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Original article

# Azoospermia factor microdeletion in infertile men with idiopathic severe oligozoospermia or non-obstructive azoospermia



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## KEYWORDS

Male infertility;  
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## Abstract

**Objectives:** To determine Y-chromosome microdeletion of azoospermia factor (AZF) loci and the concomitant testicular pathology in azoospermic and severely oligozoospermic infertile men.

**Patients and methods:** DNA from blood and semen of 50 azoospermic and severely oligozoospermic infertile men (study group) and 54 healthy fertile men (control group) was investigated for microdeletion in AZF loci, using several Sequence-Tagged Site (STS) markers for AZF. Additionally, testicular biopsies from infertile men were examined to evaluate testicular morphological changes.

**Results:** 22% (11/50) of the patients of the study group had at least one microdeletion in one or more loci of the AZF sub-regions. Sixteen microdeletions were found in the DNA extracted from blood, while 19 microdeletions were found in the DNA extracted from semen. Microdeletions were detected in the 3 AZFb loci in one case, in both AZFb and AZFc loci in another and in two AZFc loci in a third case; 8 cases had sporadic microdeletions in a single locus. Moreover, the incidence of both AZFb and AZFc microdeletions was 14% (DNA from blood) and 16% (DNA from semen), whereas AZFa microdeletions were found in only 2% (DNA from blood) and 4% (DNA from semen) of the patients. The testicular biopsies revealed variable histological changes ranging from hyalinized seminiferous tubules to arrested spermatogenesis. No microdeletion was detected in the control subjects.

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*Conclusion:* Y-chromosome microdeletions of the AZF loci are frequently seen in azoospermic and severely oligozoospermic infertile men and are usually associated with impaired spermatogenesis and variable degrees of testicular histological changes.

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## Introduction

Infertility is a major health problem affecting approximately 15% of all couples seeking to conceive a child. The male factor is assumed to be responsible for infertility in approximately 40–50% of the cases, and both qualitative and quantitative abnormalities in sperm production are present in 40% of infertile men [1,2]. A number of known risk factors such as genital infection, anatomical abnormalities, varicocele, immunological factors, genetic aberrations and others are linked to male infertility [3]. However, in a significant number of cases the causes of male infertility remain unknown; a distinction is made between idiopathic infertility with a marked reduction in semen quality and unexplained infertility with normal semen parameters [3,4].

Y-chromosome microdeletions constitute the second most common genetic cause of male infertility. The distal end of the long arm of the Y chromosome includes the azoospermia factor (AZF) locus which contains the gene(s) necessary for spermatogenesis. The AZF locus has been mapped to a region in band q11.23 of the Y chromosome [5,6]. The AZF region is divided into three sub-regions designated as AZFa, AZFb and AZFc. Spontaneous mutation or loss of one of these loci in the paternal germ line leads to severely disturbed spermatogenesis [7–9]. Also, these regions may be associated with a particular testicular histology.

The association between Y-chromosome microdeletion and defective spermatogenesis has been studied previously, and the frequency of Y-chromosome microdeletions has been reported to account for 5–10% in azoospermic and 2–5% in severely oligozoospermic men [5,10].

As the frequency of Y-chromosome microdeletions may vary among different ethnical populations, we conducted this study to evaluate azoospermic/severely oligozoospermic infertile men in our local community. Several Sequence-Tagged Site (STS) marker sequences on the long arm of the Y chromosome (AZF sub-regions) were investigated. Furthermore, testicular biopsies were processed for histological examination to evaluate possible morphological abnormalities.

## Patients and methods

After obtaining the approval from the local institutional review board, this prospective case–control study was carried out between April 2013 and June 2014. According to the previous published studies [11–17] with an expected 16% difference in the frequency of Y-chromosome microdeletion with a power of 80% and a 5% level of significance, 44 men were calculated for each group. After adding 10% of expected dropout, our study required a total of 97 men. It included 50 men with primary infertility diagnosed as azoospermia or severe oligozoospermia (study group) and 54

healthy normal fertile men (control group). Each patient agreeing to participate in the study provided a written informed consent prior to enrollment. Basic infertility evaluation included a detailed medical history and physical examination, semen analysis, hormonal assessment (serum FSH, LH, testosterone and prolactin) and scrotal color Doppler ultrasonography. Also, the patients' partners were examined by gynecologists to ensure normal results from the gynecological point of view before including the male patients in the study. Patients with secondary infertility, genital anomalies, scrotal varicocele, genital infection, and those with hormonal defects or seminal tract obstruction were excluded.

Age-matched control subjects were recruited from normal fertile men who presented to the urology outpatient clinics due to different complaints. They were subjected to thorough history taking, clinical examination and semen analysis, and only men with normal semen parameters who had fathered at least one child without assisted reproductive technologies were included.

### Study procedures

#### Semen analysis

In all participants, semen analysis was performed at least twice at one-month intervals, following 3 days of sexual abstinence. The semen samples were collected and examined after 10 min of centrifugation at  $800 \times g$ . The mean values of different semen analysis results were reported and used as average results. The reference values set by the World Health Organization (WHO) in 2010 [18] were used: a sperm count over 15 million sperms/mL was considered normal, while a sperm count  $\leq 5$  million/mL was defined as oligozoospermia and the absence of sperms as azoospermia.

#### DNA analysis by PCR

Blood and semen samples were taken from all participants for DNA analysis. The DNA was extracted using DNA extraction kits (Genorise, USA). Then DNA amplification by multiplex PCR was performed using STS primers for the AZFa sub-region (sY81, sY84 and sY86), the AZFb sub-region (sY127, sY134 and RBM1), the AZFc sub-region (sY254, sY255 and CDY) and the SRY gene (sY14). Samples showing microdeletions on the first screening were verified by subsequent multiplex PCR amplification for another two times, and the deleted loci were confirmed by simplex PCR. The complete description of primers used for detecting Y-chromosome microdeletion and the amplification sets are shown in Table 1. The multiplex PCR amplification condition was optimized as follows: initial denaturation in  $94^\circ\text{C}$  for 5 min, followed by 32 cycles of  $94^\circ\text{C}$  for 30 s,  $57^\circ\text{C}$  for 30 s and  $72^\circ\text{C}$  for 90 s; with a final extension at  $72^\circ\text{C}$  for 7 min.

#### Analysis and visualization

The PCR products were separated by electrophoresis on a 2% agarose gel stained with ethidium bromide. They were then viewed

**Table 1** The three STS primer sets used in detecting Y-chromosome microdeletions.

	STS primers	Sequences
SRYP gene on (Yp)	sY14	5'-GAATATTCCCGCTCTCCGGA-3' 5'-GCTGGTGCTCCATTCTGAG-3'
	sY81	5'-AGG CAC TGG TCA GAA TGA AG-3' 5'-AAT GGA AAA TAC AGC TCC CC-3'
AZFa	sY84	5'-AGA AGG GTC TGA AAG CAG GT-3' 5'-GCC TAC CTG GAG GAG GCT TC-3'
	sY86	5'-GTG ACA CAC AGA CTA TGC TTC-3' 5'-ACA CAC AGA GGG ACA ACC CT-3'
	RBM1	5'-ATG CAC TTC AGA GAT ACG G-3' 5'-CCT CTC TCC ACA AAA CCA ACA-3'
AZFb	sY127	5'-GGC TCA CAA ACG AAA AGA AA-3' 5'-CTG CAG GCA GTA ATA AGG GA-3'
	sY134	5'-GTC TGC CTC ACC ATA AAA CG-3' 5'-CCG TGT GCT GGA GAC TAA TC-3'
AZFc	CDY	5'-TCA TAC AAT CCA ATT GTA CTG G-3' 5'-TTC TAT CCC TCG GGC TGA GCT C-3'
	sY254	5'-GGG TGT TAC CAG AAG GCA AA-3' 5'-GAC CGT ATC TAC CAA AGC TGC-3'
	sY255	5'-GTT ACA GGA TTC GGC GTG AT-3' 5'-CTC GTC ATG TGC AGC CAC-3'

under UV trans-illumination. Negative controls with a DNA template were included with each reaction. Photographs of the gel were taken using a gel documentation system.

#### Testicular biopsy

In an open procedure and under general anesthesia unilateral testicular biopsies were taken from some infertile men. All tissue biopsies were fixed in Bouin's solution, processed, imbedded in paraffin, sectioned, stained with Hematoxylin and Eosin, and then examined for histological anomalies.

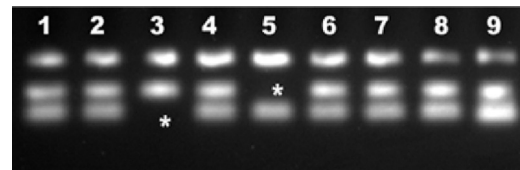
#### Data analysis

The data was presented as median and range or number and percentage. Fisher exact test was used for comparison and values of <0.05 were considered statistically significant.

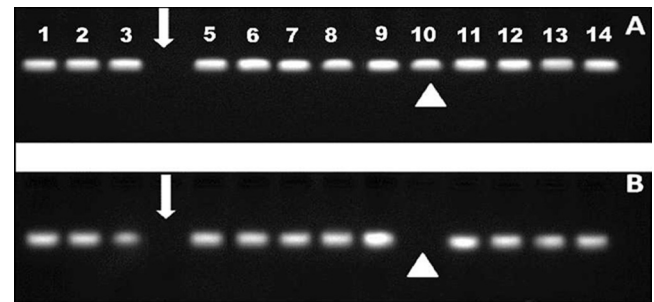
#### Results

In total, 50 infertile men were studied; 34 (68%) of them had azoospermia and 16 (32%) severe oligozoospermia. The patients' age ranged from 23 to 52 years (median: 30 years) and the duration of infertility ranged from 1 to 22 years (median: 3 years). Additionally, 54 normal fertile men aged between 22 and 46 years (median: 29 years) were studied as controls.

Sixteen microdeletions were found in the DNA extracted from blood, while 19 microdeletions were found in the DNA extracted from semen, whereas no deletion was detected in the control group. Microdeletions were detected in the 3 AZFb loci in one case, in both AZFb and AZFc loci in another and in two AZFc loci (sY254 and sY255) in a third case; 8 cases (72.7%) had sporadic microdeletions in a single locus: sY84 (2 cases), sY86 (one case), sY134 (2 cases), sY254 (2 cases), and sY255 (one case). On the other hand, SYR gene screening was normal in all infertile patients and the control



**Fig. 1** Gel photograph (STS primers for sY84, sY127 and sY254) showing microdeletion of sY254 in lane (3) and microdeletion of sY127 in lane (5) in DNA blood samples.



**Fig. 2** Gel photograph showing deletions of the sY84 loci in both DNA from blood (A) and semen (B) in case number 5 (arrows), while deletion of the same locus was detected only in DNA from semen in case number 10 (triangles).

group. Moreover, the incidence of both AZFb and AZFc microdeletions was 14% (DNA from blood) and 16% (DNA from semen), whereas AZFa microdeletions were found in only 2% (DNA from blood) and 4% (DNA from semen) of the patients. The differences between the frequency of the deletions detected in DNA from blood and DNA from semen were statistically non-significant in all cases.

Tables 2 and 3 illustrate the distribution and the percentage of all deletions detected associated with semen analysis and the possible testicular changes. Fig. 1 shows a multiplex PCR indicating microdeletions of sY254 in lane 3 and of sY127 in lane 5 in DNA from blood samples. Fig. 2 shows a simplex PCR for one of the AZFa sub-regions (sY84) in DNA from blood and semen; the deletion was detected in one locus in DNA from blood and in two loci in DNA from semen.

Testicular biopsies revealed variable testicular changes such as a mixed pattern of atrophic and hyalinized seminiferous tubules with some scattered small tubules containing Sertoli cells only, with complete or partial loss of spermatogenic cells (Fig. 3A and B); Sertoli cells only with germ cell aplasia (Fig. 4); immature germ cells, including several spermatocytes, and arrested spermatogenesis at a spermatid stage; i.e. complete absence of spermatozoa (Fig. 5).

#### Discussion

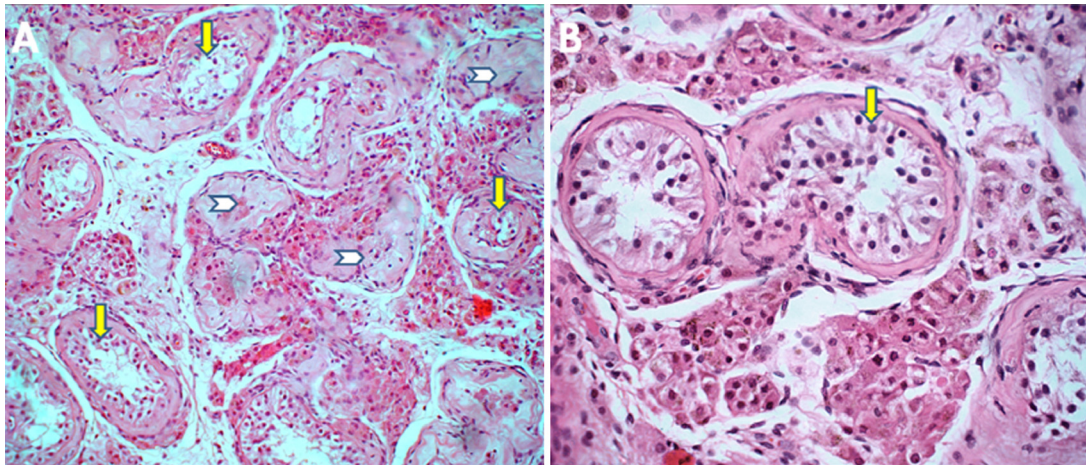
Y-chromosome microdeletion in azoospermia factor (AZF) loci is a common genetic cause of male infertility. Y-chromosome microdeletions are frequently diagnosed in a variable percentage of infertile men world-wide [19]. The frequency of Y-chromosome microdeletions in our 50 azoospermic/severely oligozoospermic infertile men was 22% (11/50). Also, the incidence of AZF microdeletions showed no variation between DNA from blood and semen (Table 3).





**Table 3** The number and percentage of each single STS locus deleted.

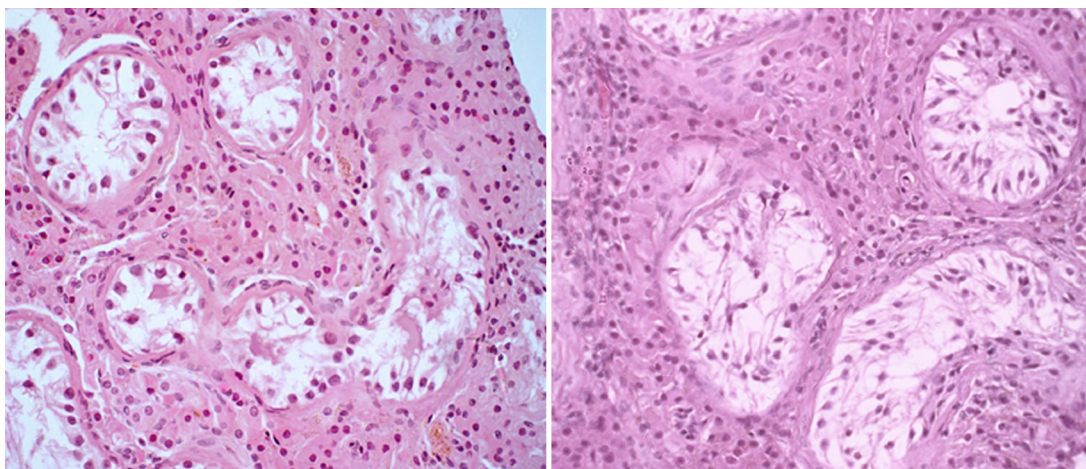
STS primers		Blood DNA (n = 50)	Semen DNA (n = 50)	P-value
AZFa	sY 81	0	0	0.500
	sY84	1 (2%)	2 (4%)	
	sY86	1 (2%)	1 (2%)	
	Total	2 (4%)	3 (6%)	
AZFb	sY127	2 (4%)	2 (4%)	0.500
	RBM1	2 (4%)	2 (4%)	
	sY134	3 (6%)	4 (8%)	
	Total	7 (14%)	8 (16%)	
AZFc	CDY	1 (2%)	1 (2%)	0.500
	sY254	4 (8%)	4 (8%)	
	sY255	2 (4%)	3 (6%)	
	Total	7 (14%)	8 (16%)	



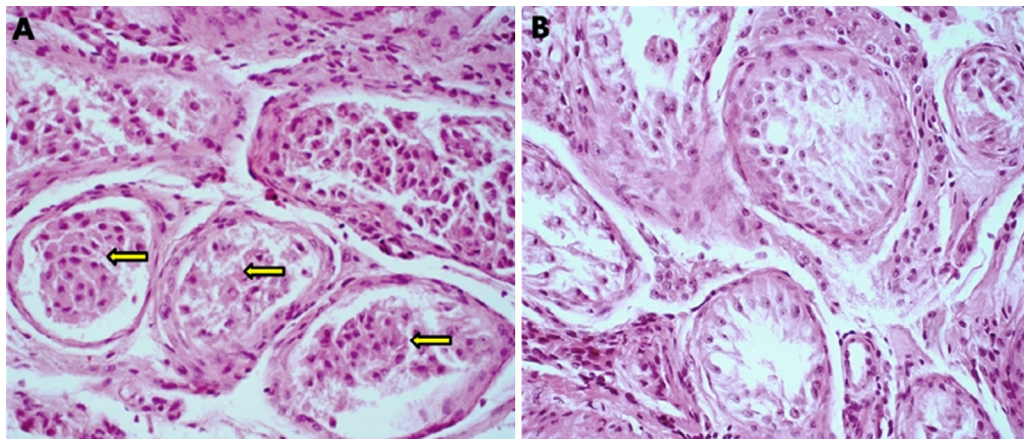
**Fig. 3** Photo-histogram from a testicular biopsy showing hyalinized seminiferous tubules with thickened basement membranes; some are occluded with complete absence of spermatogenic cells (white head arrows), and others contain Sertoli cells with complete or partial loss of spermatogenic cells (yellow arrows) [Hematoxylin and Eosin stain A =  $\times 200$ ; B =  $\times 400$ ].

Our study results differ significantly from other studies that were carried out on azoospermic/severely oligozoospermic patients. The frequency of Y-chromosome microdeletions found in our study is much higher than that reported by some investigators. For example,

Behulova et al. [11] reported a frequency of 3.35% in 226 patients, Mitra et al. [12] reported a frequency of 5.29% in 170 patients and Chiang et al. [13] reported a frequency of 8.3% in 218 patients. On the other hand, the frequency of microdeletions found in our study



**Fig. 4** Photo-histogram from a testicular biopsy showing the seminiferous tubules lined with Sertoli cells only with abundant interstitial Leydig cells. [Hematoxylin and Eosin stain.  $\times 400$ .]



**Fig. 5** Photo-histogram from a testicular biopsy showing seminiferous tubules filled with disorganized sloughing germ cells (A), or incomplete spermatogenesis (B). Also, some cells with condensed nuclei are seen in tubules, which may represent apoptotic germ cells or early spermatids. [Hematoxylin and Eosin stain.  $\times 400$ .]

is much lower than that reported by others. It was 52% in 50 patients evaluated by Malekasgar et al. [14], 36% in 75 patients as reported by Suganthi et al. [15], 24.2% in 99 patients evaluated by Omrani et al. [16] and 28.4% in 162 patients in the study of Al-Achkar et al. [17]. The heterogeneity of the results may be due to different numbers of STS primers used in mutation detection, a variation of the selective criteria used for infertile patients recruited or the fact that the studies were conducted on different ethnical populations.

The general frequency of microdeletions with respect to the AZF sub-regions has been reported to be 60% for AZFc, 16% for AZFb and 14% for AZFb + c, with great variations between different populations worldwide [20]. In fact, our results and others lie within these variations.

The distribution of sporadic AZF microdeletions in our study showed an increased frequency of deleted AZFa and AZFc loci [27.3% (3/11) each], followed by deleted AZFb loci [18% (2/11)], which is nearly similar to the findings of Sheikhha et al. [21]. On the other hand, other studies [16,22,23] reported a higher frequency of AZFc microdeletion.

Although many studies have been conducted so far, there are still heterogeneous and conflicting data on the testicular histological morphology and its correlation with the deleted AZF loci. Peterlin et al. [24] reported a marked variability with a poor correlation of testicular histology with the deleted AZF loci, while others [25,26] suggested that deletion of a particular AZF sub-region was usually correlated with specific testicular histological changes.

In the present study, a mixed pattern of hyalinized seminiferous tubules associated with some tubules containing Sertoli cells only, with complete absence of spermatogenic cells (Fig. 3), was detected in testicular biopsies from patients with deleted AZFb + c. Similarly, Brisset et al. [27] in their study had noticed atrophic and hyalinized seminiferous tubules with some sperms in the wet preparation in cases of unbalanced chromosome Y-22 translocation with large deletion of the AZF region involving AZFb, AZFc and the heterochromatic region of the long arm of the Y-chromosome. Other investigators [10,28] reported an association between the large deletion of Y-chromosome AZFb + c and the lack of sperms in testicular biopsies.

Several studies [27,29] indicated that the testicular histology of patients with complete AZFa and/or AZFb microdeletion is usually associated with Sertoli cells only, while those with partial AZFa and AZFb or those with AZFc microdeletions show a broad variation of testicular morphology ranging from Sertoli cells only or spermatogenic arrest to hypospermatogenesis with sperms in the ejaculate. In our study, we detected three cases with AZFa (single locus; sY84, sY86), two cases with AZFb (single locus; sY143) and one case with AZFb (the three loci) microdeletion. Testicular biopsies from these patients show a picture similar to the Sertoli-cell-only syndrome (Fig. 4) with complete absence of spermatogenic cells. Luetjens et al. [30] reported in their study that a testis with AZFa microdeletion showed the picture of a Sertoli-cell-only syndrome, which complies with our results, whereas AZFb microdeletion shows spermatogenic arrest at the level of the spermatocytes.

Genes in AZFa (DDX3Y) and in AZFb (KDM5D, RBMY1A1) seem to be essential for spermatogenic cell development and maturation [31]. So, deletion of AZFa or AZFb is usually associated with severe testicular damage. We detected three cases with a single locus deleted from the AZFc sub-region (sY254 or sY255) and one case with both loci deleted from the AZFc sub-region. Testicular biopsies from these patients show different patterns of spermatogenic cell maturation; some tubules contain disorganized germ cells with sloughing (Fig. 5A), and others have an incomplete or arrested spermatogenesis (Fig. 5B). This result has been found by numerous other investigators [27,30,32].

The AZFc sub-region contains several important genes for male infertility: DAZ, BPY2, CDY1 genes that control spermatogenesis [31,33]. Therefore, the pathological changes associated with AZFc microdeletion are less severe than those associated with AZFa or AZFb and show variable changes according to the deleted gene/s. Additionally, other studies indicated an association between these chromosomal anomalies and Y-chromosome microdeletions. However, several cases with chromosomal rearrangements and with Klinefelter syndrome show AZF deletions [34,35].

Spermatogenesis is regulated by a number of genes on the Y-chromosome, X-chromosome and autosomes that act at different stages of germ-cell differentiation and maturation. Several studies have demonstrated the relationship between balanced autosomal



translocation and severe oligozoospermia/azoospermia, as well as sex chromosome anomalies like Klinefelter syndrome and male infertility [36]. Other studies implied an association between these chromosomal anomalies and Y-chromosome microdeletions. However, several cases with chromosomal rearrangements and with Klinefelter syndrome show AZF deletions [33,34]. The main limitation of our study is the absence of a chromosomal analysis. Future studies should simultaneously use other measures such as semen parameters, hormonal assessments, cytogenetics, molecular genetics and testicular histology in patients with AZF deletions and severe oligozoospermia/azoospermia to assess and improve the outcome of assisted reproductive techniques.

## Conclusions

The frequency of Y-chromosome microdeletions in infertile males is significantly variable and is associated with variable testicular pathologies. Routine cytogenetic, molecular investigation of AZF loci and testicular biopsy are valuable measures in evaluating infertile men, especially those prepared to benefit from assisted reproductive technology.

## Conflict of interest

No conflict of interest.

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