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High prevalence of AZFb microdeletion in Iranian patients with idiopathic non-obstructive azoospermia

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***Background & objectives*: Genetic factors contribute about 10 per cent of male infertility. Among these, genes in azoospermia factor (AZF) region including AZFa, AZFb, AZFc and AZFd on the long arm of Y chromosome are considered most important for spermatogenesis. Deletions in these regions are thought to be involved in some cases of male infertility associated with azoospermia or oligozoospermia. We studied the incidence of AZF deletions among Iranian infertile men with idiopathic non-obstructive azoospermia.**

***Methods*: A total of 100 Iranian azoospermic infertile men were selected for the molecular study of Y chromosome microdeletions. The presence of 13 sequence tagged site (STS) markers from AZF region was investigated using multiplex polymerase chain reaction (M-PCR). One hundred fertile men were also studied as control group.**

***Results*: Twelve (12%) patients showed Y chromosome microdeletions and among these, deletion in AZFb region was the most frequent (66.67%) followed by AZFc (41.67%), AZFd (33.33%) and AZFa (8.33%), respectively.**

***Interpretation & conclusions*: Because of relatively high incidence of Y chromosome microdeletions among Iranian azoospermic patients, molecular screening may be advised to infertile men before using assisted reproductive treatments.**

Key words AZF - azoospermia - Iran - microdeletion - Y chromosome

Azoospermia is defined as complete absence of sperm from ejaculate and approximately occurs in 10-15 per cent of infertile men with abnormal semen analysis[1]. Azoospermia is classified as obstructive and non-obstructive, although in some clinical protocols aetiologies for azoospermia fall into pre-testicular, testicular and post-testicular categories[1-3]. Microdeletions on the long arm of the Y chromosome are the most frequent genetic causes of azoospermia and have been reported in 5-10 per cent of infertile men and 6-16 per cent of azoospermic men[4].

Most of the candidate genes involved in spermatogenesis have been mapped to the proximal long arm of the Y chromosome (Yq11) and are arranged in azoospermia factor (AZF) region including AZFa, AZFb, and AZFc sub regions[5]. Further, a fourth AZF region has been suggested to exist in the area where AZFb and AZFc overlap, and is termed as AZFd[6].

There are two main genes in AZFa region including *DFFRY* and *DBY*. Protein-encoding gene families on the AZFb region include *RBMY*, *PRY*, and *CDY2* which are expressed only in the testis. AZFc region contains 8 gene families including *BPY2, CDY, DAZ, CSPG4LY, GOLGAZLY, TTY3.1, TTY4.1*, and *TTY7.1* among which the 5 former are expressed only in the testis[4,5]. Microdeletions in the more proximal regions (AZFa and AZFb) cause spermatogenic arrest and sertoli cell only (SCO) syndrome, whereas partial deletions in these regions and deletions in distal regions (AZFc and AZFd) cause variable phenotypes ranging from normal to oligozoospermia and azoospermia[4,5,7].

Using assisted reproductive technologies such as intracytoplasmic sperm injection (ICSI) and *in vitro* fertilization (IVF) for treatment of male infertility may result in the transmission of Y chromosome microdeletions to the male offspring, causing the persistence of infertility problem over the next generations[8-10].

The aim of this study was to evaluate the frequency of AZF deletions among Iranian infertile men with idiopathic non-obstructive azoospermia using multiplex polymerase chain reaction (M-PCR).

Material & Methods

Patients: In a case-control study, that was carried out between June 2008 and July 2009 in National Institute of Genetic Engineering and Biotechnology (NIGEB), 100 non-obstructive azoospermic patients with a normal karyotype aged between 21 and 60 (mean ± SD = 32.41 ± 6.43 yr) were screened for the presence of Y chromosome microdeletions. The patients were candidates for ICSI and referred to NIGEB from the whole country. Semen analysis was performed according to normal standard parameters using the World Health Organization (WHO) criteria[11]. Urological examination was performed in all of the patients for anatomical integrity of genital system. Hormone analysis including folloicle - stimulating hormone (FSH) and luteinizing hormone (LH) were also done. One hundred age-matched (mean ± SD = 32.1 ± 7.06 yr) fertile men who had at least one child with no history of requiring assisted reproduction technology were considered as control group; also a female sample was used as negative control. Informed written consent was obtained from each azoospermic and fertile control man. The study protocol was approved by the ethics committee of the NIGEB.

Detection of Y chromosome microdeletions: Genomic DNA was extracted by a salting out method from peripheral blood leukocytes[12]. A series of 13 STS markers on Yq11 were used for the detection of submicroscopic deletions according to the European Academy of Andrology (EAA), the European Molecular Genetics Quality Network (EMQN), and previous protocols[7,13,14]. The STS markers included sY81, sY84 and sY86 for AZFa; sY121, sY124, sY127 and sY134 for AZFb; sY242, sY239, sY254 and sY255 for AZFc; and sY145 and sY153 for AZFd region. The primers' sequences and the size of related PCR products are shown in Table I. In addition, two sets of primers were used to amplify SRY and ZFY regions as internal controls. Five set of multiplexes were designed as follows:

Multiplex 1: SRY, sY84, sY134, sY255; Multiplex 2: ZFY, sY86, sY127, sY254; Multiplex 3: SRY, sY145, sY153; Multiplex 4: ZFY, sY81, sY121; and Multiplex 5: SRY, sY124, sY242, sY239.

Genomic DNA (100 ng) was added to a mixture of 200 mmol/l Tris–HCl (pH 8.3), 100 mmol/l KCl, 3 mmol/l $MgCl_2$, 5 mmol/l each dNTP, 10 per cent dimethyl sulfoxide (DMSO), 5 pmol/l of primer pairs, 2 U Taq DNA polymerase (Genfanavaran Co., Tehran, Iran), adjusted to a final volume of 25 µl. Amplifications were carried out on a Techne thermocycler (Techn Ltd., Cambridge, UK) with the following program: Initial denaturation at 94 °C for 5 min and a subsequent series of 35 cycles of 94 °C for 30 sec (denaturation), 57 °C for 45 sec (annealing), and 72°C for 1 min (extension). Final extension was carried out at 72°C for 5 min. All of the PCR amplification products were subjected to electrophoresis on 2.5 per cent agarose gel and/or 10 per cent acrylamide gel prepared in 0.5X TBE, stained with ethidium bromide and visualized by exposure to ultraviolet light. In order to confirm the absence of the unamplified STS markers, two additional PCRs were carried out.

Statistical analyses: χ^2 test was used to compare differences between the two studied groups. The statistical analyses were performed with SPSS 16 statistical software (SPSS Inc., Chicago, Illinois, USA). $P<0.05$ was considered significant.

Results

A total of 200 men, including 100 patients with non-obstructive azoospermia and 100 fertile controls, were analyzed for the presence of submicroscopic Y chromosome deletions. Of these, 12 patients (12%) showed Y chromosome microdeletions, while no microdeletion was detected in controls ($P<0.001$). Among the patients, deletion in AZFb region was the most common (66.67%) followed by AZFc (41.67%), AZFd (33.33%) and AZFa (8.33%) respectively (Fig. 1). Individual deletions in AZFb and AZFc regions were detected in 6 and 1 of the patients which accounted for 50 and 8.33 per cent of the total deletion respectively (Fig. 2). Combined deletions including AZFab, AZFcd, and AZFbcd were also detected in 1, 3, and 1 of the patients with microdeletion respectively (Fig. 2). Characteristics, hormonal analysis results, and treatment outcomes for the patients who showed Y chromosome microdeletion are mentioned in Table II.

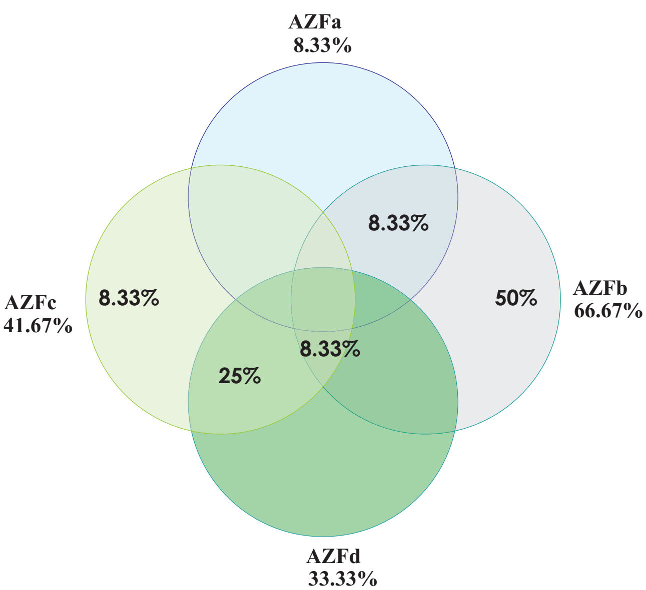


Fig. 1. Venn diagram illustrating frequencies of different types of observed Y chromosome microdeletions in AZF regions. AZFa, b, c and d deletions were involved in 8.33, 66.67, 41.67, and 33.33 per cent of total deletions respectively. Combined deletions including AZFab, AZFcd, and AZFbcd were also detected with the frequencies of 8.33, 25, and 8.33 per cent respectively. Individual deletions were also detected only in AZFb and AZFc regions with the frequencies of 50 and 8.33 per cent respectively.

Table I. STS markers and primer sequences used for screening Y chromosome microdeletions

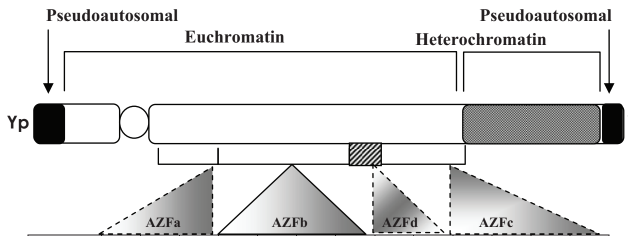
STS	Region	Size (bp)	Primer sequence
sY81	AZFa	209	5'AGG CAC TGG TCA GAA TGA AG3' 5'AAT GGA AAA TAC AGC TCC CC3'
sY84	AZFa	326	5'AGA AGG GTC TGA AAG CAG GT3' 5'GCC TAC TAC CTG GAG GCT TC3'
sY86	AZFa	320	5'GTG ACA CAC AGA CTA TGC TTC3' 5'ACA CAC AGA GGG ACA ACC CT3'
sY121	AZFb	190	5'AGT TCA CAG AAT GGA GCC TG3' 5'CCT GTG ACT CCA GTT TGG TC3'
sY124	AZFb	109	5'CAG GCA GGA CAG CTT AAA AG3' 5'ACT GTG GCA AAG TTG CTT TC3'
sY127	AZFb	274	5'GGC TCA CAA ACG AAA AGA AA3' 5'CTG CAG GCA GTA ATA AGG GA3'
sY134	AZFb	301	5'GTC TGC CTC ACC ATA AAA CG3' 5'ACC ACT GCC AAA ACT TTC AA3'
sY145	AZFd	125	5'CAA CAC AAA AAC ACT CAT ATACTCG3' 5'GGG CAT TGT ATG TTA ATA AGA GTT3'
sY153	AZFd	135	5'GCA TCC TCA TTT TAT GTC CA3' 5'ATG AGT CAC GAA AAC CCA AC3'
sY242	AZFc	233	5'ACA CAG TAG CAG CGG GAG TT3' 5'TCT GCC ACT AAA CTG TAA GCT CC3'
sY239	AZFc	201	5'CAT TCA TCT TCC CTT TTG AAG G3' 5'ATG CAA GTC GCA GGA AAT CT3'
sY254	AZFc	400	5'GGG TGT TAC CAG AAG GCA AA3' 5'GAA CCG TAT CTA CCA AAG CAG C3'
sY255	AZFc	126	5'GTT ACA GGA TTC GGC GTG AT3' 5'CTC GTC ATG TGC AGC CAC3'

STS, sequence tagged site

Table II. Clinical features of 12 azoospermia patients with Y chromosome microdeletions

Patient No.	FSH (mIU/ml)	LH (mIU/ml)	Testis biopsy	Treatment	Sperm
A1	60	12	NA	NA	NA
A8	45	10	SCO	NA	NA
Inf4	76	12	MA	TESE	-
Inf6	70	10.5	MA	TESE	-
Inf13	60	17	MA	TESE	-
Inf24	30	19	MA	TESE	-
Inf38	25	12	MA	TESE	-
Inf52	20	18	MA	TESE	-
Inf104	40	20	SCO	TESE	-
Inf121	10	7.5	SCO	TESE	-
Y2	38.5	7	NA	TESE	-
Y30	35	14	NA	TESE	-

FSH, follicle-stimulating hormone; LH, leuterizing hormone; NA, not available; SCO, sertoli cell only; MA, maturation arrest; TESE, testicular sperm extraction; -, no sperm.



Patient No.	sY81	sY84	sY86	sY121	sY124	sY127	sY134	sY145	sY153	sY242	sY239	sY254	sY255
A1	+	+	+	+	+	+	+	+	+	-	-	-	-
A8	+	+	+	-	-	-	-	-	-	-	-	-	-
Inf4	+	+	+	-	-	-	-	+	+	+	+	+	+
Inf6	+	+	+	+	+	+	+	-	+	-	-	-	-
Inf13	+	+	+	-	-	-	-	+	+	+	+	+	+
Inf24	+	+	+	-	-	-	-	+	+	+	+	+	+
Inf38	+	+	+	+	+	+	+	-	+	-	-	-	-
Inf52	+	+	+	-	-	-	+	+	+	+	+	+	+
Inf104	-	-	+	+	+	+	-	+	+	+	+	+	+
Inf121	+	+	+	+	+	+	+	-	+	-	-	-	-
Y2	+	+	+	-	-	-	-	+	+	+	+	+	+
Y30	+	+	+	-	-	-	-	+	+	+	+	+	+

Fig. 2. Schematic diagram illustrating different deletion patterns of the sequence-tagged site markers in the studied patients. +: PCR product was present; –: PCR product was not detected.

The mean value for FSH concentration in 12 patients with Y chromosome microdeletions was 44.90 mIU/ml compared with 35.50 mIU/ml in patients with no microdeletions (*P*<0.001). These values were 13.91 and 13.69 mIU/ml for LH concentration respectively (*P* = 0.178) (Table II). Nine patients who showed microdeletions underwent testicular biopsy. In six patients, maturation arrest was observed at different steps of spermatogenesis and three patients showed SCO syndrome. In all of these patients sperm retrieval failed using testicular sperm extraction (TESE) (Table II).

Discussion

Previous reports have revealed that Y chromosome microdeletions vary from 1 to 55 per cent among infertile men all over the world, but most studies have reported an incidence below 15 per cent[7]. In the present study the estimated frequency was 12 per cent among azoospermic patients that is within the range of the published data. Omrani *et al*[15] showed that the incidence of microdeletions among azoospermic patients of the Kurd and Azari ethnic groups in North West of Iran was about 30 per cent (18 of 60). Malekasgar *et al*[14] also indicated that the incidence of Yq microdeletions in South of Iran was higher than international frequency. They found microdeletions in 51.6 per cent (16 of 31) of patients with azoospermia[14]. These variations in deletion frequencies may be due to geographic and ethnic origins of the studied population and differences in the study design including the composition of the study population and sample size. However, Krausz *et al*[16,17] suggested that the major factor influencing deletion frequency was the composition of study population and ethnic or geographical differences apparently had no influence on it. They found that the highest deletion frequency was in the group defined as idiopathic azoospermic/cryptozoospermic with the incidence of 17 per cent and the incidence decreases progressively with the inclusion of less severe phenotypes[17]. It seems that the discrepancy in reported deletion frequencies in Iranian population could be explained by (*i*) differences in the sample size, and (*ii*) ethnic variations since our samples were collected from the whole country irrespective of ethnic origin.

Deletions of the AZF region occur with different frequency. It has been suggested that AZFc is the most frequently deleted region (60%), followed by deletions of the AZFb and combined deletions involving different AZF regions (35%). AZFa deletions are extremely rare (5%) and isolated deletions have been reported in this region[16].

None of the patients in the present study showed individual deletion in AZFa region using sY81, sY84 and sY86 sequence tagged sites, although deletion in this region was observed in combination with deletions in other AZF regions in one patient. In our study, AZFa region was involved in a total of 8.33 per cent of the observed Y chromosome microdeletions. This region contains single copies of *DFFRY* (USP9Y) and *DBY* (DDX3Y) genes. It is suggested that complete deletion of AZFa region may result in complete SCO syndrome and azoospermia[4,7,18,19]. Identification of deletions in this region is very important since it is impossible to retrieve testicular sperm for ICSI[4,7].

AZFb deletion was involved in 66.67 per cent of the total deletions of which AZFb alone was involved in 50 per cent. Selected STS markers for detecting deletions in AZFb region in the present study were located in the median and distal part of AZFb and in the most cases the deletion of sY127 and sY134 markers indicated a complete deletion of the AZFb region which may cause SCO syndrome or spermatogenetic arrest resulting in azoospermia[7]. Five patients showed complete deletion of AZFb whereas two patients showed partial deletion in AZFb region. *RBMY* genes, including *RBM1* and *RBM2*, located in AZFb region, are specifically expressed in testis and germ cells. The gene encodes a RNA binding protein that localizes to the nucleus of all spermatogenic cell types[4,20]. Since several copies of these genes are located in the AZFb region, it is not clear whether the loss of the *RBMY* genes in men may result in male infertility or not, therefore, the role of deletion in these genes is not clear in the process of spermatogenesis[21]. Several reports have shown that complete deletions in AZFb region will have the same results as deletions in AZFa region for testicular sperm extraction (TESE)[4,7]. Patients with complete or partial AZFb deletion in our study showed maturation arrest in their testis biopsy; therefore, attempts for retrieving sperm were not successful.

Deletions in AZFc region are the most commonly reported deletions among AZF microdeletions and its complete deletion is one of the most frequent molecular genetics causes of severe male infertility. The prevalence of this type of deletion is 5-10 per cent in cases of azoospermia and severe oligozoospermia[22]. *DAZ* gene family is the most important candidate gene for male infertility in this region and consists of four functional copies including *DAZ1, DAZ2, DAZ3* and *DAZ4* arranged in two clusters[18,23]. *DAZ* encodes for a testis specific RNA binding protein containing 8-24 copies of 24 amino acid sequences, known as '*DAZ repeat*'[24]. Deletions in *DAZ* genes may have different effects. It has been suggested that only partial AZFc deletions removing *DAZ1/DAZ2* are associated with spermatogenic impairment and male infertility, whereas those removing *DAZ3/DAZ4* are found in both fertile and infertile men[25].

In our study, deletion in AZFc region accounted for 41.67 per cent of the total deletion detected in the patients, using sY239, sY242, sY254 and sY255 sequence tagged sites which are specific for *DAZ* gene. The absence of these markers indicates deletion of the entire AZFc region, which removes all copies of DAZ[7]. Partial or complete deletions in AZFc region may result in different phenotypes vary from normal to oligozoospermia and azoospermia, so there may be a chance for retrieving sperm from testis and TESE/ICSI can be attempted in these patients. However, in our study patients with AZFc deletion showed SCO syndrome or maturation arrest in histological analysis.

None of the patients showed individual deletion in AZFd region, however deletion in this region was observed in combination with deletions in other AZF regions. It is believed that AZFd STS markers are located within the AZFc region and the existence of this region separately is seriously questioned[4]. Our results were contrary to this idea since a patient (no.A1) who showed the complete deletion of AZFc region did not show deletion of AZFd STS markers. However, three patients with AZFc deletion (Inf6, Inf38, and Inf121) also showed deletion of sY145 marker within the AZFd region. In addition, one patient (no.A8) with complete deletion of AZFbc showed deletion of both STS markers for AZFd region. Hence, our results are similar to those who consider AZFd as a separate region between AZFb and AZFc regions[6,26,27].

Finally, several studies have shown that serum FSH levels were significantly above the mean value in azoospermic patients and in patients with microdeletions[16,17,28]. Sertoli cell function depends on adequate stimulation by FSH and the elevation of FSH may result in abnormal spermatogenesis[28]. In the present study, same results were obtained, further the FSH values were significantly higher in patients with microdeletions than patients without microdeletions (P<0.001). However, this may be due to small sample size of the patients with microdeletions (n = 12) and further studies are needed to confirm these results.

Since the involvement of AZFb region was seen in 66.67 per cent of total deletions, we may conclude that genes located in the AZFb region were more involved in the fertility process in the studied patients and this is contrary to previous reports in which AZFc deletion was reported to be the most frequent deletion in azoospermic patients[4,13-15,29-31].

Due to relatively high incidence of Y chromosome microdeletions among Iranian candidates for ICSI, molecular screening for detection of these microdeletions may have diagnostic, prognostic and preventive value, and for genetic counselling in infertility clinics.

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