

## Cytogenetic and Y chromosome microdeletion screening studies in infertile males with Oligozoospermia and Azoospermia in Southeast Turkey

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### Abstract

**Purpose** In view of the genetic risks for the next generation, the importance of careful evaluation of karyotypes and AZF microdeletions in male infertility prior to assisted reproduction is evident. In the present study, it is aimed to investigate the frequency and types of both major chromosomal abnormalities by using standard cytogenetic methods and Y chromosome microdeletions of infertile males with azoospermia and oligozoospermia to give appropriate genetic counseling before assisted reproduction techniques in southeast Turkey.

**Methods** A total of 80 infertile males (52 were azoospermic, 25 oligospermic and 3 asthenospermic) were studied for the cytogenetic evaluation and molecular AZF screening program prior to use of assisted reproduction techniques. A detailed history was taken for each man. Karyotyping was performed on peripheral blood lymphocytes according to standard methods. Polymerase chain reaction (PCR) amplification by using 15 Y-specific sequence-tagged sites of AZF region was performed to screen the microdeletions in the AZF region of Y chromosome.

**Capsule** In the present study, it is aimed to investigate the frequency and types of both major chromosomal abnormalities by using standard cytogenetic methods and Y chromosome microdeletions of infertile males with azoospermia and oligozoospermia to give appropriate genetic counseling before assisted reproduction techniques in southeast Turkey.

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**Results** Of 80 cases, 71 had normal karyotype (46,XY). The total prevalence of chromosomal abnormalities was found to be 11.2% (9/80), including seven patients with Klinefelter syndromes and two patients with balanced autosomal rearrangements. All of the patients with Klinefelter Syndrome had azoospermia, but carriers with translocation had oligospermia. The deletions of Y chromosome were seen in one patient (1.3%) with features of normal karyotype and azoospermia. Microdeletions were seen in the AZFc and AZFd regions. Neither AZFa nor AZFb microdeletions were detected.

**Conclusions** The occurrence of chromosomal anomalies and Y chromosome microdeletions among infertile males strongly suggests the need for routine genetic testing and counseling prior to employment of assisted reproduction techniques.

**Keywords** Infertility · Chromosome · AZF · Azoospermia · Oligozoospermia

### Introduction

Infertility is best defined as the inability to conceive after one year of unprotected intercourse [1]. There are many cases of infertility, and this problem affects approximately 10–15% of all married couples attempting pregnancy in the general population, and male factor is responsible for approximately 40–50% of the cases [2–8]. Male infertility can be caused by a variety of factors, such as infection, varicose, endocrine disorders, spermatic duct obstruction, antisperm antibodies, cryptorchidism, retrograde ejaculation, systemic diseases, testicular cancer, testicular trauma, etc. Apart from these, in 30–40% of male infertile cases that are referred to as idiopathic, genetic abnormality is suspected [4–9].

Male infertility with genetic factors might be the result of carriers of chromosomal abnormality and/or Y chromosomal microdeletions within the Yq11 region, where the genes that control spermatogenesis, known as AZF, are located [1–4, 7–11]. The incidence of cytogenetic abnormality has been estimated as 2.1 to 28.4% in infertile men and only 0.7–1% in the general male population [5, 8, 12–14]. Somatic chromosomal anomalies in the infertile male may be numerical or structural and involve sex chromosomes (e.g., 47,XXY) or autosomes (e.g., balanced Robertsonian translocations) [3–5, 8, 12–14]. Approximately 5–10% of oligozoospermic cases and 15–20% of azoospermic cases harbor genetic abnormalities [9].

After the Klinefelter syndrome, Y chromosomal microdeletions are the most frequent genetic cause of male infertility [2, 10, 13]. Analysis of these deletions demonstrates in the AZF region four nonoverlapping loci, AZFa, AZFb, AZFc and AZFd, localized between AZFb and AZFc [2, 3, 6, 8–11, 15–17]. Deletion of these loci results in spermatogenic arrest and is associated with azoospermia and oligozoospermia [2, 3, 6, 8–11, 15–17]. These AZF genes code for RNA binding proteins and may be involved in regulation of gene expression, RNA metabolism, packaging and transport to cytoplasm, and RNA splicing [7, 10, 15, 17].

In recent years, the introduction of testicular sperm extraction (TESE) and intracytoplasmic sperm injection (ICSI) into the treatment of male factor infertility has permitted the use of sperm from oligospermic or azoospermic patients to achieve successful fertilization and pregnancies [2, 4–6, 8, 10, 11, 13, 16–19]. However, this procedure might have a potential risk of transmitting genetic abnormalities to the offspring, since the natural selection process of sperm cells is bypassed [5–8, 11, 13, 17]. In view of the genetic risks for the next generation, the importance of careful evaluation of karyotypes and AZF microdeletions in male infertility prior to assisted reproduction by ICSI is evident [5, 6, 17]. Therefore, in the present study, it is aimed to investigate the frequency and types of both major chromosomal abnormalities by using standard cytogenetic methods and Y chromosome microdeletions by using gene-based multiplex polymerase chain reaction (PCR) of infertile men with azoospermia and oligozoospermia to give appropriate genetic counseling before assisted reproduction techniques in southeastern Turkey.

## Materials and methods

### Patients

Eighty infertile men were selected prior to employment of assisted reproduction techniques because of severe male-factor infertility and past history of infertility for more than

one year. Patients were referred to Human Genetic Department from Urology Department of the University Hospital, School of Medicine, University of Dicle. Participants in this study were informed of the nature of the study, and provided informed consent. General physical and genital examination was performed by a specialist (Dr Abdullah Gedik). All of them underwent an andrological work-up, which included medical history, physical examination, and hormonal estimation. Serum concentrations of follicle stimulating hormone (FSH), luteinizing hormone (LH), prolactin (PRL) and testosterone were measured by electrochemiluminescence immunoassays (ECLIA), using Roche Elecsys 1010 (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Semen analyses (sperm concentration, motility, and morphology) were performed according to the World Health Organization Guidelines [20] for the diagnosis of azoospermia and oligozoospermia. Light microscopic evaluation of sperm concentration, motility, viability and morphology was performed. Specimens originally considered as azoospermic were centrifuged (1000 g for 20 min) and the pellets were examined for spermatozoa, before confirming azoospermia. The volunteers were requested to abstain from sexual activity for 48h to 7 days before the investigation. There were 52 men with non-obstructive azoospermia, 25 with oligospermia and 3 with asthenospermia. The diagnosis of non-obstructive azoospermia was based on clinical findings (small testicles plus elevated FSH). None of them had any history of childhood disease, environmental exposure, and radiation exposure or prescribed drug usage that could account for their infertility.

### Somatic cytogenetic analysis

All patients were analyzed cytogenetically. The somatic karyotypes of the 80 patients were studied in cultured peripheral blood lymphocytes according to the macroculture technique described by Moorhead et al. [21]. At least, 20 metaphases per subject were analyzed. The chromosomal abnormalities were described according to the norms established by the International System for Human Cytogenetic Nomenclature [22].

### Analysis of the AZF genes by PCR-sequence-tagged sites

Analysis of microdeletions in the AZF (Yq11) region was performed in these 80 clinically and cytogenetically studied patients for the presence of microdeletions in the AZF region (Yq11). Genomic DNA was extracted from peripheral blood leucocytes by standard procedures [23, 24] before being analyzed by multiplex PCR. The DNA was amplified by multiplex PCR by using 15 Y chromosome-specific STS (sequence-tagged sites according to the

method described by Henegariu et al [25]. The STS primers used were sY81, sY82, sY84 (AZFa); sY127, sY142, sY164, RBM1 (AZFb); sY254, sY255, sY257, CDY, BPY2 (AZFc); and sY145, sY152, sY153 (AZFd) (Table 1). The PCR was carried out in a 25-μL reaction volume containing: 200ng genomic DNA, 1.5mmol/L MgCl<sub>2</sub>, 200μmol/L dNTP, 0.1–0.5μmol/L of each primer, PCR buffer with adjuvants (Q-solution; Qiagen, Hilden, Germany) and 1 U HotStarTaq DNA polymerase (Qiagen, Hilden, Germany). Thermocycling (Techne Cambridge Ltd, Duxford-Cambridge U.K.) were used for multiplex PCR set. Multiplex PCR sets No. 1, No. 2, No. 3 No. 4 and No. 5 were performed together under the same PCR conditions as follows: initial denaturation at 94°C for 5 min; followed by 40 cycles of denaturation at 94°C for 40 s, annealing at 62°C for 1 min, extension at 72°C for 1.5 min and a final extension at 72°C for 10 min.

PCR products were separated on 3% agarose gel electrophoresis (Metaphor, FMC Bioproducts, Rocklands, ME, USA), stained with ethidium bromide, and visualized using gel documentation system. The deletion of one PCR fragment was confirmed in single-primer pair PCR under

the same experimental conditions. This analysis was performed at least three times on microdeleted sample. In each multiplex PCR assay, one sample from healthy female was used as negative control, and healthy fertile male was used as positive control.

Patients who were diagnosed to have chromosomal abnormalities or Y chromosome microdeletions were given genetic counseling.

Statistical analysis was carried out by the Statistical Package for Social Science for Windows, version 11.0 (SPSS; Chicago, IL, USA). The unpaired *t*-test, Mann-Whitney *U*-test and Chi-squared test were used appropriately. *P* < 0.05 was considered significantly different.

## Results

A total of 80 infertile men were studied, 52 (52/80 = 65%) of whom were azoospermic males, 25 (25/80 = 31.2%) oligozoospermic males, and 3 (3/80 = 3.8%) asthenospermic males, presenting with motility alteration. The age (mean ± SD) of infertile males was 32.79 year (range 24–45 year).

**Table 1** Sequence-Tagged Sites (STS) and gene-specific primer sequences for deletion analysis

Multiplex PCR set No	STS	Region	Primer sequence	Size (bp)
1	sY142	AZFb	Forward 5' AGC TTC TAT TCG AGG GCT TC-3 Reverse 5' CTC TCT GCA ATC CCT GAC AT-3'	182 bp
	sY164	AZFb	Forward 5' AAT GTG CCC ACA CAG AGT TC-3 Reverse 5' TGG AAG ACC AGG ATT TCA TG-3'	590 bp
	sY277	AZFc DAZ3	Forward 5' GGG TTT TGC CTG CAT ACG TAA TTA-3' Reverse 5'CCT AAA AGC AAT TCT AAA CCT CCA G-3'	275 bp
	sY153	AZFd	Forward 5' GCA TCC TCA TTT TAT GTC CA-3' Reverse 5' CAA CCC AAA AGC ACT GAG TA-3	135 bp
2	RBM1	AZFb	Forward 5' ATG CAC TTC AGA GAT ACG G-3' Reverse 5' CCT CTC TCC ACA AAA CC A ACA-3'	800 bp
	sY254	AZFc DAZ1	Forward 5' GGG TGT TAC CAG AAG GCA AA-3' Reverse 5'GAA CCG TAT CTA CCA AAG CAG C-3'	350 bp 380 bp
	sY82	AZFa	Forward 5' ATC CTG CCC TTC TGA ATC TC-3' Reverse 5' CAG TGT CCA CTG ATG GAT GA-3'	280 bp
	sY152	AZFd	Forward 5' AAG ACA GTC TGC CAT GTT TCA-3' Reverse 5' ACA GGA GGG TAC TTA GCA GT-3'	120 bp
3	sY127	AZFb	Forward 5'GGC TCA CAA ACG AAA AGA AA-3' Reverse 5'CTG CAG GCA GTA ATA AGG GA-3'	282 bp
	sY81	AZFa	Forward 5' AGG CAC TGG TCA GAA TGA AG-3' Reverse 5' AAT GGA AAA TAC AGC TCC CC-3	200 bp
	sY145	AZFd	Forward 5' CAA CAC AAA AAC ACT CAT ATA CTC G-3' Reverse 5' TTG AGA ATA ATT GTA TGT TAC GGG-3	125 bp
4	sY84	AZFa	Forward 5' AGA AGG GTC TGA AAG CAG GT-3' Reverse 5' GCC TAC TAC CTG GAG GCT TC-3'	295 bp
	BPY2	AZFc	Forward 5' CAG CGT ATC ATA GAA AAT GT-3' Reverse 5' AGT ACT TTA TTT GCA GGT TCT G-3'	142 bp
	sY255	AZFc DAZ2	Forward 5' GTT ACA GGA TTC GGC GTG AT-3' Reverse 5' CTC GTC ATG TGC AGC CAC-3'	120 bp
5	CDY	AZFc	Forward 5' TCA TAC AAT CCA ATT GTA CTG G-3' Reverse 5' TTC TAT CCC TCG GGC TGA GCT C-3'	132 bp

There was no significant difference in the mean ages of men with oligozoospermia, azoospermia and asthenospermia ( $31.6 \pm 3.2$  and  $33.1 \pm 5.3$  and  $33.50 \pm 12.02$  years, respectively). The average duration of infertility was 8.85 years (range 1–28 years). None of the cases reported previous exposure to gonadotoxins, such as radiation treatment or cancer chemotherapy.

The levels of FSH and LH in azoospermic males ( $16.4 \pm 9.2$  and  $10.3 \pm 7.5.0$  IU /L, respectively) were significantly higher than those in the oligozoospermic males ( $8.9 \pm 8.1$  and  $7.5 \pm 6.8$ , respectively,  $P = 0.001$ ). However, the levels of testosterone ( $3.9 \pm 1.52$  vs.  $4.1 \pm 2.9$  ng/mL;  $P = 0.749$ ) were not significantly different.

Of 80 cases with male infertility, 71 (88.8%) had normal karyotype ( $46,XY$ ) and nine cases (11.2%) had abnormal karyotype. Numerical and structural chromosomal abnormalities, which were detected in nine patients, are summarized in Table 2. Seven of the chromosomal abnormalities were gonosomal in patients with Klinefelter Syndrome and two were balanced reciprocal translocations involving autosomes. One of the translocations was  $46,XY,t(3;7)(q24;q36)$  and the other was  $46,XY,t(5;6)(q35;q21)$ . All of those with Klinefelter Syndrome had azoospermia, but translocation carriers had oligospermia. Seven of 52 azospermic patients (13.5%) and two of 25 oligozoospermic patients (8%) had chromosomal abnormalities.

In the current study, Yq microdeletion was detected in 1 (1.9%) of 52 azoospermic cases, but not seen in other cases. Azoospermic patient Md-58 presented the loss of sY277, DAZ3 and sY153 in AZFc and AZFd regions, respectively (Fig. 1). Neither AZFa nor AZFb deletions were detected in any participant. The case with deletions had normal karyotype ( $46,XY$ ). Combination of chromosomal abnormality and Y chromosome microdeletion was not seen in any case. The father and brothers of deleted patient were also investigated, and no deletions were found. It is possible to conclude that these represent de novo deletions and may be considered to be an etiological factor for the spermatogenic defect.

Including Y chromosome microdeletions and chromosomal abnormality, a total genetic abnormality rate was detected in 13.5% of azoospermic cases (7/52) and 8% of oligospermic cases (2/25), but not seen among the cases of oligoasthenospermic.

Cytogenetic abnormalities observed in infertile men with their semen analysis as well as Y chromosome microdeletion status are shown in Table 2.

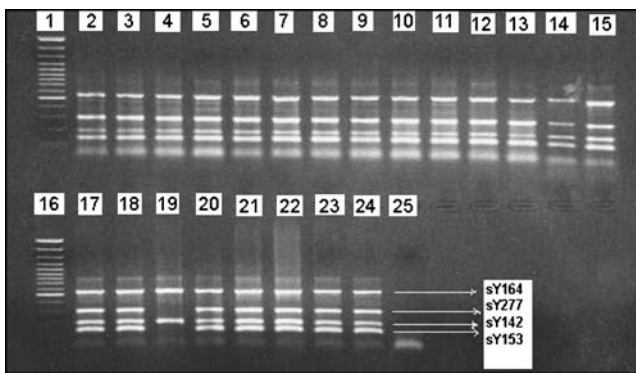
## Discussion

Among numerous etiologic factors, genetic plays a primary role in male infertility with abnormal semen parameters

**Table 2** Cytogenetic abnormalities observed in infertile men with their Y chromosome microdeletion and semen analysis and hormone profiles and, A:azoospermia, B:oligozoospermia

Patient number	3	45	48	53	58	71	72	77	79	80
Karyotyping	47,XXX	47,XXX	46,XY,t(3;7)	47,XXX	46,XY sY277 sY153	46,XY,t(5;6)	47,XXX	47,XXX	47,XXX	47,XXX
AZF deletion	None	None	None	None	A	None	None	None	None	None
Semen analysis	A	A	O	A	A	O	A	A	A	A
Testosterone (ng/ml)	0.59	0.48	3.14	0.49	2.26	2.87	2.41	0.48	0.78	5.32
FSH (IU/L)	43.49	44.35	3.15	37.72	8.77	72.97	29.75	55.04	39.61	23.42
LH (mIU/ml)	27.32	26.15	4.28	18.26	72.91	3.47	13.70	30.63	19.54	16.31





**Fig. 1** Results from multiplex polymerase chain reaction analysis; lanes 1 and 16 show 100-bp molecular weight markers; lanes 2–15 and 17–18 and 20–23 show undelated samples, lane 19 show deletions of sY277 and sY153 in the patient Md-58; lane 24 show positive control (normal male); lane 25 show negative control sample of female DNA

[5, 6, 9]. Spermatogenesis is regulated by a number of genes on the Y chromosome and by autosomes that act at different stages of germ cell development [9]. In the present study, the frequency of chromosomal aberrations (11.2%) exceeded the incidence of microdeletions of the Y chromosome (1.3%). While nine cases had numerical or structural chromosomal anomaly, only one case had Yq microdeletions. These findings are very similar to those reported in earlier studies [2, 13, 14].

Sex chromosome abnormalities, among which Klinefelter syndrome is the commonest, are the most frequent chromosome-related cause of hypogonadism and infertility [4, 5, 26, 27]. This abnormality is associated with severe spermatogenic failure, causing a marked reduction in testicular size and azoospermia resulting in infertility [4]. In our study, we found seven men with 47,XXY karyotype i.e., Klinefelter's syndrome and azoospermia. It has been shown that the possibility to retrieve sperm by TESE in a proportion of XXY patients who can be candidates for ICSI and preimplantation genetic diagnosis [18, 19].

A relationship between balanced autosomal translocation and infertility has been reported among severely oligozoospermic and azoospermic men [4–6, 8]. In our study, reciprocal translocations [t(3;7) and t(5;6)] were seen in two oligozoospermic male cases. It is hypothesized that balanced translocations interfere with normal chromosome pairing and segregation at meiosis I, thus providing a potential for formation of unbalanced gametes and subsequent unbalanced abnormal offspring [4, 27]. Another hypothesis acts on the assumption of potential autosomal genes involved in male gametogenesis that might be deregulated by chromosome breakpoints. The relation between chromosomal breakpoints and male infertility has been investigated, and it has been found that there is a non-random distribution of breakpoints associated with infertility [3, 27]. Further research in this direction is necessary.

The frequency of autosomal chromosome anomalies detected in the present study was 2.5% (2/80 patients), only in the oligospermic group. These anomalies were balanced reciprocal translocations. The frequency of sex chromosome anomalies (47,XXY) was seen in the group of azoospermic patients as 8.75% (7/80 patients). These findings are compatible with those of previous studies [5, 10, 12, 13, 26–29].

In conclusion, the occurrence (11.3%) of chromosomal anomalies among infertile males strongly suggests genetic testing and counseling prior to the ICSI treatment.

The levels of FSH and LH in azoospermic males were significantly higher than those in the oligozoospermic males. However, the levels of testosterone were not significantly different. This is consistent with a large study by Vutyavanich et al. [8].

The frequency of AZF microdeletions detected in our sample was only 1.3% (1/80 patients), a value similar to that in the studies previously reported [16, 29–31]. However, our finding is lower than that in some previous studies that indicated it between 3 and 55% [2, 15, 17, 27, 31].

Yq microdeletion was detected in 1 (1.9%) of 52 azoospermic cases, but not seen in other cases. This result is similar to those of previous studies [29, 31]. It has been shown by Ferlin et al [15] that chromosome Y microdeletions are much less frequent when sperm concentration is less than 2 millions/ml. This may be one of the reasons for the failure to detect Y microdeletions in the non-azoospermic groups. An effect of the small size and slightly unbalanced nature of our sample (azoospermic patients = 52, oligospermic patients = 25, asthenospermic = 3), ethnic differences, different patient selection criteria, methodological aspects, other additional genetic, epigenetic, nutritional and local factors can contribute to the difference between the present and previous studies. Furthermore, the type and number of markers used in the studies may have further contributed to the variation [8].

The DAZ gene family is located in the AZFc region, and it is reported to be the most frequently deleted AZF candidate gene in infertile males [1, 2, 10, 15]. The DAZ genes are encoding proteins in testicular tissue that contain RNA-binding motive, which has a regulatory role in RNA metabolism [2, 10, 15]. AZFc deletions appear to remove the DAZ gene cluster and have been associated with a variety of spermatogenic alterations, ranging from azoospermia due to Sertoli cell-only to oligozoospermia with different testicular phenotype [1, 2, 10, 15]. AZFd deletions may present with mild oligospermia or even normal sperm counts with abnormal sperm morphology such as severe teratozoospermia phenotype [2, 10]. In the present study, the azoospermic patient presented the loss of sY277 (DAZ3) and sY153 in AZFc and AZFd regions, respectively. Neither AZFa nor AZFb deletions were

detected in any participant. Our result confirms the role of deletions in the AZFc region overlapping the DAZ gene family among other Yq regions in determining male infertility.

Most deletions are de novo in origin, and the transmission of these deletions to the male offspring is 100% [6]. The deletions in our study were de novo too, and were not found in the father and brothers of deleted patient.

However, Y chromosome microdeletions were found only in one azoospermic patient in our population; the occurrence of Y chromosome microdeletions among infertile men strongly suggests genetic testing and counseling prior to the ICSI treatment, because studies have indicated that deletions on the long arm of the Y chromosome involving a particular and consistent segment might lead to azoospermia and sometimes to severe oligozoospermia [4].

In conclusion, the occurrence of chromosomal anomalies and Y chromosome microdeletions among infertile males strongly suggests genetic testing and counseling prior to employment of assisted reproduction techniques in southeastern Turkey.

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