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Determination of Y Chromosome Microdeletions in Infertile Men at Cukurova Region in Turkey

Türkiye'nin Çukurova Bölgesinde İnfertil Erkeklerde Y Kromozom Mikrodelesyonlarının Saptanması

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ABSTRACT

Purpose:Y Chromosome infertility is inherited in a Y-linked manner. Three different regions have been mapped on the long arm of the Y chromosome, named ''Azoospermic Factor'' (AZFa, AZFb and AZFc) are involved in the control of spermatogenesis. Microdeletions in these gene loci may result in azoospermia or severe oligozoospermia. The aim of this study is to establish the prevalence of Y chromosomal microdeletions in infertile men at the Cukurova Region in Turkey.

Material and Methods: We evaluated the frequency of Y chromosome deletions in 63 infertile men (38 azoospermic and 25 severe oligozoospermic) and 10 fertile men as a control group by using multiplex polymerase chain reaction (PCR) analyis. Plasma hormone concentrations of all patients including FSH, LH, testosterone, prolactin and leptin were measured by radioimmunoassay

Results: Microdeletion frequency detected in all cases was 6.3% (4/63). The values for azoospermic group and severe oligozoospermic group were 7.8% (3/38) and 4% (1/25) respectively. Deletions were found at AZFb, AZFc and proximal AZFc/d regions in infertile group. However, no microdeletions were detected at the AZFa region. No deletions were found in the control group. FSH and LH levels were significantly elevated in azoospermic group than control and severe oligozoospermic groups (p:0.000). Prolactin levels were significantly elevated in azoospermic group than control group (p:0.000).

Conclusion: Detection of Y Chromosome deletions in infertile males in routine clinical diagnosis may suitable for counseling prior to assisted reproduction.

Key Words: Y chromosome microdeletions, male infertility, AZF genes, PCR.

ÖZET

Amaç: Y kromozomuna bağlı infertilite Y-bağlı kalıtım gösterir. Y kromozomunun uzun kolunda, spermatogenezden sorumlu ''Azospermik Faktör'' olarak anılan, AZFa, AZFb ve AZFc şeklinde üç farklı bölge tanımlanmıştır. Bu bölgelerin delesyonu azospermi ya da şiddetli oligospermi ile sonuçlanabilmektedir. Bu çalışmanın amacı, Türkiye'nin Çukurova Bölgesi'ndeki infertil erkeklerde Y kromozom mikrodelesyon sıklığının saptanmasıdır.

Materyal ve Metod: Çalışmada, 63 infertil erkekte (38 azospermik, 25 şiddetli oligospermik) ve 10 fertil kontrol grubunda Y kromozom mikrodelesyonu multipleks PCR yöntemi ile araştırılmıştır. Tüm olguların plazma FSH, LH, testosteron, prolaktin ve leptin düzeyleri "radiyoimmünoassay" yöntemiyle çalışılmıştır.

Bulgular: Mikrodelesyon görülme sıklığı tüm olgularda %6,3 (4/63) iken, azospermik grupta %7,8 (3/38), şiddetli oligospermik grupta %4 (1/25) bulunmuştur. İnfertil grupta, AZFb, AZFc ve proksimal AZFc/d bölgelerinde mikrodelesyon saptanırken AZFa bölgesinde delesyon saptanmamıştır. Kontrol grubunda delesyon görülmemiştir. Azospermik grupta FSH ve LH düzeyleri şiddetli oligospermik ve kontrol gruplarına göre anlamlı derecede yüksek bulunmuştur (p.0000). Azospermik grupta prolaktin düzeyi kontrol grubuna göre yüksek saptanmıştır (p:000).
Sonuç: İnfertil erkeklerin rutin klinik tanısında, Y kromozom mikrodelesyonlarının tayini, yardımcı üreme yöntemlerinden önce danışmanlık amaçlı kullanılabilir.
Anahtar Kelimeler: Y kromozom mikrodelesyonları,erkek infertilitesi, AZF genleri, PCR.

INTRODUCTION

Approximately 15 % of couples attempting to conceive over a period of 1 year are unable to become pregnant and 20 % of those cases are exclusively attributable to male factor. Male infertility can be categorized as pretesticular, testicular and post-testicular[1,2]. The aetiology of male factor infertility has often been poorly understood. Y chromosome plays a fundemental role not only for sex determination but also in the control of spermatogenesis[3]. The most common chromosomal disorder in male infertility is Klinefelter syndrome and the second most common genetic cause is microdeletions of the Y chromosome[4]. The Y chromosome is the smallest chromosome in the human genome[5-7]. Use of intracytoplasmic sperm injection (ICSI) may allow Y chromosome defects to be inherited[8-13]. Recent information has supported the importance of Y chromosome in the control of spermatogenesis[14-20]. The AZF region of the Y chromosome has been described previously by Tiepolo and Zuffardi in 1976 regarding to cytogenetic deletions in infertile men[21]. Three different regions named AZFa, AZFb and AZFc, involved in the control of spermatogenesis have been mapped on the long arm of the Y chromosome (Yq11.22-23)[22-26]. Two candidate genes have been identified: the RNA-binding motif (RBM) gene which is isolated from the AZFb region, and the deleted in Azoospermia (DAZ) gene identified in several copies in the AZFc region[27-30]. Microdeletions most frequently involve AZFc region (60%) less frequently the AZFb region (16%) and only rarely the AZFa interval (5%)[18]. AZFc region includes three important protein coding gene families: DAZ, BPY2 (Basic Protein on Y) and CDY1 (Chromodomain on Y)[15,16,18,19,7,29,31]. Deletions in AZFa region have been associated with Sertoli cell-only syndrome I (SCOS I), those in AZFb leads to variable Phenotypes such as hypospermatogenesis or Sertoli cell-only sydrome II. Moreover, deletions including AZFc region (such as AZFb+c or AZFa+b+c) are associated with a total absence of testicular spermatozoa[32-39,17]. Until now, screening for Y chromosome microdeletions in men with oligozoospermia and azoospermia has been performed and different deletion ratios were found[32,40-43,38,44,31,39,45-52]. The incidence of Y chromosome microdeletions varies between studies, ranging from 1% to 35% in azoospermic or oligospermic men[8,28]. These variations have different explanations. First of all, the study groups are not the same since the underlying aetiology differ from oligozoospermia to azoospermia. Secondly, AZFa, AZFb and AZFc regions have been studied by different molecular techniques and different series of sequence-tagged sites (STSs) on Yq were used for the detection of submicroscopic deletions. Thirdly, ethnical differences besides the lack of agreement concerning genotype/phenotype correlations or the frequency and/or position of Yq microdeletions were noted[18,53,54].

In this study, we determined the existance of Y chromosome microdeletions in cases of azoospermia and severe oligozoospermia by using polymerase chain reaction method in infertile men at the Cukurova Region which lies at the eastern Mediterranean coast of Turkey.

MATERIALS and METHODS

Patients

63 Infertile (38 azoospermic, 25 severe oligozoospermic) and 10 fertile men (control group) were evaluated for the frequency of Y chromosome microdeletions by multiplex PCR (polymerase chain reaction) method using 20 gene-spesific primers (STSs). Semen specimens were provided from patients who attended to the Assisted Reproduction Unit of the department of Obstetrics and Gynecology, Medical Faculty, University of Cukurova for infertility evalution. Written informed consents were obtained from all patients, according to the criteria of the Ethical Committee of the Medical Faculty.

Semen analysis

At least two microscopic semen analysis, at 3-week intervals following 3 to 5 day sexual abstinence were assessed according to WHO (World Health Organization) criteria. Sperm concentrations were measured after liquefaction by the use of Mackler chamber. Patients with concentrations of less than $1x10^6$/ml were considered as severe oligospermic while specimens considered as azoospermic were centrifuged (1000g for 10 min) and the pellets were examined for spermatozoa for the confirmation of azoospermia .

Molecular-genetic methods

Genomic DNAs were isolated from peripheral leucocytes by the salting out method. Isolated DNA was stored at -40 ◦C. Y chromosome microdeletions were evaluated by multiplex PCR with the Promega version 2.0 Y chromosome deletions detection system. 20 Gene-spesific primers (STSs) within the long arm of the Y chromosome were selected emphasizing the AZFa, b, c and proximal AZFc/d regions. For each participiant, Y chromosome-spesific STSs that spanned the AZFa (sY81, sY84, sY86, sY182), AZFb (sY121, sY124, sY127, sY128, sY130, sY133, sY134, SYPR3), AZFc (sY157, sY208, sY242, sY254, sY255) and proximal AZFc/d (sY145, sY152) subregions were used. Testing of the presence of the short arm of the Y chromosome (Yp) was performed with the STS sY14, located within the sex-determining region on the Y chromosome (SRY gene) (Promega ver 2.0).

The primers were combined into five sets for multiplex PCR for the purpose of determining the presence of all 20 sequence-tagged sites by performing five parallel PCR amplifications. This system covers all of the loci recommended by the European Academy of Andrology (EAA) and the European Quality Monitoring Network Group (EMQN)[53,54]. Four of the multiplex primer sets contain a control primer pair that amplifies a fragment of the X-linked SMCX locus. One of the multiplex primer sets (Multiplex E Master Mix) contains a control primer pair that amplifies a unique region in both male and female DNA (ZFX/ZFY). Finally, a primer pair that amplifies a region of the SRY gene has been included in Multiplex E Master Mix as a control for the testis-determining factor on the short arm of the Y chromosome to detect XX males arising from Y to X translocations. The primers used for each multiplex PCR set were as follows:

- Multiplex A: sY254, sY157, sY81, sY130, sy182
- Multiplex B: SYPR3, sY127, sY242, sY208
- Multiplex C: sY128, sY121, sY145, sy255
- Multiplex D: sY133, sY152, sY124
- Multiplex E: sY14, sY134, sY86, sY84

Amplifications were carried out on a thermocycler (Eppendorf master cycler) with the following program: initial denaturation at 94°C for 2 min and subsequent series of 35 cycles at 94°C for 1 min (denaturation), 58 °C for 30 s (annealing) and 72°C for 1 min (extension). A final extension was carried out at 72°C for 5 min. PCR amplification product was electrophorised on a 4% gel with NuSieve agarose (sigma) gel prepared in 1XTBE buffer containig ethidium bromide (10 mg/ml) and visualized by exposure to ultraviolet light (Promega ver 2.0).

Hormonal evaluation

Plasma hormone concentrations of all patients including FSH, LH, testosterone, prolactin and leptin were measured by radioimmunoassay.

Statistical analysis was carried out by Statistical Package for Social Science (SPSS) for Windows, version 12.0. Results were analyzed with the Mann-Whitney U test. A P value of less than 0.05 was considered statistically significant.

RESULTS

In this study, 63 infertile and 10 fertile men were examined and four AZF deletions were detected in infertile groups. All deletion regions were shown in figure 5. No microdeletions were dedected in fertile control group. Microdeletion frequency detected in all infertile cases was 6.3% (4/63). The values for azoospermic group and severe oligozoospermic group were 7.8% (3/38) and 4% (1/25) respectively. Deletions were dedected in AZFb, AZFc and proximal c/d regions (fig. 1). Among these, one deletion in azoospermic group was involving complete AZFb and AZFc regions and incomplete proximal c/d regions. Another deletion in azoospermic group, involved AZFb and AZFc except DYS221 locus (fig.2). One individual in azoospermic group and another individual in severe oligozoospermic group involved only AZFc regions and always included the DAZ gene (fig.3 and 4). A year later, no sperms were detected in ejaculates of severe oligospermic men with deletions in AZFc region.

Plasma concentrations of follicle stimulating hormone (FSH), luteinizing hormone (LH), prolactin, testosterone and leptin were measured by radioimmunoassay and their relation to Y chromosome microdeletions were investigated (table 1). FSH and LH levels were significantly elevated in azoospermic group when compared to the severe oligozoospermic group (p:0.000) and control cases. Prolactin levels were significantly elevated in azoospermic group than control group (P:0.000). Plasma testosterone and leptin levels varied between individuals significantly.

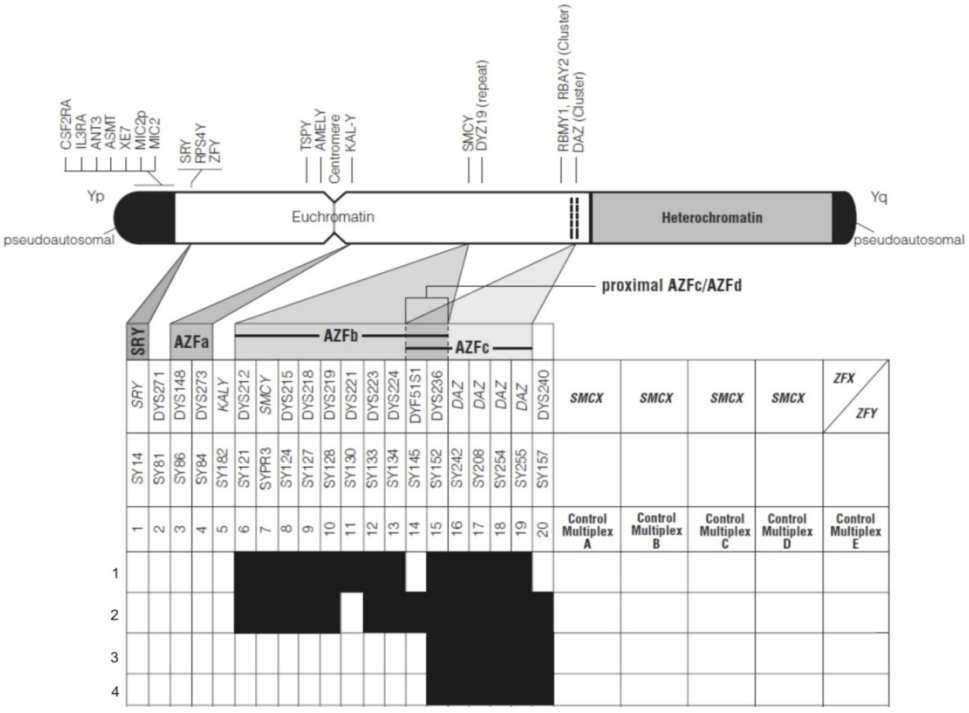

Figure 5. Y chromosome deletions regions of four patients with AZF deletions

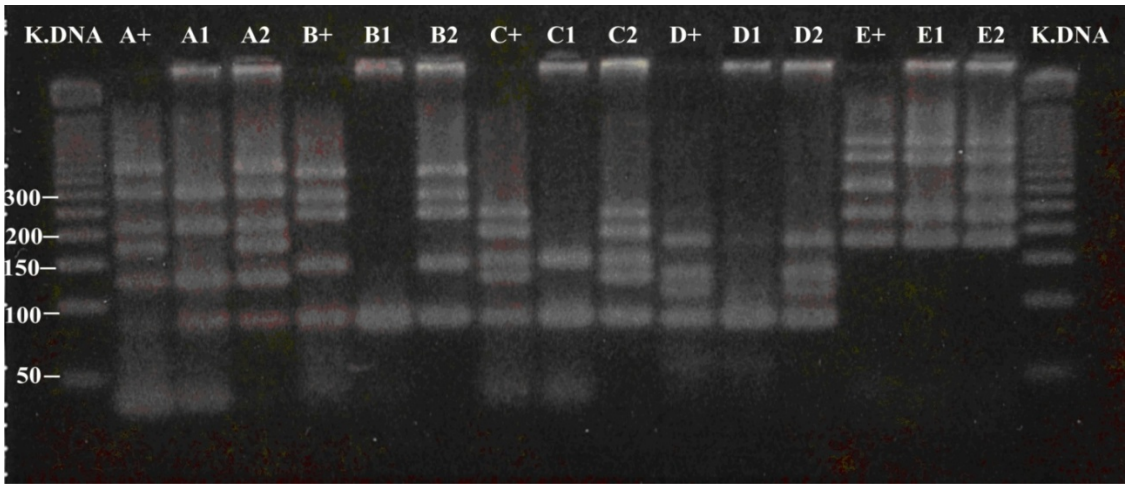

Figure 1. Y chromosome deletion analysis of complete AZFb and AZFc region and incomplete proximal c/d regions. The amplification products from multiplex A master mix, multiplex B master mix, multiplex C master mix, multiplex D master mix and multiplex E master mix reactions are shown. Lane:K.DNA contain the 50bp DNA step ladder. Lane A+, male genomic DNA control (no deletions) of multiplex master mix A. Lane 1: man with no AZF deletions, lane 2: azoospermic man with AZF deletions.

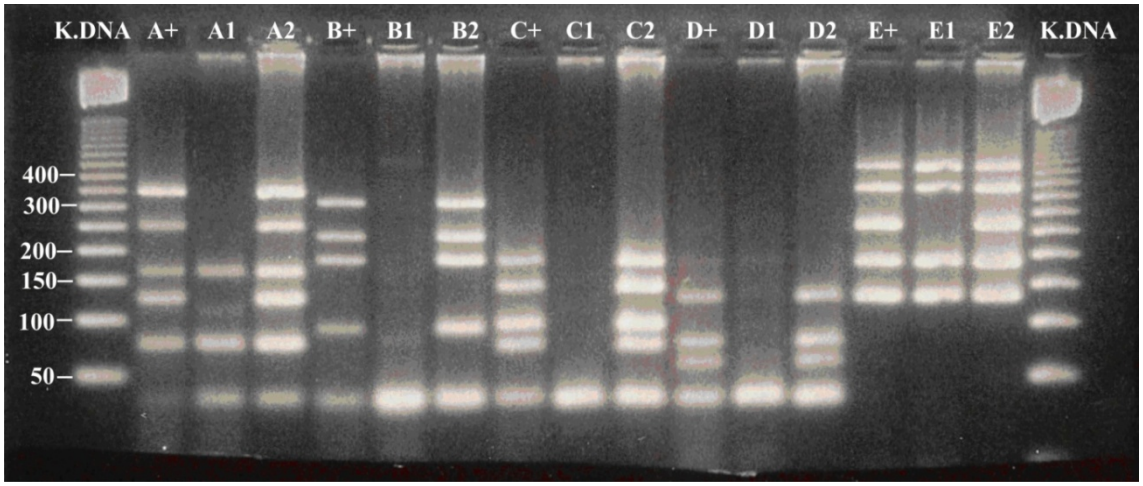

Figure 2. Y chromosome deletion analysis of AZFb and AZFc regions except DYS221 locus and complete proximal AZFc/d region. Lane 1: azoospermic man with AZF deletions. Lane 2: man with no AZF deletions

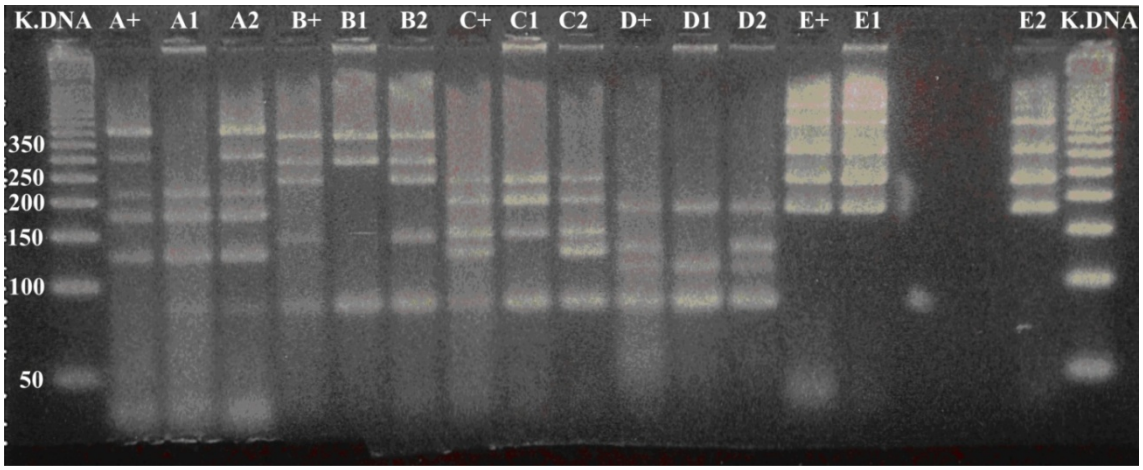

Figure 3. Y chromosome deletion analysis of complete AZFc region and incomplete proximal AZFc/d region. Lane 1: azoospermic man with AZF deletions. Lane 2: man with no AZF deletions,

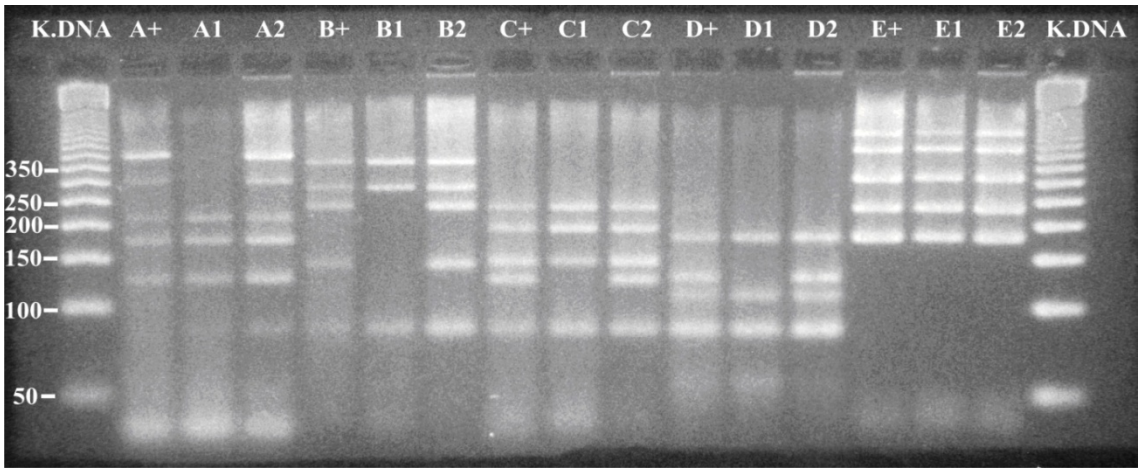

Figure 4. Y chromosome deletion analysis of complete AZFc region and incomplete proximal AZFc/d region. Lane 1: azoospermic man with AZF deletions. Lane 2: man with no AZF deletions,

Table 1. Plasma hormone levels of control group and patients with Y chromosome mictodeletions

Patients No	FSH (mlU/mlj	LH (mIU/ml)	Testosterone (ng/ml)	Prolactin (ng/ml)	Leptin (ng/ml)
1	9.0	9,1	3,.6	15,2	13,2
2	9,4	12,4	1,8	6,6	9,5
3	7,5	7,2	2,0	9,1	5,3
4	8,0	5,4	2,0	26,8	0,9
Control (X±SD) (min-max)	3,54±1,51 0.80-5.70	6,58±2,30 2.50-8.80	3,51±0.98 2,30-5.60	7,49±2,21 3,60-11,20	5,23±2,66 1,5-9,5

DISCUSSION

Anatolia is a multi-ethnic region. Cukurova region located at the Mediterranean coast of Anatolia-Turkey is a cosmopolitan center because of the population movements, as a key force in the social transformation of Turkey and some other countries. For this reason, hemoglobinopathies such as sickle cell anemia traits (10 %) and ß-thalassemia traits (3.7 %) are the most common genetic diseases in this region [55-57]. So; Y chromosome microdeletions were thought to be an interesting topic in infertile men of this area.

In this study, four patients with AZF deletions were detected in 63 infertile males. The general frequency of Y chromosome microdeletion was 6.3% (4/63). The frequencies in azospermic and severe oligozoospermic groups were respectively 7.8% (3/38) and 4% (1/25). No microdeletions were found in fertile control group.

Most of the genes related to spermatogenesis, encode proteins involved in post-transcriptional gene expression and therefore could participate in the sperm maturation process[58,22,23,16,56,18,6]. Deletions of the three AZF regions occur with different frequency; AZFa 3%, AZFb 9%, AZFc 79%, AZFb+c 6%, AZF a+b+c 3 % [54].

In this study, we found deletions at the AZFb, AZFc and proximal AZFc/d regions. No microdeletions were detected that affected the AZFa region. The diagnosis of complete deletions of the AZFa region implies the virtual impossibility to retrieve testicular sperm for ICSI. Large deletions in the AZFa region are associated with SCOS phenotype[23,33-39]. In AZFa region, the DFFRY gene which encodes the protein involved in desubiquitination has been proposed to play role in gametogenesis [22,59,27,6,26].

AZFc deletions are associated with variable histological pictures .The clinical conclusions derived from the literature is that at least a few spermatozoa are detected in most infertile men with standard AZFc deletions as an explanation to the fact that deletions of AZFc region alone are generally not convenient to prevent complete spermatogenesis [22,37, 18,60].

Spermatozoa are generally not detected in men with larger deletions encompassing AZFc plus more proximal and distal regions of the Y chromosome[22,23,60,38,47]. Deletions in AZFb region result in spermatogenic arrest, usually at the spermatocyte stage. Therefore, deletion of functional members of RNA-binding motive (RBM) gene family, or of some of the other genes found in AZFb region[58], e.g.EI1AY, PRY and TTY2, might contribute to the enhanced severity of spermatogenic defects observed in patients whose deletions extend proximal to the AZFc region[22,14-16,25,18,31,30]. According to the studies performed in Turkey about Y chromosome microdeletions, AZFa deletion was detected in the west while it was not found in the eastern part of the Mediterranean

region[61]. Also the reported prevalence of Y chromosome microdeletions in 18% of azoospermic and 21% of oligospermic groups were related to AZFa, AZFb and AZFc regions. In central Anatolia 9.1% deletion at the AZFc locus was evaluated[62]. In another survey the incidence of Y chromosome microdeletions was 14.7 % and the deleted regions were AZFa and AZFb, c, d in the northwest part of Turkey [63]. In a study carried out in Iran the reported prevalence of Y chromosome microdeletions was 3.9 % in azoospermic and cryptorchidisim groups and all had occupied the AZFc region[64]. The incidence of Y chromosome microdeletions in 3.9 % of azoospermic and oligospermic men in Saudi Arabia was found in AZFa, AZFb and AZFc regions[65] and was 5,29% in azoospermic man in Indian men [66]. Ioulıanos et al. reported the incidence of Y chromosome microdeletions as 5% in azoospermic and oligospermic Greek-Cypriot men in AZFb and AZFc regions [42].

AZFa deletions seems to be detected more frequently in American and European populations compared to middle east countries like Turkey, Iran, Saudi Arabia and Cyprus where AZFb and AZFc deletions were more frequent. Coplete AZFa deletion is rare and usually associated with azoospermia and absence of sperm cells in teticular tissue[67].

We found one deletion in severe oligozoospermic group involving complete AZFb and AZFc region and incomplete proximal c/d regions. Interestingly, this patient had a few spermatozoa in his first sample, but none were found 6 months later. Even in testicular biopsies, no sperm cells were found. This observation implies that deletions with complete AZFb, AZFc and incomplete proximal c/d regions could lead to germ cell destruction and cause spermatogenic failure in the course of time. Also it is possible that, for this patient spermatogenesis might be still ongoing in a few tubuli as the TESE procedure may not represent the whole testis. These results suggest that severe oligozoospermic men with Y chromosome microdeletions should be directed to an ICSI programme as soon as possible and any spermatozoa found in the testicular tissue must be frozen to be used for his future ICSI applications. Moreover; micro TESE could be the best choice for these patients.

FSH and LH levels were significantly elevated in azoospermic group when compared to the severe oligozoospermic group and control cases. Prolactin levels were significantly elevated in azoospermic group than control group. Plasma testosterone and leptin levels varied between individuals significantly. High plasma FSH and LH levels indicated inefficient response of Sertoli and Leydig cells to these hormones[2]. Tomasi et al, found no difference in the function of pituitary-testicular axis in men with and withouth Y-chromosome microdeletions [68].

Our results emphasize that Y chromosome microdeletion analysis should be carried out in all patients with idiopathic azoospermia or severe oligospermia who are candidates for intracytoplasmic sperm injection. We believe that, if the functions of the genes that play role in spermatogenesis are deciphered, it will offer decisive solution to the etiology of male infertility.

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