Cytogenetic Abnormalities and Y Chromosome Microdeletions in Azoospermic and Oligospermic Infertile Males from West of Iran

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ABSTRACT

About 15% of couples have infertility problems, half of which are related to male factors. Cytogenetic and genetic disorders account for about 10% of the male infertility problems. The aim of this study was to determine the frequency and types of both cytogenetic abnormalities and AZF microdeletions of Y chromosome in idiopathic azoospermic and oligospermic infertile men in west of Iran. In this case-control study, a total of 108 infertile men including 62 azoospermic and 46 oligospermic men were studied for the cytogenetic and AZF microdeletions. Moreover, 90 fertile men served as a control group. Detailed clinical and laboratory examination was done for all participants. Karyotyping was done on peripheral blood lymphocytes to detect the cytogenetic abnormalities; likewise, multiplex-PCR method was performed to identify the presence of microdeletion in AZFa, AZFb or AZFc regions. Chromosomal abnormalities were detected in 6.5% (7/108) of cases, including two oligospermic men with balanced autosomal rearrangements, one oligospermic and four azoospermic men with Klinefelter syndrome. Y chromosome microdeletions were detected in 4.6% (5/108) of infertile men (AZFc: 3.7%, AZFbc: 0.9%). No AZFa deletion was detected in any of the patients. No chromosomal abnormality and Y chromosome microdeletion was detected in control group. The prevalence of chromosomal abnormalities and Y chromosome microdeletions shows the importance of genetic factors in male infertility. The analysis of karyotype and Y microdeletions in infertile men provide a proper understanding about the causes of infertility, the choice of the appropriate assisted reproduction technique and reducing the risk of transmission of these genetic defects to the future generation.

Keywords: Male infertility; Chromosomal abnormalities; AZF microdeletions; Oligozoospermia; Azoospermia

INTRODUCTION

Infertility is a global medical problem. According to World Health Organization (WHO) definition, infertility is a disease of reproductive system, defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse [1,2]. Approximately 15 to 20 % of all couples are infertile. It is suggested that 30 to 40% of infertile couples suffer from male factor infertility, while 30 to 40% suffer from female factor infertility and the rest are due to factors involving both partners or due to unexplained causes [3]. Male infertility is a multifactorial syndrome, including

a wide range of disorders and despite great advances in the field of infertility, 30% of male infertilities are still defined as idiopathic [4,5]. Male infertility is often caused by impairment of spermatogenesis. Sperm abnormalities include azoospermia (not having any measurable sperm in semen), oligospermia (low concentration of sperm), asthenospermia (reduced sperm motility), teratospermia (reduced levels of normal shaped sperm) and aspermia (complete lack of ejaculation) [6]. Several factors are involved in male fertility which can be broadly divided into non-genetic factors (include varicocele, ejaculatory duct obstruction, sperm agglutination, sexual dysfunction and hormonal imbalance), epigenetic and genetic factors [7, 8]. According to the latest findings, genetic factors account for 10 to 15% of male infertility and include chromosomal aberrations and single gene mutations. About 30% of the cases of male infertility associated with impaired spermatogenesis (azoospermia or oligospermia) have no known cause (a condition defined as idiopathic male infertility); however, in 30 to 40% of these cases, genetic abnormality is suspected [9]. Chromosomal abnormalities are among the most important genetic causes of male infertility whose prevalence is estimated between 2.1 to 28.4%; this prevalence increases as the number of sperms decreases. Chromosomal abnormalities may be numerical or structural and involve sex chromosomes or autosomes. Klinefelter syndrome, also known as 47, XXY or XXY, is the most important genetic cause of male infertility [10]. The Y is one of the smallest human chromosomes spans (more than 58 million base pairs) and represents almost 2% of the total DNA in human genome. The main role of this chromosome is to create sperm; however, molecular mechanisms of spermatogenesis and its relationship with Y chromosome are not completely understood. After the Klinefelter syndrome, microdeletions of Y chromosome are the second most frequent genetic cause of infertility in men [11]. Polymerase chain reaction (PCR) studies of the sequence tagged site (STS) of Y chromosome in infertile men indicate the occurrence of microdeletions in a region of Y chromosome which are not detectable by karyotype analysis. These studies have led to the identification of three non-overlapping loci in Yq11, named as the azoospermia factor (AZF) which carry those genes involved in the control of spermatogenesis and are divided into AZFa, AZFb, and AZFc regions [12]. Microdeletions within the AZFa and AZFb regions leads to sertoli cell only (SCO) syndrome and spermatogenic arrest, whereas deletions in AZFc can cause different phenotypes ranging from normal to oligozoospermia and azoospermia [13]. The genes located in AZF region code proteins are involved in the regulation of gene expression, RNA splicing and metabolism and packaging and transport to cytoplasm [14]. In recent years, using assisted reproductive technologies (ART) such as testicular sperm extraction (TESE) and intracytoplasmic sperm injection (ICSI) for treatment of male infertility has allowed the use of sperm from oligospermic or azoospermic males to obtain successful fertilization and pregnancies [15]. This procedure has raised some concerns about the potential transmission of genetic abnormalities to the next generation, since the natural selection process of sperm cells is bypassed [16]. So this study aims at determining the prevalence and types of chromosomal abnormalities and Y chromosome microdeletions in infertile men with idiopathic oligo and azoospermia in order to provide appropriate genetic counseling to patients referred for assisted reproductive treatment in west of Iran.

MATERIALS AND METHODS

Study design

This study was carried out on men referred to the Motazedi Infertility Teaching Clinic of

Kermanshah University of Medical Sciences to employment of assisted reproduction techniques because of severe male factor infertility, between November 2014 and June 2016. After detailed clinical examination, patients were subjected to semen and endocrinological examinations. Diagnosis of azoospermia and oligospermia was made according to WHO guidelines in at least two ejaculates [17]. Endocrinological investigations include measurement of serum Follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH) and Testosterone levels were determined using ELISA method (Roche Elecsys, Germany). All subjects were of Iranian ethnic origin and gave relevant informed consent. This research was approved according to official Iranian law and regulations by the Institutional Review Boards of each participating teaching and referral hospital. The present study also complies with the Declaration of Helsinki. Patients having any cryptorchidism, congenital absence of vas deferens, seminal tract obstruction, varicocele, testicular tumors or undergoing chemotherapy and radiotherapy were excluded from this study and finally, 108 men with idiopathic non obstructive azoospermia and oligosperma were included. These patients were separated into two groups: group A: men with non-obstructive azoospermia (n= 62) and group B: men with severe oligospermia (n= 46). Ninety age-matched normozospermic fertile men who had at least one child were considered as control group (group C). Statistical analysis was carried out using SPSS for windows 16.0 software and $P<0.05$ was considered statistically significant.

Chromosomal analysis

All patients and controls were analyzed cytogenetically. Karyotype analysis was performed on blood lymphocyte cultures using standard cytogenetic methods. Briefly, blood cells were cultured in RPMI 1640 medium containing 20% fetal bovine serum (FBS) and 10 µg/ml phytohaemagglutin as the mitogen and were incubated at 37°C for 68 to 72 hours. Afterwards, colcemid was added to the medium and incubated at 37°C for 10 minutes. The medium was centrifuged and the pellets were resuspended in 5 to 10 ml KCl (0.075 M) for 20 minutes at 37°C. After centrifugation, the cells were resuspended in a fixative solution (methanol: acetic acid, 3:1). The fixative was replaced for a minimum of 3 times. By using a Pasteur pipette, drops of the solution were poured on slides for morphological studies. The chromosomes were viewed under a phase-contrast microscope to assess metaphase quality and the nuclei. The chromosomes were stained by GTG banding technique and karyotyped after aging. A 400-band stage resolution was applied for analysis. A minimum of twenty metaphases were counted for each sample. The chromosomal abnormalities were described according to the guideline by the International System for Human Cytogenetic Nomenclature [18].

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

1 ml of blood samples were taken from all the patients and controls and collected in tubes containing EDTA. Genomic DNA was isolated from peripheral blood leukocyte using QIAGEN DNA extraction kit and stored in –20°C. The PCR comprised a total volume of 25 µL, of which 10 µL was the master mix (2X) (Amplicon, Denmark), 0.1–0.5µmol/L of each primer, 100 ng of genomic DNA, and 8 µL of enzyme-free water. A series of 6 STS markers on the long arm of Y chromosome were used for detection of microdeletions according to the European Academy of Andrology (EAA) and European Molecular Genetic Quality network (EMQN). The markers consisted of sY84 and Sy86 for AZFa, sY127 and sY134 for AZFb, sY254 and sY255 for AZFc regions. Also two primer pair that amplifies specific STS of SRY and ZFY gene was used as positive control. The sequence and size of all of primers are shown in table 1. DNA was amplified by multiplex PCR method. Two sets of amplification reactions were used. In each PCR, three STS primers and two internal controls were used. Amplification was carried out for 35 cycles in a Eppendorf Mastercycleras as follows: denaturation at 94°C for 30 s, annealing at 57°C for 90 s and extension at 72°C for 60 s. Amplification cycles were proceeded by initial

denaturation for 15 min at 95°C and final extension at 72°C for 5 min. In each PCR reactions, DNA from normal fertile males was used as normal control, while water served as negative control. The PCR products were separated on a 2% agarose gel prepared in 1× TBE buffer containing GelRed dye by electrophoresis [8]. A 100bp DNA marker (Fermentas) was loaded with PCR products to confirm the band size. A positive result was scored when the amplification product of expected size was obtained. Deletion of PCR fragments was confirmed by three repetitions.

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

Reaction A and B	STS	Sequence	Product length [bp]
ZFY	ZFY-F	5-ACC RCT GTA CTG ACT GTG ATT ACA C-3	495
	ZFY-R	5-GCA CYT CTT TGG TAT CYG AGA AAG T-3	
SRY	sY14-F	5-GAA TAT TCC CGC TCT CCG GA-3	472
	sY14-R	5-GCT GGT GCT CCA TTC TTG AG-3	
Reaction A			
AZFa	sY86-F	5-GTG ACA CAC AGA CTA TGC TTC-3	318
	sY86-R	5- ACA CAC AGA GGG ACA ACC CT -3	
AZFb	sY127-F	5-GGC TCA CAA ACG AAA AGA AA-3	274
	sY127-R	5-CTG CAG GCA GTA ATA AGG GA-3	
AZFc	sY254-F	5-GGG TGT TAC CAG AAG GCA AA-3	380
	sY254-R	5-GAA CCG TAT CTA CCA AAG CAG C-3	
Reaction B			
AZFa	sY84-F	5-AGA AGG GTC CTG AAA GCA GGT-3	326
	sY84-R	5-GCC TAC TAC CTG GAG GCT TC-3	
AZFb	sY134-F	5-GTC TGC CTC ACC ATA AAA CG-3	301
	sY134-R	5-ACC ACT GCC AAA ACT TTC AA-3	
AZFc	sY255-F	5-GTT ACA GGA TTC GGC GTG AT-3	123
	sY255-R	5-CTC GTC ATG TGC AGC CAC-3	

RESULTS

Patients

This study was performed on 108 men who were candidate for ICSI which were separated into two groups: group A: men with non-obstructive azoospermia (n = 62; 57.4 %), and group B: men with severe oligospermia (n= 46; 42.6 %). The average duration of infertility was 7.2 years (range 1–20 years). Characteristics of age and hormonal analysis results of infertile males and controls are mentioned in Table 2.

Table 2. Information of age and hormone levels of men participating in the study.

	Group A (n=62)	Group B (n=46)	Group C (n=90)	Normal Ranges
Age (years)	32.4±6.4	31.5±7.2	34±5.9	
Testosterone	4.9±2.8	5.0±2.5	5.4±2.6	2.8-8 ng/ml
LH	12.1±6.3	10.5±6.7	4.9±2.1	1.7-8.6 mIU/ml
FSH	19.8±4.6	14.6±7.5	8.2±5.3	1.5-12.4 mIU/ml

Cytogenetic analysis

Cytogenetic analysis showed that from 108 infertile men, 100 males (92.6 %) had normal karyotype (46; XY), and 7 men (6.5 %) had various chromosomal abnormalities. Four chromosomal anomalies were detected in the azoospermic patients (4/62 = 6.4%) and three in the oligospermic patients (3/46 = 6.5%). The most frequent chromosomal anomaly was Klinefelter syndrome (5/108= 4.6%) (Figure 1).

Four of Klinefelter syndrome KS cases were in the azoospermic group (4/62 = 6.4%) and one was in the oligospermic group (1/46 = 2.8%); moreover, in oligospermic group, one case of balanced Robertsonian translocation 45, XY,der (13q,14q) and one case of balanced reciprocal translocation 46,XY,t(11;22) (q23.3;q11.2) were found.

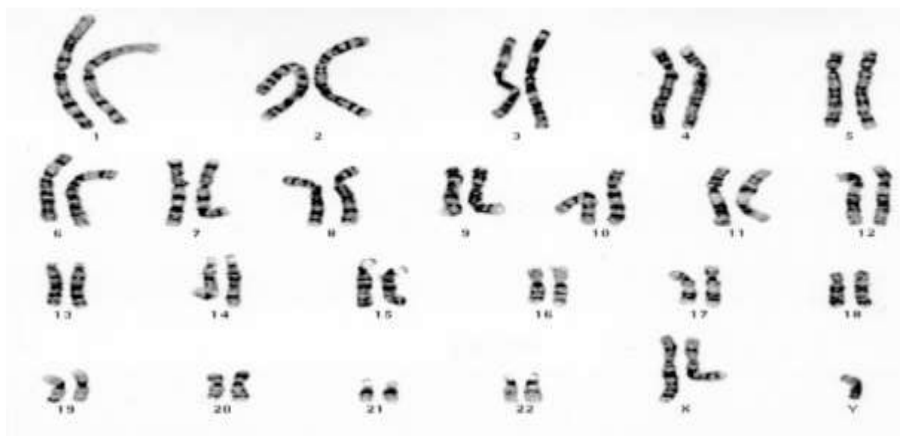

Figure 1. Karyotype of an azoospermic patient with klinefelter syndrome

Microdeletions in the Yq11 region

The total frequency of microdeletions detected in infertile men was 4.6% (5/108). Y chromosome microdeletions were detected in 3 of 62 (4.8%) azoospermic and 2 of 46 (4.3%) oligospermic men. Frequency and types of deleted region are shown in table 3. No AZFa microdeletion was detected in in infertile men. All the men with AZF microdeletions had normal karyotype (46,XY) and combination of chromosomal abnormality and Y chromosome microdeletion was not seen in any infertile men.

Table 3. Cytogenetic abnormalities and Y chromosome microdeletion observed in infertile males

Phenotype	patient number	karyotype	Deleted AZF		
Azoospermia			AZFa	AZFb	AZFc
	6	46,XY	-	-	sY254 sY255
	19	47,XYY	-	-	-
	22	46,XY	-	-	sY254 sY255
	30	47,XYY	-	-	-
	41	47,XYY	-	-	-
	46	47,XYY	-	-	-
	58	46,XY	-	sY134	sY254 sY255
Oligospermia					
	9	46,XY	-	-	sY254
	15	45, XY,der (13q,14q)	-	-	-
	22	46,XY	-	-	sY254
	34	47,XYY	-	-	-
	43	46,XY,t(11;22)(q23.3;q11.2)	-	-	-

Total genetic abnormality rate

The results of cytogenetic and Y chromosome microdeletions analysis showed that from 108 infertile men included in the study, 12 3males (11.1 %) had chromosomal abnormality or Y chromosome microdeletion. Also total genetic abnormality rate (include chromosomal abnormality and Y chromosome microdeletions) was 11.2 % in azoospermic males (7/62) and 10.8 % in oligospermic males (5/46). No men in control group show chromosomal abnormality or AZF micro deletion.

DISCUSSION

Male infertility has attracted a great deal of attention from many genetic and biology researchers and until now, many genetic aberrations have been identified, which are involved in spermatogenesis impairment and fertility [1]. In the present study, the frequency of chromosomal abnormalities is more than the frequency of the Y chromosome microdeletions (6.5% vs 4.6%). These findings are in accordance with those reported in earlier studies [5,11,12]. Sex chromosome abnormalities are the most common genetic cause of infertility. These abnormalities are associated with severe spermatogenesis impairment, leading to a significant reduction in testis volume and azoospermia or severe oligospermia [3,4]. In the present study, five males were proved to have Klinefelter syndrome (47,XXY karyotype), four of which had azoospermia and one had severe oligospermia. A relationship between balanced autosomal translocation and infertility has been reported in azoospermic and severely oligospermic men. In the present study, the frequency of autosomal chromosome abnormalities was 1.8% (2/108). These abnormalities include one case of reciprocal translocation [t(11;22)] and one case of Robertsonian translocation [der (13q,14q)], detected in oligospermia group. It is believed that the balanced translocations may disrupt normal chromosome pairing and disjunction during meiosis I, which can lead to the production of gametes with unbalanced genetic makeup and subsequent apoptosis of gametes. Another possible mechanism, based on possible autosomal genes is involved in spermatogenesis which might be disrupted by chromosome breakpoints [4]. In this study, sex chromosome aberrations are more frequent than autosomal abnormalities. These results are similar to those reported in other studies [17,18]; Amouri et al. (2014) reported that aberrations of sex chromosomes are more common in azoospermic men, while aberrations of autosomal chromosomes are more common in severe oligospermic ones [10]. AZF regions are located in the long arm of Y chromosome. It is believed that deletions in these regions can affect genes controlling spermatogenesis process. The frequency of AZF microdeletions are different from one study to another; it may be due to the different factors such as ethnicity of the study population, selection of inclusion criteria and variation in the number of STS markers used in various studies [1]. In this study, we used six primer pairs according to EAA/EMQN guideline and reported 3.7% of microdeletions in AZFc region and 0.9% in AZFb,c regions. Previous studies have reported that the frequency of Yq microdeletions varied from 1% to 55% among infertile men, but the majority of studies have mentioned that this ratio is below 15% [2, 13]. In our study, the frequency of microdeletion was 4.8% and 4.3% among azoospermic and oligospermic men respectively, which is similar to the majority of previously published studies [3, 7]. None of the patients in the present study showed deletion in AZFa region using sY84 and sY86 STS. AZFa region includes single copies of DDX3Y and USP9Y genes. Detection of deletions in the AZFa region is very crucial because it is impossible to retrieve testicular sperm for TESE/ICSI procedure [4]. In the present study, one azoospermic male showed partial deletion in AZFb region in combination with AZFc deletion, whereas no infertile men showed complete deletion in AZFb region, either alone or in combination with other deletions. Deletions of AZFb region may cause SCO syndrome or spermatogenetic arrest in azoospermic males. RBM1 and RBM2 genes are located in AZFb region and are specifically expressed in testicular tissues and germ cells. These two genes encode RNA binding proteins that are localized to the nucleus of spermatogenic cells [5]. Deletions in the AZFc region are the most commonly reported deletions among AZF regions. The most important candidate gene in AZFc region is the two clusters of DAZ gene family, BPY2 and CDY1 genes which control spermatogenesis. Deletions of AZFc region may have variable effects on male fertility potential according to the deleted genes [6].

In our study, deletions detected in AZFc region using sY254 and sY255 STS, account for 4.6 % of total AZF deletions were found in patients. Partial or complete deletions in AZFc region may result in different phenotypes, varying from normal sperm count to oligospermia and

azoospermia; thus, there is a chance for retrieving sperm from testis and performing TESE/ ICSI for these patients [11]. Studies performed in different part of Iran have reported different results compared to our study. Omrani and colleagues (2009) reported that the frequency of Y chromosome microdeletions among azoospermic men of the Kurd and Azari ethnic origin in North West of Iran was about 30% [2]. Mirfakhraie and colleagues (2010) showed that the frequency of Y chromosome microdeletion was 12% among azoospermic and oligozoospermic men from central of Iran; they also reported that the most frequent microdeletions were in the AZFb region followed by AZFc region [16]. Malekasgar and colleagues (2008) also indicated that the frequency of microdeletions in Y chromosome in azoospermic men from South West of Iran was 51.6% which is higher than the international frequency [9]. These variations in frequencies of deletion may be due to ethnic and geographic origins of the studied population or differences in the study design including the sample size and different STS selection.

CONCLUSION

The analysis of karyotype and Y microdeletions among infertile men is highly recommended for the purpose of providing a proper understanding of the causes of infertility, choosing the appropriate assisted reproduction technique and reducing the risk of transmission of these genetic defects to the future generation.

"The authors declare no conflict of interest"

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