Common Tag STSs in the AZF Region Associated with Azoospermia and Severe Oligospermia in Infertile Egyptian Men

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Abstract: Screening of *Yq* has become one of the most frequently performed postnatal molecular genetic tests in Egypt. Our purpose was to determine the tag sequence-tagged sites (STSs) in the AZF -region of *Yq* associated with azoospermia and severe oligospermia in infertile Egyptian men. We analyzed blood samples from 49 infertile men (28 with azoospermia and 21 with severe oligospermia) using multiplex PCR for six common AZFa, AZFb, and AZFc STS markers,, as recommended by the European Academy of Andrology. Twenty-four (37%) microdeletions with five separate deletions were identified. We found 66.7% of the deletions in the AZFb locus, 20.8% in the AZFa locus, and 12.5% in the AZFc locus. Some common haplotypes (7 of 10) were identified in our sample population. Haplotypes H3 (corresponding to sY127) and H4 (corresponding to sY134) were the most common. We suggest that screening with a minimum of three STSs-sY86, sY127, and sY134-would provide the highest level of clinical sensitivity in genetic testing among infertile Egyptian men. Moreover, separate microdeletions were localized in infertile Y-chromosome patients.

Keywords: AZF region, Azoospermia, Male infertility, Microdeletions, Oligospermia, Y Chromosome.

INTRODUCTION

The 10-Mb AZF region on the q arm of the Y chromosome is frequently deleted in men with unexplained spermatogenic failure. Microdeletions are linked to AZF loci in 20-30% of patients with non-obstructive azoospermia and in 3-7% of patients with severe idiopathic oligospermia [1]. AZF microdeletions are associated with varied testicular histology, ranging from Sertoli-cell-only (SCO) syndrome to hypospermatogenesis to maturation arrest [2]. Although documentation of the prevalence of infertility is lacking in some countries, the Egyptian IVF Center estimates the prevalence to be about 15%. A survey sponsored by the World Health Organization estimated the prevalence of infertility among married Egyptian couples to be 12% (4.3% for primary infertility and 7.7% for secondary infertility) [3]. In addition, Boivin *et al.* [4] stated that the prevalence of infertility ranged from 3.5% to 16.7% in more developed nations and from 6.9% to 9.3% in less-developed nations, with an estimated overall median prevalence of 9%.

The sequence-tagged site (STS) markers of the AZF region have been useful for identifying microdeletions in DNA from blood [5] and sperm [6] of infertile men. Most studies have focused on men with low sperm counts, so most of the deletions that have been found are associated with azoospermia and severe oligospermia. The European Academy of Andrology and the European Genetics Quality Network recommend using six STSs to screen the AZF region [7].

Egypt has been the interest of many conquerors since the time of the ancient Pharaohs, including during the Osmani Empire, the French campaign, British domination, and the Arab-Israeli conflict. As a consequence, much social intermarriage between populations [8] admixed the Y chromosomes. In this study, we identified common tag STS markers in azoospermia and severe oligospermia to screen them for Y chromosome microdeletions before couples initiated assisted reproduction. We extended our work to analyze the testicular phenotypes and the frequency of *Yq* microdeletions in infertile Egyptian men.

MATERIALS AND METHODOLOGY

Patients

Forty-nine infertile men with oligozoospermia or non-obstructive azoospermia (age, 22-45 y; median, 33 y) who were seeking andrologic investigation for male-factor infertility at the Andrology Clinic-Faculty of Medicine, Ain Shams University-Cairo, were included in our study. Non-

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1876-827X/10

obstructive azoospermia was considered as a result of spermatogenic defects on testicular biopsy or an elevated serum FSH level, total testicular volume <30 ml, and no other applicable diagnosis [9]. These men had a history of at least one year of infertility. Only patients with a normal 46,XY karyotype, as shown by conventional GTG-banding, were included in this study. Moreover, a man was excluded from the study if any problems were found in the female partner. All participants signed a consent form before the study began. Patients were informed about the purpose of the study, blood samples were taken on a single occasion, and all the steps of the tests were explained to the patient along with their possible complications.

Molecular Analysis

Genomic DNA was isolated from peripheral blood using the QIAamp DNA Blood kit (Qiagen, Hilden, Germany) or from buccal cells [8]. DNA was then analyzed using multiplex PCR to amplify six STSs from the AZF region. Primers covering *only regions considered to be hotspots for microdeletions* were chosen [10]. These primers corresponded to AZFa (sY84, sY86), AZFb (sY127, sY134), and AZFc (sY254, sY255) (Table **1**). We created modified primer mixes for sY84, sY134, and sY255 (mix I), and sY254, sY86, and sY127 (mix II) using sY14 (*Yp*, internal positive control).

Genomic DNA (250 ng), 5.5 µ*M* of multiple primer mix (Metabion, GmbH, Germany), and 5% DMSO were added to Ready-*Taq* DNA enzyme mix (Fermentas, GmbH, Germany). We performed 45 rounds of PCR with annealing at 54°C and elongation at 65°C for 45 s each. The PCR products were separated on a 3% MetaPhor gel (BMA, Rockland, ME) stained with ethidium bromide (Fig. **1**). All deletions were confirmed by conventional Southern blotting [11].

Haplotype Analysis

We defined a haplotype as a specific pattern of deletions within a group of STS markers. Based on prior understanding of this region of the Y chromosome, a minimum number of certain STS markers can be used as tags for specific haplotypes. Using this concept, we estimated haplotype frequencies in our samples, as previously described [12].

Clinical Analyses

A detailed medical and surgical history, including history of orchitis, mumps, testicular maldescent, testicular injuries, chemotherapy, radiotherapy, smoking, and alcohol habits was taken for each participant. The size, volume, and consistency of the testes, hydrocele, and varicocele were determined, and secondary sexual characteristics were examined. Ultrasonography of the testes was also performed to detect subclinical varicocele and parenchymal lesions compatible with neoplasms.

The diagnosis of azoospermia was established by analyzing the pellet after semen centrifugation [13]. Levels of LH, FSH, and testosterone were measured using a commercial RIA kit (Pharmacia, GmbH, Germany). Testicular biopsy and histological analysis of spermatogenesis

Table 1. Sizes, Positions, and Primer Sequences of Sequence-Tagged Site (STS) Markers on the Y Chromosome

Locus	Deletion sub-Interval	Marker	Primer Sequences	Physical Position*	Distance between Markers	Amplicon size (nt)	Chromosomal Locus
AZFa (DYS148)	5C	sY86	F: 5`-GTGACACACAGACTATGCTTC-3` R: 5`-ACACACAGAGGGACAACCCT-3`	13918257 to 13918574	70782	320	*Yq*11.21
AZFa (DYS273)	5C	sY84	F: 5`-AGAAGGGTCTGAAAGCAGGT-3` R: 5`-GCCTACTACCTGGAGGCTTC-3`	14100173 to 14100518	66560	326	*Yq*11.21
AZFb (DYS218)	5Q	sY127	F: 5`-GGCTCACAAACGAAAAGAAA-3` R: 5`-CTGCAGGCAGTAATAAGGGA-3`	21717107 to 21717380	6418023	274	*Yq*11.223
AZFb (DYS224)	6A	sY134	F: 5`-GTCTGCCTCACCATAAAACG-3` R: 5`-ACCACTGCCAAAACTTTCAA-3`	22702751 to 22703053	613504	301	*Yq*11.23
AZFc (DAZ)	6D	sY255	F: 5`-GTTACAGGATTCGGCGTGAT-3` R: 5`-CTCGTCATGTCATGTGCAGCCAC-3`	24160002 to 24160125	851595	126	*Yq*11.233
AZFc (DAZ)	6D	sY254	F: 5`-GGGTGTTACCAGAAGGCAAA-3` R: 5`-GAACCGTATCTACCAAAGCAGC-3`	24161378 to 24161757	1253	350	*Yq*11.233
SRY		sY14	F: 5`-GAATATTCCCGCTCTCCGGA-3` R: 5`-GCTGGTGCTCCATTCTTGAG-3`			472	Yp

* As reported in release July 2003 of the UCSC (http://genome.ucsc.edu/).

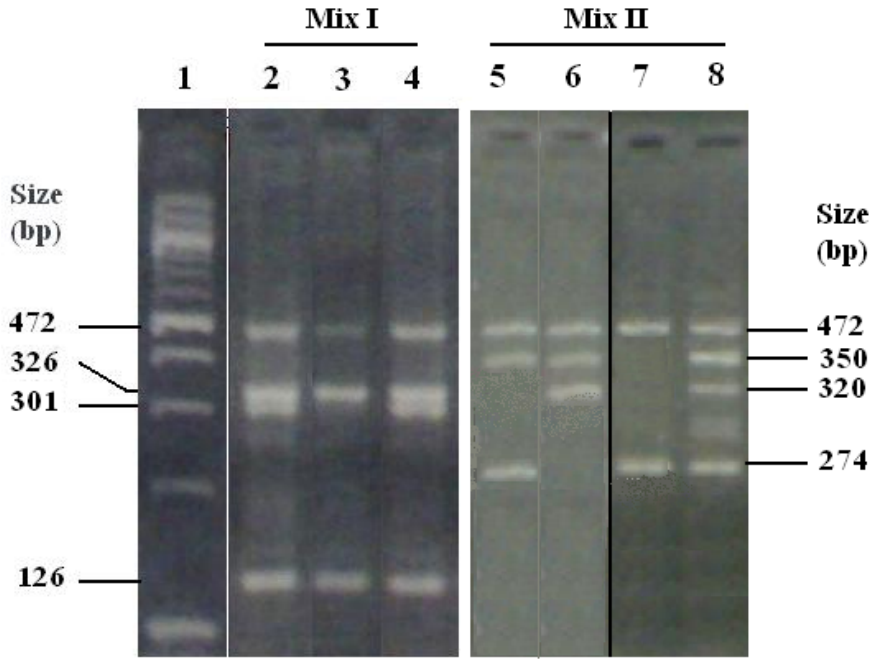

Fig. (1). PCR analysis on DNA of infertile men using sY84, sY134, sY255 (mix I), and sY86, sY127, sY254 (mix II). The fragment due to sY14 'SRY' (472-bp) was used as the internal control. Lane 1, molecular weight (100-bp DNA ladder marker); lanes 2 and 8, normal men with no deletions; lane 3, deletion of sY134; lane 4, infertile man with no deletions. Lanes 5 and 6, patients with deletions of sY86 and sY127, respectively; lane 7, a patient with large deletions of sY86+sY254.

was performed on haematoxylin- and eosin-stained sections. Four sections were examined per patient. The diameter of the seminiferous tube of each section was measured using an ocular micrometer. The involvement of Sertoli cells in different spermatogenesis impairment was also studied by immunohistomorphometric technique using vimentin as a marker of immature Sertoli cells (Fig. **2**). Graphical analyses were performed using Microsoft Excel and SPSS Statistics 17.0.

RESULTS

Prevalence of Yq Microdeletions

To determine the tag STSs associated with azoospermia and severe oligospermia in our population of 49 infertile Egyptian men, we amplified six common STSs on the AZF loci using two multiplex PCRs. We detected 18 (37%) microdeletions in 49 samples. Eleven (39%) of the samples were from men with azoospermia and 7 (33%) from men with severe oligospermia. AZFb (sY127, sY134) was the most common loci, involved in 16 (66.7%) of the 24 microdeletions. The AZFa locus (represented in sY86) was involved in 5 (20.8%), and the AZFc locus (represented in sY254) was involved in 3 (12.5%) (Fig. **3**, Table **2**).

With regard to severity of disease, 9 (56%) of the 16 microdeletions in azoospermic men and 7 (56%) of 8 micro-deletions in oligospermic men were in the AZFb locus. AZFc microdeletions were found in 12.5% of men with azoospermia and 12.5% of men with oligospermia. All of the AZFa deletions were found in patients with azoospermia (Table **2**).

Among the 49 men with infertility, 32 (65%) had idiopathic infertility and 17 (35%) had varicocele. Twenty-

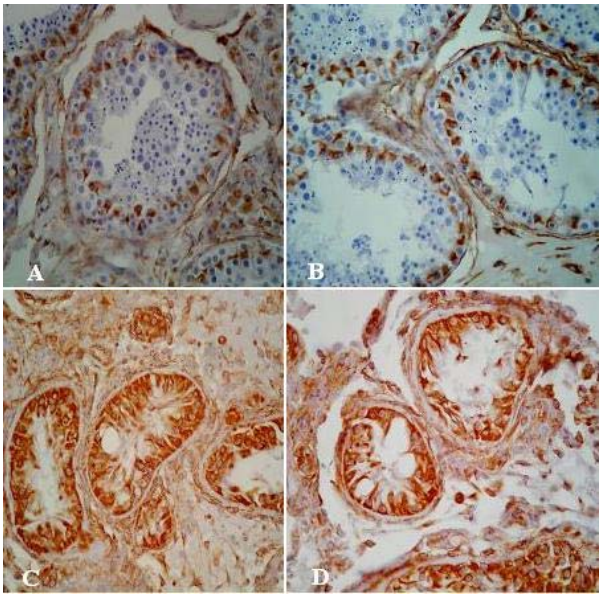

Fig. (2). Immunohistochemical staining for vimentin intermediate filaments within the seminiferous epithelium. The cytoplasm of Sertoli cells was uniformly immunopositive for vimentin in tubules with normal spermatogenesis (**A**) as well as in tubules with maturation arrest (**B**) and Sertoli cell-only syndrome (**C** and **D**). Patients with idiopathic Sertoli cell-only syndrome had a mosaic pattern of differentiated and undifferentiated Sertoli cells. Nevertheless, such patients had a much smaller tubule diameter compared with patients with complete spermatogenesis. Original magnification x200. Scale bar = 150 μm.

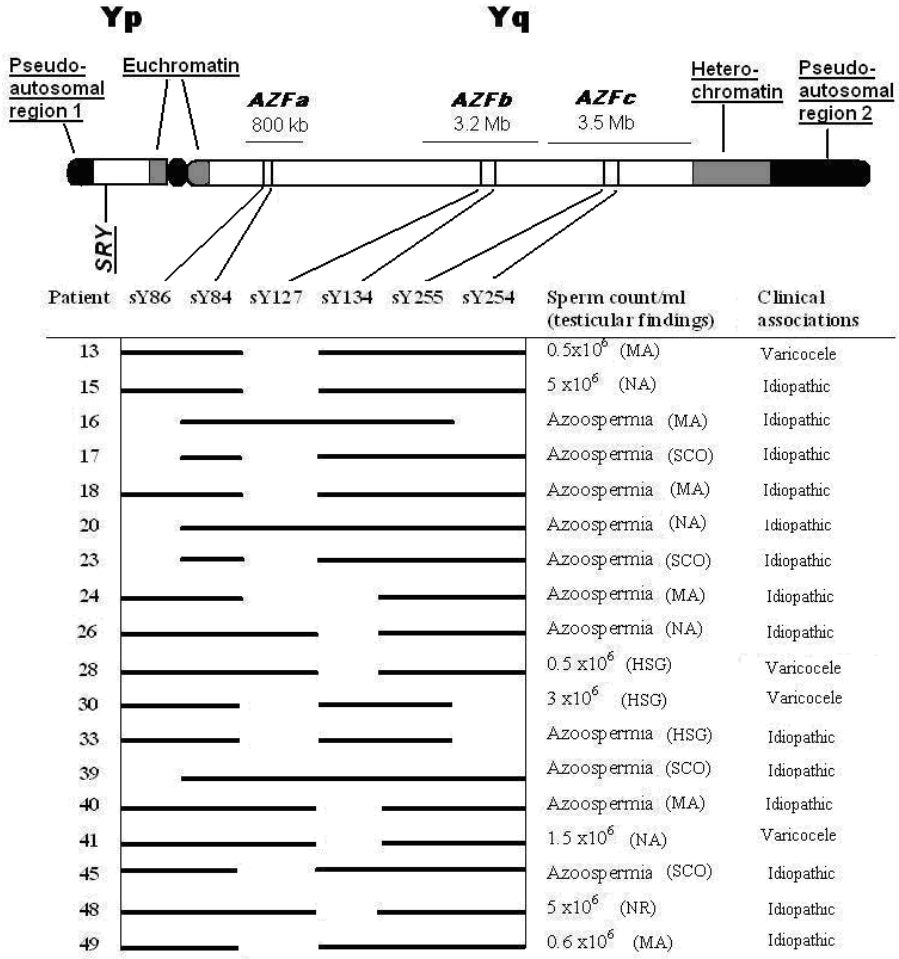

Fig. (3). Y chromosome ideogram represented spermatogenesis in Egyptian men with AZF loci deletions. The solid line represents the presence of the STS locus, while the blank is the deletion of the STS locus. MA= maturation arrest, SCO= Sertoli cell-only syndrome, NA= not available, HSG= hypospermatogenesis, NR= normal testicular findings.

Table 2. Frequency of Microdeletions According to STS and Sperm Status

Phenotype	Number of Deletions	*AZFa*		*AZFb*		*AZFc*	
		sY84	*sY86*	*sY127*	*sY134*	*sY254*	*sY255*
Azoo-spermia	16 (66.7%)	0	5 (31.3%)	6 (37.5%)	3 (18.8%)	2 (12.5%)	0
Oligo-spermia	8 (33.3%)	0	0	4 (50%)	3 (37.5%)	1 (12.5%)	0
Subtotal	24 (100%)	0	5 (20.8%)	10 (41.7%)	6 (25%)	3 (12.5%)	0
Total		20.8%		66.7%		12.5%	

eight men (57%) had non-obstructive azoospermia, and 21 (43%) had severe oligospermia. Fourteen (78%) of the 18 men with microdeletions had idiopathic infertility. The position and extent of the deletions are schematically diagrammed in Fig. **3**.

Five (28%) of the 18 infertile men with microdeletions had two separate deletions (cases #16, 17, 23, 30, 33) (Fig. **3**) localized on the Y chromosome, as validated by conventional Southern blotting (unpublished data). Azoospermia of maturation arrest was found in one of these five cases, SCO syndrome in two of the cases, and hypospermatogenesis in one case. The remaining case with two deletions was an infertile man with severe oligospermia (varicocele).

Haplotypes

A wide range of combinations of microdeleted STSs were analyzed separately in infertile men. Each combination of deletions was considered a marker haplotype. Table **3** shows the most common 10 haplotypes (H1–H10) observed in infertile men. In the present study, we observed 7 of these 10 haplotypes in our patients. However, haplotypes H2, H5, and H6 (corresponding to single deletions in sY84, sY255, and sY254, respectively) were not detected in any of our patients. Although deletions in sY84 (H2) and sY254 (H6) did not occur *per se*, they did occur in combination with other deletions as part of haplotypes H8 and H9. H3 and H4 (corresponding to single deletions in sY127 and sY134, respectively) were the most common haplotypes. Of the four haplotypes consisting of multiple deletions, H7 (corresponding to sY86 and sY127) and H8 (corresponding to sY127 and sY254) were the most common (Table **3**).

Testicular Phenotypes

Of the 18 men with microdeletions, 9 of 11 with deletions were azoospermic and five of seven with deletions were oligospermic. Of the nine patients with azoospermia, four had SCO syndrome, four had maturation arrest, and one presented with hypospermatogenesis. Of the five oligospermic patients, two displayed maturation arrest, two had hypospermatogenesis, and one had normal spermatogenesis. Most patients with a deletion that included the AZFa region had SCO syndrome. One patient with microdeletions in both the AZFa and AZFc loci had maturation arrest. The patients with deletions in the AZFb region showed a range of histological findings (Table **4**). Immunohistochemical analyses showed that the cytoplasm of Sertoli cells was uniformly immunopositive for vimentin in all the biopsies, independent of spermatogenic impairment or the state of Sertoli cell differentiation. Patients with idiopathic SCO syndrome had a mosaic pattern of differentiated and undifferentiated Sertoli cells (Fig. **2**). Nevertheless, these patients had a much smaller tubule diameter than patients with complete spermatogenesis.

Hormone Levels

For all patients, the levels of LH and testosterone were in the normal range (.2-12 IU/L and 7.4-52.4 IU/L, respectively). Although the mean FSH levels were elevated in the study population (15.9±7.4mIU/ml), no significant differences in FSH levels were found between the patients with deletions and those without them.

DISCUSSION

Screening of *Yq* is one of the most frequently performed molecular genetic tests in Egypt [14]. However, the impressive demand for *Yq* screening deserves validation. In this regard, some important issues can be answered only by careful clinical assessment of a group of men with well-characterized microdeletions.

The reported frequencies of deletions in the AZF region of *Yq* vary from 1% to 55%, depending on a study's inclusion criteria and possibly on the STS markers used for screening [15]. Using six common STSs to screen the AZF region of *Yq*, we found a microdeletion frequency of 37% among 49 infertile Egyptian men. Microdeletion frequencies of 39% for azoospermic men and 33% for severely oligospermic men were also observed. The prevalence in azoospermic men in the present study was similar to that reported by Foresta *et al.* [16]. The prevalence in oligospermic men was higher than what most studies have reported [17], but it was similar to that reported in a Tunisian population [18].

In monogenic disorders, some double deletions are present and may help to restore the reading frame of the mRNA transcript and consequently promote milder phenotypes [19, 20]. The five cases of double deletions

Table 3. Composition of Haplotypes in the Study Population

Locus (Haplotype)	STSs in the Haplotype	No. of Patients with *Yq* Deletions		Total no. of Patients with Deletions (%)
		Azoo Group	Oligo Group	
AZFa (H1)	sY86	2	0	2 (11%)
AZFa (H2)	sY84	0	0	0
AZFb (H3)	sY127	2	3	5 (28%)
AZFb (H4)	sY134	2	3	5 (28%)
AZFc (H5)	sY255	0	0	0
AZFc (H6)	sY254	0	0	0
AZFa,b (H7)	sY86+sY127	2	0	2 (11%)
AZFb,c (H8)	sY127+sY254	1	1	2 (11%)
AZFa,c (H9)	sY86+sY254	1	0	1 (5.5%)
AZFb (H10)	sY127+sY134	1	0	1 (5.5%)
Total		**11 (61%)**	**7 (39%)**	**18 (100%)**

Table 4. Testicular Histopathology of the 18 Men with Microdeletions, According to Sperm Status

Locus (STS)	No. of Deletions	Azoospermia				Oligospermia
		SCO	HSG	MA	NA	
AZFa						
(sY86)	5	3	0	1	1	0
AZFb						
(sY127)	10	3	1	2	0	1 (HSG), 2 (MA), 1 (NA)
(sY134)	6	0	0	2	1	1 (HSG), 1 (NR), 1 (NA)
AZFc						
(sY254)	3	0	1	1	0	1 (HSG)
Total	24	6	2	6	2	8

SCO= Sertoli cell-only syndrome, HSG= hypospermatogenesis, MA= maturation arrest, NA= not available, NR= normal testicular findings.

identified within the AZF region of the Y chromosome in this study clearly increased the severity of the clinical phenotypes, as four of the five cases showed azoospermia on clinical investigation. These separate deletions might be due to the creation of alternative initiation and termination codons in the coding sequence of the mRNA transcript. Further investigation could help someone to develop procedures for reducing the severity of clinical phenotypes, by disrupting the translational fidelity and consequently restore the correct frame [8, 19, 20].

Most studies have found the highest frequency of microdeletions in the AZFc locus, followed by the AZFb locus [21, 22] and, less commonly, the AZFa locus [23, 24]. In the present study the highest frequency of microdeletions was found in the AZFb locus, followed by the AZFa (20.8%) and AZFc (12.5%) loci. Our findings are similar to those [25] that, in contrast to most reports, found that 54% of patients with *Yq* microdeletions had a deletion involving the AZFb region. Our results, combined with those of Brandell *et al.* [25] suggest that the AZFb locus is a good candidate for future functional studies.

Generally, ethnic differences might be associated with variations in both the frequency and pattern [26] of Y-chromosome microdeletions. For instance, an unusually low frequency of microdeletions (3.3%) has been reported in infertile Turkish men [27]. In terms of patterns, the STSs sY240 and sY129 are commonly deleted in infertile Japanese men [28], while sY269 [29] and USP9Y [30] are more commonly deleted in Italian men and sY100 in French men [31].

Although STSs sY84 (AZFa) and sY255 (AZFc) are frequently recommended for traditional analyses, we did not detect deletions in either of these loci in our population. More deletions would be found if more primer sets for multiplex PCR were used [32]. However, this would contradict the recommendations of the European Academy of Andrology [33], which suggest that more than 90% of microdeletions can be detected using six STSs to screen the AZF region (i.e., two STSs for each AZF locus).

We also found that only azoospermic patients had microdeletions in the AZFa locus and that most of these patients were found to have SCO syndrome. Patients with isolated AZFb microdeletions had histological differences ranging from SCO syndrome to normal spermatogenesis. This result was consistent with published data showing that patients with AZFb deletions are as phenotypically heterogeneous as those with AZFc deletions [34]. The finding of normal spermatogenesis in the presence of Y-chromosome microdeletions is an extremely rare finding, which has been previously reported by Pryor *et al.* [35] to be associated with spermatic duct obstruction. The possibility of areas of focal spermatogenesis in the presence of testicular pathologies [36] may explain this finding. Our findings support the view that the worst spermatogenic defects are caused by deletions of the AZFa region [37]. Identical microdeletions may be associated with diverse types of tubular damage, as recently reported [38], but the only method for distinguishing the specific tubular alterations in azoospermic or severely oligospermic men is to directly analyze their testicular structures by diagnostic open biopsy or fine-needle aspiration.

In our study, we detected microdeletions in 4 (23%) of 17 of patients with varicocele. Moro *et al.* [39] found a microdeletion frequency of 17.5% among patients with varicocele and severe oligospermia, although no genetic abnormalities in patients with varicocele and only mild oligospermia were noted. It has been found that 8% of infertile men with varicocele have microdeletions of STSs in the AZF region [40]. In contrast, Kleiman *et al.* [24] did not detect any *Yq* deletions in men with varicocele, regardless of their sperm counts. Although varicoceles are associated with infertility, only 1 (~17%) of 6 men with a varicocele presents with infertility [41]. Microdeletions may have been the primary cause of infertility in our four patients with varicoceles, although the varicocele itself can worsen the testicular alteration.

Our immunohistochemical analyses showed that the cytoplasm of Sertoli cells was uniformly immunopositive for vimentin in all of the biopsies performed, independent of

spermatogenic impairment. Kleiman *et al.* [5] studied the involvement of Sertoli cells in different spermatogenesis impairment by an immunohistomorphometric technique using cytokeratin-18 (CK-18) and vimentin [42], which are co-expressed in the cytoplasm of Sertoli cells during prenatal and pubertal differentiation [43]. They came to the conclusion, after examining testicular biopsies from nine men with microdeletions, that AZF deletions have no impact on the Sertoli cell maturation process. They also concluded that there is no association between spermatogenesis and the maturation state of the Sertoli cells.

Finally, no significant differences in the mean levels of testosterone, LH, or FSH were found between patients with deletions and those without them. Similarly, other recent studies comparing FSH levels between patients with and without deletions found no significant differences [37]. Together, these results suggest that hormone levels are not a likely indicator of who may be at risk of a deletion.

Intracytoplasmic sperm injection (ICSI) now provides fertility in many cases of severe idiopathic spermatogenic failure and obstructive azoospermia. Genetic causes must be sought by systematically evaluating infertile men and affected couples who are informed about the implications of diagnoses for assisted reproduction and their potential offspring [44].

Partial deletions of the AZFc region might prospectively influence the fertility status of the patients. It remains unclear which of the genes located in the deleted regions are important for the progression of spermatogenesis. X-linked genes can also affect male infertility. For example, mutations in X-linked genes crucial for spermatogenesis will have an immediate impact on sperm production. The X-linked genes NXF2, USP26, and TAF7L were previously reviewed for the presence of mutations [45]. Recently, Stouffs *et al.* [45] have studied five autosomal genes: SYCP3, MSH4, DNMT3L, STRA8, and ETV5. They detected changes in the latter two genes that were absent in a control population of men with normozoospermia. Functional analysis of the changes in ETV5 and the localization of the change observed in STRA8 showed that these alterations were probably not the cause of the fertility problems in these men. These reports could be concluded that mutations in X-linked genes in humans, presumed to be important for spermatogenesis, have been disappointing.

Based on the results of our study, we recommend that all patients with a sperm concentration lower than $5x10^6$/ml—especially those with severe testiculopathy—must be screened for Y-chromosome microdeletions, regardless of other concomitant causes of testicular damage. The detection of a deletion allows the clinician to avoid unnecessary and expensive treatments for determining the cause of infertility in a patient.

CONCLUSION

We conclude that the tag STSs from our study can be used to screen infertile Egyptian men for *Yq* microdeletions before assisted reproduction is initiated as a treatment. To efficiently detect these deletions in the Egyptian population, we recommend performing multiplex PCR with a combination of three STSs—sY86, sY127, and sY134—as a first step.

ACKNOWLEDGEMENTS

We thank Kerry Aradhya for help with English. The authors are grateful to the staff of the Department of Andrology, Faculty of Medicine-Cairo, for their fruitful services to fulfill patients' records.

LIST OF ABBREVIATIONS

AZF = Azoospermia factor CK-18: cytokeratin-18

FSH = Follicle stimulating hormone

GTG = G-banding by Trypsine-Giemsa

HSG = Hypospermatogenesis

ICSI = Intracytoplasmic sperm injection

IVF = *In vitro* fertilization

LH = Leutinizing hormone

MA = Maturation arrest

PCR = Polymerase chain reaction

SCO = Sertoli-cell-only

STS = Sequence-tagged site

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Received: December 21, 2009 Revised: February 15, 2010 Accepted: April 13, 2010