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Prevalence of Y Chromosome Microdeletion in Males with Azospermia And Severe Oligospermia in Egypt

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Abstract: Microdeletions of the so-called azospermia factor (AZF) locus of the Y chromosome long arm (Yq) are an etiological factor of severe oligozospermia or azospermia. Patients affected are infertile unless assisted reproduction techniques are used. Most of the microdeletions that cause azospermia or oligospermia occur in the non-overlapping regions of the long arm of the Y chromosome. These regions, also called azospermia factor regions (AZF), are responsible for spermatogenesis. The loci ae termed AZFa, AZFb and AZFc from proximal to distal Yq. This study aims at PCR-based rapid analysis of Y chromosome microdeletions, which is a cause for male infertility. We studied 100 infertile men compared to 20 normal fertile men. PCR amplification using Y specific sequence tagged sites of the AZF regions for AZFa: sY86 – sY84, AZFb: sY134 – sY127, AZFc: sY255 – sY254 were conducted. Results: of the 100 infertile men 68 were oligospermic and the remaining 32 were azospermic. Severe oligospermia was diagnosed in those patients who produced only one-third the concentrations of the sperm of that found in fertile men. Among the 100 infertile men 4 had microdeletions (4%) The frequency of microdeletions in oligospermic patients was 2/68 (2.9%) (AZFb: sY134 – sY127). The frequency of microdeletions in azospermic patients ws 2/32 (6.2%) (AZFa sY86 & AZFb 134). Microdeletion analysis using PCR helps to determine the frequency and site of gene deletion and also contributes to the determination of an accurate prognosis and ultimately to valuable counseling for couples diagnosed with azospermia microdeletions

Key words: male infertility, male factor, oligozoospermia, azoospermia, microdeletion.

INTRODUCTION

Infertility affects about 15% of couples and in 40% - 50% of cases, the male partner has quantitative or qualitative abnormalities of sperm production [1]. In more than 60% of cases the origin of reduced testicular function is unknown. Several studies have reported a marked decline in male reproductive health and an increase in the population of subfertile males. Both genetic and environmental factors are believed to be responsible for this decline. Another factor that is reported to cause DNA damage in the sperm and Y chromosome is oxidative stress, in which there is a decline in the total antioxidant capacity in the semen of infertile males and increased levels of reactive oxygen species [2].

Germ cell development is under the control of a large number of genes on autosomes and on the Y chromosome. The long arm of the Y chromosome genes families is involved in spermatogenesis and is critical for germ cell development and differentiation. In 1992, Vollrath and colleagues [3] constructed a 43 – interval deletion map of a human Y chromosome that contained an ordered array of sequence tagged sites (STS) that spanned the entire length of the Y chromosome. The genes critical for spermatogenesis are located on the long arm of the Y chromosome in deletion interval 5 and 6 (Figure 1). This region is referred to as the Azospermia factor (AZF), as the most severe phenotype associated with its deletion is azospermia. It has been known that deletion of the long arm of the Y chromosome is associated with spermatogenic failure and leads to partial or complete spermatogenic arrest [4].

The AZF region is subdivided into three non-overlapping sub-regions called AZFa in the proximal portion, AZFb in the intermediate region nd AZFc in the distal region. Each of these regions contains several genes involved in male fertility that are in the euchromatic region of Yq; they are strongly associated with spermatogenic defects, such as azospermia and oligozospermia [5].

The microdeletions that occur in the AZF region affect genes that are involved in spermatogenesis. Microdeletions in the AZF region are frequently found in patients with azospermia and severe oligospermia,

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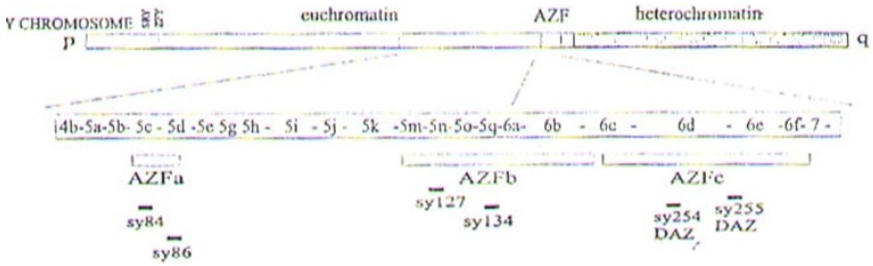


Fig. 1: Long arm of the Y chromosome (Vogt *et al.,* 1996)[4].

the incidence of these microdeletions has been found to vary from 3 to 55 % in Yq of patients with infertility problems. A relatively high frequency of de novo deletions could be due to spontaneous susceptibility to loss of genetic material in the Y chromosome[6].

Deletion of the AZFa region (least common class of deletion) is most frequently associated with azospermia manifested by the sertoli cell only syndrome (SCOS) and less often with oligospermia. Deletion of the AZFb region is associated with azospermia, oligospermia and normozospermia. Deletion of AZFc interval is associated with azospermia, severe to mild oligospermia, and the production of insufficient mature sperm to enable reproduction [7].

The intense effort of many laboratories in last years contributed to a significant understanding of the clinical and molecular significance of this genetic alteration and nowadays Y chromosome microdeletions are routinely screened worldwide.

Thus the identification and analysis of Y chromosome deletions is an important research tool for studying male infertility.

Patients with azoospermia or severe oligospermia who may be candidate for intracytoplasmic sperm injection (ICSI) or testicular sperm extraction (TESE/ICSI) should be offered deletion screening because TESI should not be recommended in cases of complete deletion of the AZFa region, or complete deletion of AZFb or deletions of the AZF b+c regions. Moreover, microdeletions of the AZFc region are transmitted to the male offspring if assisted reproduction is performed. Therefore, the diagnosis of a deletion has prognostic value and can influence therapeutic options [8].

Aim of the Work: To examine and report the prevalence of microdeletions of the Y chromosomes in infertile men with azospermia and severe oligozospermia in an assisted reproduction clinic. This study also aims at a polymerase chain reaction (PCR)-based rapid analysis of Y chromosome microdeletion. which is a cause for male infertility.

Subjects: We studied 100 infertile men compared to 20 normal fertile men. The 100 infertile men, who attended Heliopolis Fertility and genetic centre in Egypt from 2004 to 2007, were screened for Yq microdeletions. Only those with normal karyotypes were selected. An informed consent was taken from all participants to be submitted to genetic testing and were screened for Yq chromosome microdeletions. As controls we recruited 20 subjects with proven fertility and normal sperm concentration (> 20 million / ml).

Patients were excluded if they had (i) varicocele (ii) undergone treatment with chemotherapeutic agents or radiotherapy or they had testicular tumors, (iii) karyotype abnormalities including Y chromosome alterations (iv) obstruction in the seminal tract.

Methods: All patients underwent a comprehensive examination including a detailed history, physical examination, two consecutive semen analyses, endocrinology (FSH, LH testosterone) and chromosome analysis. Serum levels of FSH [9] and LH [10] were measured by enzyme linked fluorescent assay (minvidas biomieureux) (Normal reference ranges for FSH 1.4 – 18.1 mIU/ml, LH 1.1 – 7 mIU/ml and testosterone [11] (2.41 – 8.27 ng / dl). Peripheral blood cultures were set up for chromosomal analysis using G-banding karyotype [12].

Semen samples were obtained on two different occasions, with each collection being performed after a 3-day period of sexual abstinence and separated by a 3-week interval. A complete semen analysis was performed according to World Health Organization guidelines [13]. Semen was analyzed for motility, morphology, count, ph, and viability of sperm. The diagnosis of azospermia was established by pellet analysis after semen centrifugation. (1000 g, 20 minutes).

In 100 infertile men with a normal 46 XY karyotype molecular analysis for AZF loci was done using PCR. Each patient was examined for six AZF loci that mapped to interval 5 and 6 of the Y chromosome.

Genomic DNA was extracted from peripheral venous blood using a salting out protocol, as described by Miller *et al.,* 1988 [14]. The polymerase chain reaction (PCR) was used to detect the 6 different sites loci corresponding to different azoospermia factors (AZFs) loci. These include: For AZFa region: sY86, sY84, for AZFb region: sY134, sY127and for AZFc region: sY255, sY254. Amplification was carried out with sequence-specific primers [7] Table (1).

The reaction was comprised of 50-100 ng of genomic DNA in 25 ul PCR reactions(10 mM Tris HCL pH 8.3, 50mM KCL, 1.5mM MgCl2, 2mmol dNTPs, 20 p mol of each primer and 1 units of *Taq* DNA polymerase. Initial denaturation step at 94° C for 5 min, followed by 27 cycles of 94 °C for 30 sec,55 °C for 30 sec, and 72 °C for 4 min ending with a final extension of 5 min at 72 °C. The PCR products were separated on 2% agarose gels stained with ethidium bromide, on the basis of the size of the product obtained. A ØX marker ladder helped identifying the different band sizes.

Specimen Collection: Ten cc venous blood was collected and divided into two parts:

- Eight cc venous blood was incubated at 37 ^{0} C waterbath for 10 minutes and centrifuged at 3000 g for 10 minutes. Serum was divided into aliquots and stored at – 20 ^{0} C until assay for hormones.
- Two ml was collected in a tube containing ethylenediamine tetraacetate (EDTA) as an anticoagulant for DNA extraction.

Statistical Analysis: The SPSS software (version 10.0) was used for data management and analysis. Quantitative data was presented as mean ± SD. As most of the data were normally distributed continuous variables after being subjected to Kolmogorov-Smirnov test, student's *t* test was used to assess whether a statistical significance is present between the studied groups. Test was two-tailed and considered statistically significant when $p < 0.05$. Categorical variables were presented as percentages.

RESULTS AND DISCUSSION

The age of patients and controls were 34.31 ± 4.72 years & 32.0 ± 6.48 years respectively ($p > 0.05$).

Sixty eight of the 100 infertile men were oligospermic so sperms were taken for assisted reproduction. The remaining thirty two patients were azospermic. Their testicular biopsy showed that 22 patients of the 32 (70%) had motile sperms and 10 patients of the 32 had no motile sperms in the testicular biopsy (30 %).

Among the100 infertile patients 4 had microdeletions (4%).

Among the 68 oligospermic men 2 had microdeletions in AZF b (Sy134) & AZF b (Sy127). Among the 32 azospermic patients 2 had microdeletions in AZF a (Sy86) & AZF b (Sy134). So if we consider the frequency of micrdeletion only for men truly candidates for Y micrdeletions screening (azospermia and oligospermia) the frequency for oligospermia is 2 / 68 (2.9 %) and for azospermia is 2 / 32 (6.2 %).

Hormonal data showed no differences in LH and testosterone plasma concentrations between men with and without Yq microdeletions. FSH levels in men with Yq microdeletions were higher than controls, but lower than men without deletions but with no significance differences (Table 2).

Discussion: Y microdeletions are specific for spermatogenic failure since no deletions have been reported in a large number of normospermic men. Although fertility can be compatible with Y deletions, it simply reflects the fact that natural fertilization may occur even with relatively low sperm counts depending on the female partner's fertility status. For this reason, it is more appropriate to consider Y deletions as a cause of oligo / azospermia rather than cause of infertility [15].

Spermatogenesis is regulated by a number of genes on the Y chromosome and autosomes. Y chromosome deletions are emerging as a prevalent cause of male factor infertility. The frequency of the Y chromosome deletion increases with the severity of the spermatogenic defect. These Y chromosome microdeletions cannot be predicted cytogenetically, on the basis of clinical finding or by semen analysis. In the past, the diagnosis of a genetic etiology had little clinical significance. Today, the assisted reproductive technology helps in overcoming this infertility problem, but there is still transmission of the genetic defects, like microdeletion, to their offsprings. Hence, this diagnosis will provide the information necessary to counsel these couples effectively, particularly with regard to the birth of an infertile male offspring, who may have the same or secondary, larger deletions with more severe testicular phenotype[1].

Thus, the aim of this study is a PCR-based screening of AZF regions for microdeletions on Y chromosome in infertile men.

Studies on microdeletions of the Y chromosome have been carried out with a variety experimental design, and variation in the prevalence of the microdeletions in the Y chromosome from 1 – 55.5% has been reported[16]. A number of factors have been proposed to explain the wide variation in Y deletion frequencies, including population, ethnic variation, environmental influence, patient selection criteria and classification values used to define severe oligospermia of 5 χ, 2 χ or 1 χ 10^6 sperm/ml. Also if blood or semen DNA was used[17].

Table 1: Primer Sequence used in the molecular study of microdeletions in the AZF region of the Y chromosome

Region	STS	Primer sequence
AZF a	sY86	F 5' – GTC ACA CAC AGA CTA TGC TTC – 3' R 5' – ACA CAC AGA GGG ACA ACC CT – 3'
	sY84	F 5' – AGA ACG GTC TGA AAG CAG GT – 3' R 5' – GCC TAC TAC CTG GAG GCT TC – 3'
AZF b	sY127	F 5' – GGC TCA CAA ACG AAA AGA AA – 3' R 5' – CTG CAG GCA GTA ATA AGG GA – 3'
	sY134	F 5' – GTC TGC CTC ACC ATA AAA CG – 3' R 5' – ACC ACT GCC AAA ACT TTC AA – 3'
AZF c	sY254	F 5' – GGG TGT TAC CAG AAG GCA AA – 3' R 5' – GAA CCG TAT CTA CCA AAG CAG C – 3'
	sY255	F 5' – GTT ACA GGA TTC GGC GTG AT – 3' R 5' – CTC GTC ATG TGC AGC CAC – 3'

STS = sequence tagged sites

Table 2: Age and Reproductive hormones in azospermic and severe oligospermic men with microdeletion, without microdeletions and normozoospermic controls

	Age (years)	FSH (mIU/ml)	LH (mIU/ml)	Testosterone (ng/dl)
Control (N 20)	32.0 ± 6.48	12.75 ± 3.54	6.64 ± 2.03	6.27± 2.96
Infertile men without microdeletion (96)	33.96± 4.98	13.71 ± 3.35	6.95± 2.53	5.73 ± 1.66
Infertile men with microdeletion (4)	33.44 ± 4.66	12.97± 1.16	6.36 ± 3.97	6.84 ± 0.96

Our study utilized ologospermic patients with values below $2 \chi 10^6$ sperm/ml. These values were the same values used by Vogt *et al.,*[19] who considered the values for oligozospermia below $2 \chi 10^6$ sperm/ml. However Carrara *et al.,*[19] and Hellani *et al.,*[20] established values for oligozospermia of $< 1 \chi 10^6$ sperm/ml. Dada *et al.,*[21] only indicated oligozospermia without specifying the number of spermatozoa.

The withdrawal of FSH action from Sertoli cells and the reduction in testicular testosterone levels resulting from LH withdrawal from leyding cells markedly suppress spermatogenesis[22]. In our study hormonal data are not useful to identify a priori patient with higher risk of Yq microdeletions. We found lower plasma FSH concentrations in men with Yq microdeletions with respect to men without deletion, and higher FSH serum levels than controls but with no significant differences and serum concentrations of FSH were not significantly different between patients with and without microdeletions of the Y chromosome according to previous preliminiary study by Foresta *et al.,*[23] and Tomasi *et al.,*[24]. The range of FSH concentrations is highly overlapping between men with and without microdeletions. Therefore, individual data cannot be used as indicators of Yq abnormalities. However, we believe that an increase in patient number would clarify this aspect, because we observed a trend characterized by lower FSH levels in deleted patients than nondeleted patients.

Y microdeletions are specific for spermatogenic failure since no deletion have been reported in a large number of normospermic men [15]. Each AZF locus acts at a different phase of spermatogenesis, and deletion of each locus causes spermatogenic arrest at a particular stage. On the basis of testicular histology, the deletion of AZFa was associated with the complete absence of germ cells and the presence of Sertoli cells in the seminiferous tubules, characteristic of SCO syndrome and was associated with azospermia. Deletion of the AZFa region, no sperm are found upon attempts of testicular sperm extraction (TESE) in the patients[25]. The deletion of AZFb was associated with the developmental arrest of germ cells at the pachytene stage and led to meiotic maturation arrest. The deletion of AZFc was associated with developmental arrest of germ cells at the spermatoid stage, but was also found to be associated with hypospermatogenesis or maturation arrest and was associated with low sperm counts. In men with azospermia and AZFc deletion there is a fairly good chance of retrieving sperm from TESE for ICSI. Thus, deletion of a particular AZF locus results in a characteristic phenotype, and genes at each locus act at a particular stage of germ cell differentiation [1].

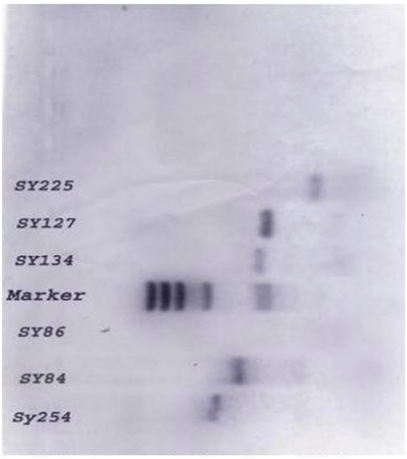
In our study, a genotype-phenotype correlation has not been objectively demonstrated.

The frequencies of deletions of Yq, reported in different studies, range between 3% and 18% of males with non-obstructive azospermia or severe oligospermia [26] and [27]. In our screening of the 100 infertile males, we found 4 patients carrying microdeletions corresponding to a frequency of 4 % (4 out of 100 patients had microdeletions). In comparison to the statistical values obtained from all the surveys,[27] some

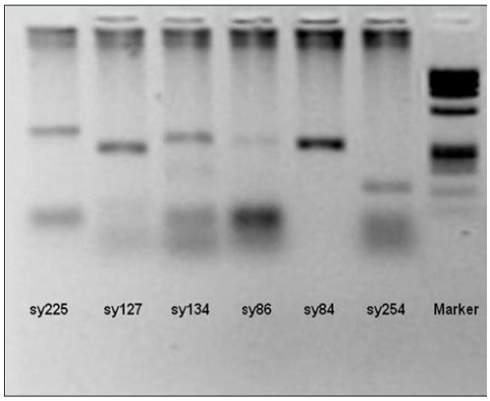
studies reported 13% of infertile microdeletions in the Y chromosome[28] and [29]. While others, less than 5% [30]. Another study showed an incidence of microdeletion between 5.1% and 9.6% in the infertile males [31]. However, 55.5% of infertile males with Y microdeletions were found in Italy [32]. Our results are in accordance with the reported results of between 3% - 18%.[26] and [27].

Deletion of the AZFc region was not detected any patient. This is in disagreement with earlier studies, which showed that deletion in the AZFc region was high when compared with that in the AZFa and AZFb regions [33]. In our study, 25% (1/4) of the total Y chromosome deletion was in the AZFa region and 50 %(1/2) of azospermic patients. This result is similar to another report that 24.2% of total Y chromosome deletion was in the AZFa region [34]. Sun et al, 1999 reported that point mutation in the genes present in the AZFa region can cause azospermia [35]. This is in agreement with the present observation that the deletion in the AZFa region was very small, and that all individuals with deletion in the AZFa region were azospermic. In the AZFb region, there was 50 % (1/2) deletions from that of total deletions in azospermic men and ologospermic patients showed 100% deletions (2/2). Deletions in the AZFb region have been found to be associated with azospermia, oligozospermia, and normozospermia.

Conclusion: Microdeletion analysis using PCR helps to determine the frequency and site of gene deletion, and also contributes to the determination of an accurate prognosis and ultimately to valuable counseling for couples diagnosed with AZF microdeletions and enables unnecessary medical and surgical treatment to be used.



gel photograph showing deletion in the AZFa region (sy 86) with the other regions present



gel photograph showing all regions with no deletion.

Recommendation: Screening for Y deletions would be indicated in patients undergoing intracytoplasmic sperm injection (ICSI) programs and counseling on the risks of transmitting Y microdeletions and other Y chromosomal abnormalities to the offspring should be provided.

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