the potential of the most common genetic and non-genetic causes of oligozoospermia and azoospermia in our region for the first time.

MATERIAL AND METHODS

Peripheral blood samples in K2-EDTA tubes by filling in informed consent form 58 patients who came to the Urology Polyclinic of Yozgat Bozok University, Medical Faculty Hospital due to primary infertility and had azoospermia/oligozoospermia in their semen analysis. The ethical approval of the current study was granted by the Clinical Research Ethics Committee of Faculty of Yozgat Bozok University (Date: 25.09.2019, Decision No: 2017-KAEK-189_2019.09.25_20).

This study was conducted in line with the principles of the "Helsinki Declaration". DNA isolation was performed from peripheral blood samples from 58 patients using commercial kits (PureLink™ Genomic DNA Mini Kit-Invitrogen). Polymerase chain reaction of the regions of interest was performed on a thermal cycler (SimpliAmp-Applied Biosystems Thermo Fisher Scientific) in accordance with the kit manual (GT AZFScreen Plus). FSH, LH and testosterone hormone levels were measured by Cobas series equipment (Roche Diagnostics, GmbH, Mannheim, Germany).

PCR conditions of initial denaturation (95°C- 20 mins), 30 cycles of (95°C- 1 min; 63°C- 90 sec; 70°C- 2 mins), final elongation (70°C- 20 mins), storage (4°C- ∞) was employed in thermal cycler. Samples were loaded into ABI Prism 3130 XL Genetic Analyzer and analyzed based on the pics of the fragments.

The spermiogram values of the patients; were divided into 3 groups and evaluated as azoospermia (zero sperm count), severe oligozoospermia (<5 million sperm/mL), and oligozoospermia (5-20 million sperm/mL). Figures were created and analyzed by choosing One-way-ANOVA (analysis of variance) and/or Kruskal-Wallis methods in the GraphPad Prism Version 8 software.

RESULTS

24 out of 58 patients who accepted to participate in the study had azoospermia, 22 had severe oligozoospermia, and the remaining 12 patients had oligozoospermia phenotype (Figure 1). The hormone levels we used as a reference in our project are given in table 1. The majority of the patients (n=47) were born in Yozgat and its surrounding districts (81.03%) (Supplemental Table 1).

When the patients were examined in 3 groups according to their sperm count, we found a significant difference between all groups (Figure 2). Accordingly, the mean sperm count of the patients with oligozoospermia was 13.4 ± 4.1 million/mL, which was higher than the mean of the patients with severe oligozoospermia, which 2.2 ±1.3 million/mL. The difference between the azoospermia group (zero sperm) and both severe oligozoospermia and oligozoospermia groups was also statistically significant ($p < .05$).

Table 1. Male reference hormone values

Hormon	Minumum	Maximum	Unit
FSH	0.95	11.95	mIU/mL
LH	0.57	12.07	mIU/mL
Total testosteron	1.4	9.2	ng/mL
Estradiol(E2)	11	44	pg/mL
Prolactin	3.46	19.40	ng/mL

FSH, LH, testosterone values were examined according to the groups, we found a significant difference between azoospermia and FSH levels (Figure 3). The mean FSH (15.0 ± 9.4 mIU/mL) in patients with azoospermia was higher than both the mean FSH in the patient group with oligozoospermia (5.5 ± 4.5 mIU/mL) and the mean FSH in the patient group with severe oligozoospermia (8.5 ± 7.2 mIU/mL). The mean FSH level of the patient group with severe oligozoospermia the was higher than the mean FSH level of the patient group with oligozoospermia, but it was not statistically significant (p> .05). Differences between mean LH or testosterone levels were also not statistically significant (p> .05). The mean FSH level was 10.5 ± 8.5 mIU/mL, the mean LH level was 6.2 ± 3.2 mIU/mL, and the mean testosterone level was 5.4 ± 2.9 ng/mL (Figure 4).

It was found that 5 of the married couples were consanguineous (8.8%). When evaluating male infertile patients by occupation, it was noted that 5 patients (8.8%) were security guards and 4 patients (7.0%) were construction workers. Additionally, a smoking history was observed in 16 patients (28.1%), with 12 of them (75%) consuming more than one pack of cigarettes per day.

Fifteen patients (26.3%) had a history of varicocele with a grade lower than three. In 6 patients out of 15 with varicocele had a history of varicocele operation. In addition to the 15 patients mentioned earlier, another patient (patient #22) experienced physical trauma to his testicles in his youth. Fifteen patients had a history of undergoing TESE/mTESE (microsurgical testicular sperm extraction). Among these 15, sperm retrieval was unsuccessful in 5 patients, all of whom were diagnosed with azoospermia.

A non-smoker patient without a history of varicocele had a history of pituitary adenoma (prolactinoma) and was followed for a long time using psychiatric drugs (patient #13). Despite being azoospermic, blood tests showed relatively normal follicle-stimulating hormone (FSH) at 2.6 mIU/mL and luteinizing hormone (LH) at 0.9 mIU/mL, while testosterone was low at 3 ng/mL. Long-term use of high-dose colchicine due to familial mediterranean fever (FMF) was observed in a patient with oligozoospermia (patient #58). A patient with azoospermia working in the construction industry (patient #30), who stated that he smoked 1 pack of cigarettes a day, and he had received chemotherapy 10 years ago for lung cancer. A patient with

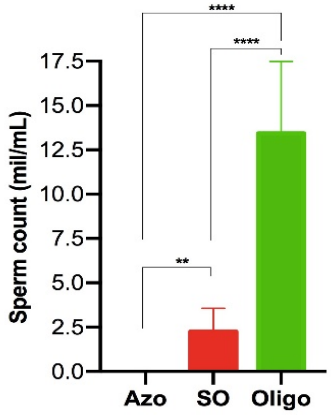

Figure 2. Demonstration of the difference between the sperm counts of the patients (** p = .0094, ****, p < .0001, Kruskal-Wallis)

azoospermia (patient #9) had a history of left orchiectomy in 2014 for sertoli cell only syndrome (SCO) and testicular cancer.

TESE history was followed up in 15 patients (26.3%). One of these patients (patient #48) had a healthy baby with the ROSI (round sperm injection) method. Apart from this patient, it was learnt that a patient with a sperm count of 100 thousand/mL (patient #57) fathered a healthy girl later on in a normal way. Excluding these two cases, we learned about two additional patients who achieved successful pregnancies with ART (assisted reproductive techniques) despite having azoospermia and severe oligozoospermia, respectively. Notably, these outcomes were achieved without the use of TESE. Additionally, it was observed that a patient with oligozoospermia (patient #35) successfully fathered a daughter naturally, without the need for ART.

KS (47, XXY) was detected in 2 patients (patient #27 and #49). Patient 27 did not present the phenotypic features of KS such as enlarged breasts and lack of facial and body hair. The patient was not tall (1.70 meter) either. Additionally, no microdeletions were found in the AZF regions of the Y chromosome in any of the patients, and none exhibited XYY syndrome.

Patient #45 underwent a male infertility panel that evaluated five genes: *CFTR*, *CATSPER1*, *LHCGR*, *AR*, and *FSHR*. This analysis revealed a heterozygous pathogenic mutation (p.G178R) in the *CFTR* gene. Additionally, conventional cytogenetic testing confirmed a normal chromosomal karyotype (46, XY) for this patient.

When providing counseling upon receiving the genetic results, it was realized that the azospermic patient 21 did not fully meet the criteria for primary

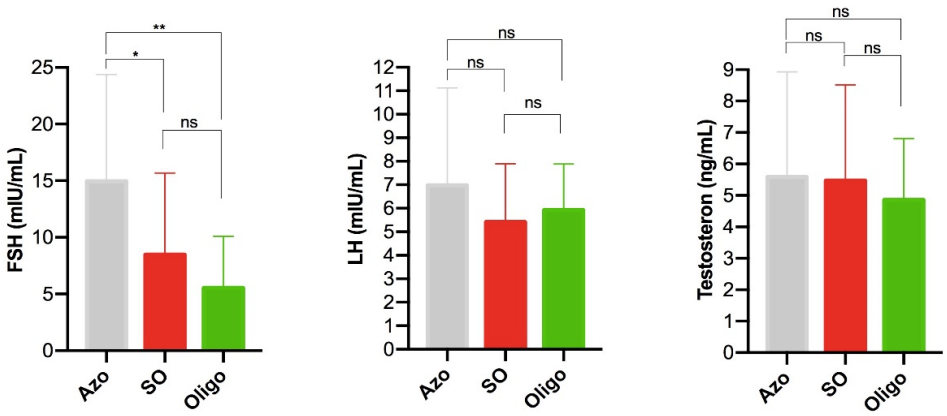

Figure 3. FSH, LH and testosterone hormone levels according to the sperm count profile (FSH group * p = .0195, ** p = .0036, ns: not significant = not significant, p = .5491, Ordinary one-way ANOVA, expressed as ns: p < .05 for LH and testosterone groups)

infertility (duration is 10 months but not a year). Therefore, the patient was not included in the results section. So, we decided to give patient number as 57 while calculation the ratios.

DISCUSSION

Sperm count was below 5 million in 79% of primary infertile men in this current study. When the patients are grouped in terms of their hormone profiles, we can say that high FSH value can be used as a predictive factor in azoospermia (Figure 4). The same is not the case with the other two hormones (LH and testosterone).

In line with the tests we conducted within the scope of the project, we determined genetically factors that could explain infertility in 2 patients (3.5%). These patients were also azoospermic. In the literature, the incidence of KS in azoospermic cases is up to 12.6% and its frequency has been reported as up to 3% in infertile men (21–23). Considering our 23 cases of azoospermia, our rate is slightly low (8.7%), and it is almost the same when considering our 57 infertile cases. In a retrospective study, in which only cases with azoospermia and severe oligozoospermia were evaluated, KS was found to be 8% in total, 3.2% in men with azoospermia, and 11.5% in men with severe oligozoospermia (24).

One distinctive feature of our study is the existence of unexpected combination of relatively short stature in one patient with KS (BMI-body mass index; 24.2). Short stature which was explained by partial growth hormone deficiency in some previous pediatric KS cases (25–27) , was observed in the adult patient in our case (170 cm). Enlarged breast structure and lack of hair growth, which are specific findings of KS, were not present in our case either. The hormone profile of this patient was high FSH (21.8 mIU/mL), and borderline testosterone 152.4 ng/dL while there is normal LH (8.7 mIU/mL) level. One very rare adult case report was also found in literature that acromegaly can accompany with Klinefelter syndrome as a result of high growth hormone concentration (15.1 ng/mL, reference range is 0.01-3.6 ng/mL) due to the existence of pituitary adenoma (28). The height of this adult patient (age 27) was found to be 2 cm taller than our patient but was considered as short compared to the patients with KS. We couldn't find any documentation in the patients' medical records regarding growth hormone testing or its results. The unusual presentation of KS in this patient may have led to a delayed or missed diagnosis by their physicians. This could be due to the presence of unexpected and uncommon features associated with the syndrome.

AZF region deletion was not found in any of the infertile male patients included in our study. Our findings suggest that Y-chromosome microdeletions are not a major contributing factor to male infertility in our patient population. Deletions in the AZF regions of the Y chromosome are the second most frequent genetic cause of male infertility, following KS, with a reported prevalence of up to 10% (29). Among Y-chromosome microdeletions, the most common condition is AZFc deletions. In a study originating from Turkiye and finding the lowest rate of AZFc deletions (in only 1 azoospermic patient), this rate was found to be 1.3%, while AZFa and AZFb region deletions were not observed (30). The prevalence of Y-chromosome microdeletions can vary across populations. An Iranian study, for instance, found a higher frequency of AZFb deletions (66.7%)

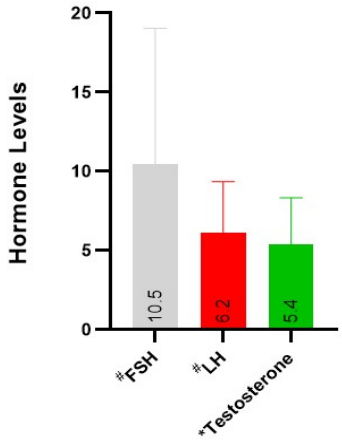

Figure 4. FSH, LH and testosterone hormone levels in primary infertile male patients (#:mIU/mL for FSH and LH levels, * ng/mL for testosterone levels)

compared to AZFc deletions (41.7%), with an overall microdeletion rate of 12% (31).

While our study did not identify the most common genetic contributors to infertility in this patient group, it does not rule out a genetic cause altogether. Further investigation into chromosomal abnormalities, such as Robertsonian and reciprocal translocations, is necessary. Techniques such as conventional cytogenetics or array CGH (comparative genomic hybridization) can be employed for this purpose. Emerging research suggests a significant role for de novo mutations in male infertility. Studies have identified at least 94 genes potentially contributing to this condition (32).

CONCLUSION

We can conclude that etiological factors like varicocele (28%), smoking (28%), working in intensive jobs (15.8%), long-term drug use (1.8%) such as cholchisine, chemotherapy history (1.8%) have more potentials to explain male infertility in our primary infertile patient group than the genetic factors such as XXY syndrome and Y chromosome microdeletions. Since ratio of smokers is significantly higher in Türkiye than western countries, conducting studies with a larger number of patients divided into two distinct groups, smokers and non-smokers, would yield healthier results. To prevent missed/overlooked diagnoses of KS in patients presenting with atypical symptoms, physicians should consider including common causes of primary male infertility in their differential diagnoses.

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Conflict of Interests: The authors declare no conflict of interest.

Ethical Approval: The ethical approval of the current study was granted by the Clinical Research Ethics Committee of Faculty of Yozgat Bozok University (Date: 25.09.2019, Decision No: 2017-KAEK-189_2019.09.25_20).

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Supplemental Table 1. The hormone levels and some clinical outputs of 58 patients in current study

Patient No	Infertility (month)	Relativity status	Sperm million/mL	LH mIU/mL	FSH mIU/mL	T. ng/mL	Smoking status	Var*/operation torsion-trauma	TESE status
1	36	No	20	3.7	18.5	2.1	1 pack	operated	No
2	18	Yes	0	6.5	15	5.8	No	No	No
3	18	No	3	2.8	5.8	3.1	No	No	No
4	36	No	0	6.8	21.1	4.3	No	No	No
5	48	No	3	3.4	3	2.3	No	No	No
6	36	Yes	2.5	7.1	25.6	5.3	No	No	No
7	72	No	0	4.8	7.6	8.7	No	No	No
8	18	No	2	5.9	8.5	5.3	No	No	No
9	12	No	0	14.3	38.4	3.1	No	left orchiectomy	Yes
10	12	No	2	4.4	18.9	7	No	No	No
11	24	Yes	0.5	5.2	7.3	5.7	No	Var.	No
12	24	No	0	5.8	5.3	2.4	No	No	Yes
13	36	No	0	0.9	2.6	3	No	No	Yes
14	12	No	0.5	7	6	7.7	No	No	No
15	12	No	2.5	4	3.9	3.1	No	operated	No
16	84	No	4	4.3	2.7	6	No	No	No
17	12	Yes	0	1.8	3.5	5.8	No	Var.	Yes
18	48	No	0	2.6	5.3	3.8	No	operated	No
19	24	No	4	4.7	5	5.8	No	No	No
20	12	No	0.1	2.6	3.6	4.2	No	No	No
21	NA	NA	0	69.1	136	1.6	No	torsion	No
22	18	No	0	11.5	19.3	3.3	3-4 butt	trauma	No
23	30	No	12	8.2	5.8	4.9	No	No	No
24	18	No	2	4.5	3.5	3.3	No	No	No
25	48	No	1	5.4	24.1	6.9	1 pack	No	No
26	72	Yes	3	3	4.5	3.5	1 pack	Var.	No
27	276	No	0	12	20	166	4 packs	operated	Yes
28	12	No	12	5.4	1.9	2.2	No	No	No
29	42	No	0	4	22.2	3.7	No	No	Yes
30	60	No	0	6.3	10.5	4.6	1 pack	No	No
31	18	No	14	6.6	5.2	5.6	3-4 butt	No	No
32	18	No	18	6.8	5	7	No	Var.	No
33	12	No	3	11.9	21.7	4.8	No	No	No
34	12	No	0.1	9.1	4.3	4	No	No	No
35	12	No	14	5.1	5.3	3.9	1 butt	No	No
36	12	No	0	7.9	15.7	7.4	No	No	No
37	18	No	4	5	5.6	5.2	No	operated	No
38	12	No	15	7.6	2.3	8	No	No	No
39	12	No	3	8.8	3.4	3.7	No	No	No
40	24	No	6	4.5	2.8	5.5	No	operated	No

Supplemental Table 1 Continue.

Patient No	Infertility (month)	Relativity status	Sperm million/mL	LH mIU/mL	FSH mIU/mL	T. ng/mL	Smoking status	Var*/operation torsion-trauma
41	24	No	14	5.7	3.9	6.2	No	No
42	36	No	4	2.7	7.3	2.9	No	Var.
43	156	No	7	9.9	3.7	5.4	No	No
44	96	No	0	6.6	19.9	2.3	No	Var.
45	72	No	0	8.6	12.5	2.8	No	No
46	96	No	12	3.9	2.7	5.5	30 butt	Var.
47	20	No	17	3.7	9.3	2	5-6 butt	Var./right orchiectomy
48	180	No	0	1.5	2.8	14.7	1 pack	No
49	12	No	0	18.4	12.1	9.4	No	No
50	180	No	0	8	12	5.5	1 pack	No
51	18	No	2	6.7	6.6	5.2	1 pack	Var.
52	60	No	0	6.4	12.5	12.4	No	No
53	40	No	0	6.3	30	6	1 pack	No
54	72	No	0	7.1	5.3	2.8	1 pack	No
55	150	No	0	8.8	25.5	8.7	1 pack	No
56	120	No	0	3.6	24.8	6.2	1 pack	No
57	24	No	0.1	8.2	11.8	8.3	No	No
58	24	No	2.8	2.5	3.2	17	No	No

Abbreviations: T.:Testosteron,*Var. represents the varicocele status is present but patient has not been operated. Rows in green show patients with KS.