Original Communication

Y chromosome microdeletions in Turkish infertile men

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AIMS: To detect the frequency and types of both chromosomal abnormalities and Y chromosome microdeletions in infertile men attending to our university intracytoplasmic sperm injection ICSI/IVF centre and fertile control subjects in our patient population.
SETTINGS AND DESIGN: A total of 50 infertile men who were referred to IVF center of Meram medical faculty were selected for the molecular azospermia factor (AZF) screening program.
MATERIALS AND METHODS: Karyotype analysis and polymerase chain reaction amplification using 15 Y-specific sequence-tagged sites of AZF region were done.
RESULTS: The total prevalence of chromosomal abnormalities was found to be 10% (5/50), including 4 patients with numerical and 1 patient with structural abnormalities. Overall, 4 of the 50 patients tested (8%) exhibited deletions of the Y chromosome, 3 of them being azospermic and 1 of them oligospermic men. The frequency of the microdeletions in subgroups with azospermia and oligozoospermia was found to be 10.7% (3/29) and 4.7% (1/21) respectively. Microdeletions of AZFb and AZFc regions were detected in all of the 4 patients. Neither AZFa nor AZFd microdeletions were indicated.
CONCLUSIONS: Our findings suggest that one must know whether there is a genetic cause for male infertility before patients can be subjected to ISCI or testicular sperm extraction (TESE)/ISCI treatment.

Key words: AZF region, azospermia, chromosomal abnormality, male infertility, oligozoospermia, Y-chromosome

About 15% of couples attempting pregnancy have reduced infertility; and in approximately 40-50% of all cases, the reason is male-factor infertility.[1,2] Contributing factors such as infection, varicocele, endocrine disorders, spermatic duct obstruction, antisperm antibodies, etc., are described as idiopathic male infertility, accounting for over 20% of all male-factor infertility cases. However, in 60% of cases, male infertility could be the result of genetic factors, including cytogenetic abnormalities and microdeletions of the Y chromosome.[2,3] Tiepolo and Zuffardi[4] have postulated the presence of a spermatogenesis locus located at Yq11, which is named 'azospermia factor (AZF) locus.' Molecular analyses of infertile men has identified four close subregions: AZFa, AZFb, AZFc and AZFd, within interval V and VI on Y q11.22-23.[5] In each region, candidate genes have been proposed, i.e., DFFRY in AZFa region, RBM (RNA binding motif) in AZFb region and DAZ (deleted in azospermia) in AZFc region.[6] Recent genetic studies have identified nearly 15 novel genes or gene families in the human Y chromosome, some of which located within AZF intervals.[7] Gene deletions in these regions have shown to be pathologically involved in male infertility associated with azospermia and severe oligozoospermia.[7-9] The new technique of intracytoplasmic sperm injection (ICSI) has allowed many infertile men to achieve their dreams of fatherhood. However, it might be a potential risk of transmitting this defect to future offspring.[10]

In this study, we evaluated the prevalence of chromosome abnormalities and Y chromosome microdeletions by polymerase chain reaction (PCR) in a Turkish population of azospermic and oligozoospermic men attending to our university ISCI/IVF center.

Materials and Methods

Patients

A total of 50 infertile men who were referred to IVF center of Meram medical faculty were selected for the molecular AZF screening program. Twenty-nine of them (58%) had non-obstructive azospermia and 21 (42%) had severe oligospermia (< 1 x 10^6 spermatozoa per milliliter of ejaculate). Semen analyses were performed according to the 1992 World Health Organization guidelines.[11]

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Chromosome Analyses

All patients were analyzed cytogenetically. Metaphase cells were obtained from phytohemagglutinin-stimulated blood lymphocytes and stained by Seabright's modified GTG-banding method.[12] Twenty metaphase spreads were routinely analyzed from each patient. Fifty cells were examined in a case that was numerical mosaic for sex chromosomes. Numerical abnormalities in at least three cells were considered as mosaics. Karyotypes were reported according to ISCN.[13] Locus-specific probe (SRY/X) (Vysis) and whole chromosome painting probe (Y) (Vysis) were used for the patient with 46,X,inv(Y)(p11.2q11.23) karyotype in FISH technique.

Molecular Analyses

Genomic DNA was isolated from peripheral blood lymphocytes by screening for microdeletions on the Y chromosome according to standard protocols. Ten healthy fertile men were selected for the control data and five healthy women were used as negative controls.

In this study, 15 sets of sequence-tagged sites (STS) - primers which amplify 15 different loci distributed along the length of the AZF locus - were used to screen for Y chromosome microdeletions. The STS primers used were - for AZFa: sy81p1, sy81p2, sy84p2; AZFb: sy127p1, sy142p1, sy164p2, rbm1p2; AZFc: cdyp1, bpy2p1, sy255p1, sy254p1, sy277p2; AZFd: sy145p2, sy152p1, sy153p1. The sequences of these primers are shown in Table 1. Each reaction was performed with 200 ng of genomic DNA, 1.5 to 2.0 mmol/l $MgCl_2$, 10 mmol/l dNTPs, 50 pmol/l of each primer, PCR buffer and 5 IU of Tag DNA polimerase in a reaction volume of 50 μl. Amplifications were carried out on MWC Biotech Primus 96 thermalcycler, with the program consisting of initial 5

Table 1: Sequence-tagged sites and primer sequences used for Y chromosome microdeletion analysis

STS	Region	Primer sequence	Size (base pair)
sY81	AZFa	Forward 5' AGGCACTGGTCAGAATGAAG	200 bp
		Reverse 3' AATGGAAAATACAGCTCCCC	
sY82	AZFa	Forward 5' ATCCTGCCCTTCTGAATCTC	280 bp
		Reverse 3' CAGTGTCCACTGATGGATGA	
sY84	AZFa	Forward 5' AGAAGGGTCTGAAAGCAGGT'	295 bp
		Reverse 3' GCCTACTACCTGGAGGCTTC'	
sY127	AZFb	Forward 5' GGCTCACAAACGAAAAGAAA'	282 bp
		Reverse 3' CTGCAGGCAGTAATAAGGGA'	
sY142	AZFb	Forward 5' AGCTTCTATTCGAGGGCTTC'	182 bp
		Reverse 3' CTCTCTGCAATCCCTGACAT'	
sY164	AZFb	Forward 5' AATGTGCCCACACAGAGTTC'	590 bp
		Reverse 3' TGGAAGACCAGGATTTCATG'	
rbm1	AZFb	Forward 5' ATGCACTTCAGAGATACGG'	800 bp
		Reverse 3' CCTCTCTCCACAAAACCAACA'	
sY145	AZFd	Forward 5' CAACACAAAAACACTCATATACTCG'	125 bp
		Reverse 3' TTGAGAATAATTGTATGTTACGGG'	
sY152	AZFd	Forward 5' AAGACAGTCTGCCATGTTTCA'	120 bp
		Reverse 3' ACAGGAGGGTACTTAGCAGT'	
sY153	AZFd	Forward 5' gCATCCTCATTTTATGTCCA'	135 bp
		Reverse 3' CAACCCAAAAGCACTGAGTA'	
sY254	AZFc	Forward 5' GGGTGTTACCAGAAGGCAAA'	350-380 bp
	(DAZ-1)	Reverse 3' GAACCGTATCTACCAAAGCAGC'	
sY255	AZFc	Forward 5' GTTACAGGATTCGGCGTGAT'	120 bp
	(DAZ-2)	Reverse 3' CTCGTCATGTGCAGCCAC'	
sY277	AZFc	Forward 5' GGGTTTTGCCTGCATACGTAATTA'	275 bp
	(DAZ-3)	Reverse 3' CCTAAAAGCAATTCTAAACCTCCAG'	
cdy	AZFc	Forward 5' TCATACAATCCAATTGTACTGG'	132 bp
		Reverse 3' TTCTATCCCTCGGGCTGAGCTC'	
bpy2	AZFc	Forward 5' CAGCGTATCATAGAAAATGT'	142 bp
		Reverse 3' AGTACTTTATTTGCAGGTTCTG'	

The size of the expected amplified DNA product is indicated for each STS, STS - Sequence-tagged sites

min of denaturation at 95°C; 40 cycles of incubation at 95°C for 35 s (denaturation), at 58°C for 35 s (annealing) and at 72°C for 40 s (extension) and a final extension step at 72°C for 5 min.

The PCR products were separated on 2% agarose gel prepared in 1 x TBE buffer containing ethidium bromide at a concentration of 1μg/ml by electrophoresis. A STS was considered absent only after testing the patient at least three times to provide accuracy and reproducibility. DNA from 10 fertile men and 5 women served as normal and Y deleted negative controls.

Patients who were diagnosed as having chromosomal abnormalities or Y chromosome microdeletions underwent genetic counseling.

Results

Cytogenetic Results

The total prevalence of chromosomal abnormalities was found to be 10% upon screening 50 patients with male infertility. Numerical and structural chromosomal abnormalities, which were detected in 5 patients, are summarized in Table 2. Three of them had azospermia, Md-25 and Md-40 had previously undetected Klinefelter syndrome and Md-6 were 45, X/46, XY mosaic. The remaining 2 patients had oligospermia; one had 47, XXY karyotype and the other had a pericentric inversion of Y chromosome. As a result, 3 cases out of 29 azospermic patients (10.7%) and 2 cases out of 21 oligozoospermic patients (9.5%) had chromosomal abnormalities. Y chromosome microdeletions were indicated in only 1 of these patients, MD-6. Locus-specific probe (SRY/X) (Vysis) and whole chromosome painting probe (Y) (Vysis) were used for the patient with 46, X, inv(Y) (p11.2q11.23) karyotype in FISH technique. It was shown that the inversion did not change the position of SRY region.

Table 2: Cytogenetic abnormalities found from G-banded analysis of peripheral blood lymphocytes

Case number	Karyotype
Md-6	45,X/46,XY
Md-15	47,XYY
Md-25	47,XXY
Md-40	47,XXY
Md-49	46.X,inv (Y) (p11.2q11.23)

Molecular Results

In total, 4 (8%) patients of the tested 50 infertile men exhibited Y chromosome microdeletions. PCR results of the 4 patients are summarized in Table 3. All the 4 patients represented AZFb and AZFc microdeletions corresponding to sy127(AZFb), rbm1 (AZFb), cdy (AZFc), sy254 (AZFc,DAZ-1), sy255 (AZFc, DAZ-2) and sy277 (AZFc,DAZ-3) primers. Neither AZFa nor AZFd microdeletions were detected. AZFb microdeletions sy127 and rbm1 were found in all the 4 patients. Three of them had microdeletions in the region of AZF candidate gene, DAZ, at the same time. Only one of these patients (Md-6) presented a 45,X/46,XY karyotype. The frequency of microdeletions in subgroups with azospermia and oligozoospermia was found to be 10.7% (3/29) and 4.7% (1/21), respectively.

Discussion

In approximately 60% of cases, male factor infertility can be diagnosed as carriers of a chromosomal abnormality and Y chromosome microdeletions, which is probably related to spermatogenic failure.[14,15] The incidence of chromosomal abnormalities in general male population is reported to be 0.7-1%.[14,15] When this incidence was compared with infertile males, such incidence in infertile males was found to be 10- to 20-fold higher. Several studies have shown a high incidence

Table 3: Characterization of Y chromosome deletions in four patients

STS sites	Region		Md-3	Md-6	Md-7	Md-13
sY81	AZF-a					
sY82	AZF-a					
sY84	AZF-a					
sY127	AZF-b		•	•	•	•
sY142	AZF-b					
sY164	AZF-b					
rbm1	AZF-b		•	•	•	•
sY145	AZF-d					
sY152	AZF-d					
sY153	AZF-d					
sY254	AZF-c	DAZ-1		•		
sY255	AZF-c	DAZ-2	•			•
sY277	AZF-c	DAZ-3		•		
Cdy	AZF-c				•	
bpy2	AZF-c					

STS - Sequence-tagged sites

of chromosomal abnormalities in infertile men, ranging from 2.2 to 14.3%.[3,14-17] Chiang *et al.* showed that the prevalence of chromosomal abnormalities is 28.4% upon screening 220 patients with male infertility.[18] In another study, Vicdan *et al.* found that 3.4% of infertile men had a chromosomal abnormality, and it was 4.2% in azospermic patients and 2.2% in patients with severe oligoasthenoteratoazospermia.[17] Van Assche *et al.* reported that 13.7% of men with azospermia and 4.6% of men with oligospermia have chromosomal abnormalities affecting approximately 5.1% of the total number of infertile men. Furthermore, they indicated that sex chromosome abnormalities predominated in azospermic group (12.6%), whereas autosomal abnormalities were the most frequent in the oligospermic group.[16] In our study, we indicated that 10% (5/50) of infertile men had chromosomal abnormalities, and it was 10.7% (3/29) in azospermic patients and 9.5% (2/21) in oligospermic patients.

According to the new advances in molecular genetics, Y chromosome microdeletions are the most important cause of male infertility. AZF regions located on the euchromatic region of the long arm of the Y chromosome play an essential role for spermatogenesis. There are four regions in AZF: AZFa, AZFb, AZFc and AZFd. Microdeletions in these regions have been shown to be related to infertility associated with azospermia or severe oligozoospermia.[6] Azospermia was found in men with AZFa and AZFc microdeletions.[5] Deletions including and extending beyond the AZFc region (AZFb+c, AZFa+b+c) are associated with total absence of testicular spermatozoa.[19] In ~50% of azospermic patients with AZFc deletions, mature spermatozoa have been found.[20] AZFa and AZFb deletions are associated with severe defects in spermatogenesis and Sertoli cell-only syndrome.[21,22] Partial AZFb and complete AZFc deletions can be associated with oligospermia.[5,7] In different studies, the frequency of Y chromosome microdeletions varies extensively (3-18%).[5,9,17,23-26] In our study, 4 (8%) of the tested 50 infertile patients exhibited Y microdeletions. Our result is lower than some previous studies with incidences up to 18%.[5,6,27] We found the frequency of microdeletions in the subgroups with azospermia and oligospermia to be 10.7% (3/29) and 4.7% (1/21) respectively. In azospermic males, Mulhall *et al.* showed this value was 9.6%, while others indicated between 3 and 23%.[20,25,28-30] However, Foresta *et al.*[6] reported 55% of azospermic males with Y chromosome microdeletions. In oligospermic men, Pryor *et al.*[25] reported 9.7% frequency of microdeletions and Foresta *et al.,*[6] 22%; but some other studies indicated less than 6%.[9,31]

The DAZ gene family is located in the AZFc region and it is reported to be the most frequently deleted AZF candidate gene. This gene family consists of four copies. The DAZ genes are encode proteins in testicular tissue that contain RNA-binding motif, which have a regulatory role in RNA metabolism.[19,32] A high percentage of sequence homology with mouse gene *dazla* and the drosophila gene *boule* had led to the hypothesis that DAZ has been conserved throughout evolution and performs similar roles as dazla and boule, which seems to regulate the meiotic cell cycle. This would mean that all men with DAZ deletions would be incapable of producing mature sperm; however, some oligospermia have been shown to carry DAZ deletions.[7,19,32] The RBMY genes (RBMY1 and RBMY2) include 20-50 genes and pseudogenes The multicopy nature of RBMY1 has made it difficult to assign the gene a specific function in spermatogenesis.[28] The AZFb region may contain the only functional copies of the RBM1 gene, and their presence in the nucleus of human male germ cells indicates their testis-specific expression. It is probable that the RBM1 genes are required for normal infertility in men. However, whether the loss of the RBM1 genes in men with AZFb deletions causes infertility is not clear.[7,32]

In the present study, the severely oligospermic patient Md-7 presented the loss of sy127, rbm1 and cdyp bands in AZFb and AZFc regions. He did not reveal DAZ microdeletions. Two of azospermic patients, Md-3 and Md-13, had shown the same microdeletions in AZFb and AZFc regions, including the loss of sy127, rbm1 and sy255 (DAZ-2) bands. The third azospermic patient with 45,X/46,XY karyotype (Md-6) has shown the absence of sy127; rbm1 bands of AZFb; and sy254 (DAZ-1), sy277 (DAZ-3) bands of AZFc region. As a result, three azospermic patients had microdeletions in DAZ locus, and all the patients presented rbm1 deletions. Neither AZFa nor AZFd deletions were detected in any participant.

The majority of Y chromosome microdeletions occurred in *de novo* events. The origin of these

microdeletions is not clearly understood. Microdeletions may arise in the testis, in fertilized eggs and embryos; microdeletions prevent the formation of spermatogonia in the fetus, resulting in impaired spermatogenesis in the adult.[30,31] The high frequency of Y microdeletions suggests that the Y chromosome is susceptible to spontaneous loss of genetic material. Aberrant recombination events occur between areas of homologous or similar sequence repeats between X and Y chromosome or within Y chromosome itself by unbalanced sister chromatid exchanges.[30,33] The stability of the Y chromosome may be related to a high frequency of repetitive elements clustered along the length of the chromosome.[21]

The development of ISCI has allowed many males with severe male factor infertility to have a child. This technique however cannot cure the underlying spermatogenic problem, and it is possible that the genetic defect is transmitted to his offspring by virtue of the ICSI.[34]

Conclusion

Both structural and numerical cytogenetic abnormalities and Y chromosome microdeletions can cause spermatogenic failure at various points, which results as Y chromosome derived infertility. Routine screening of Y chromosome microdeletions in all male patients before ICSI treatment is important, and counseling on the risks of transmitting Y microdeletions and other Y chromosomal abnormalities to the offspring should be provided.

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Conflict of Interest: None declared.