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Research Article
Molecular Screening for Y Chromosome Microdeletions in Egyptian Infertile Men with Severe Oligozoospermia

[1]Zakaria Mahran, [1]Rabie Bedir, [2]Mekky Abdel Monem and [1]Osama Shahat

[1]Department of Dermatology, Al-Azhar University, New Damietta, Egypt
[2]Department of Clinical Pathology, Al-Azhar University, New Damietta, Egypt

Abstract

Background: Male infertility is a multifactorial syndrome. Genetic factors contribute about 10-15% of cases. Among these factors, Y chromosome microdeletions are the second most frequent genetic cause after Klinefelter's syndrome. **Objective:** The aim of the study was to detect azoospermia factor (AZF) region microdeletions and their locations on Y chromosome in infertile men with severe oligozoospermia and to correlate these microdeletions with the patient's semen analysis, hormonal profile and testicular volume. **Materials and Methods:** In this cross sectional study, 50 infertile severe oligozoospermic patients (<5 million) were included, their ages ranged from 20-50 years. The patients included in this study were selected from Damietta Outpatient Clinic of Dermatology and Andrology Department, Al-Azhar Faculty of Medicine from March, 2014-2016 and were subjected for history, general and local examinations, semen analysis, FSH, LH and testosterone levels and PCR examination. **Results:** The mean age of studied cases was 34.26 years with mean duration of infertility 8.16 years. Normal FSH, LH and testosterone was found in 74, 96 and 88%, respectively. The AZF deletion was reported in 4 cases. Regarding relation to studied hormones and testicular size, there was significant association between AZF deletion with both FSH ($p = 0.02$) and LH ($p = 0.025$), while there was no significant association with testosterone ($p = 0.44$) or testicular size ($p = 0.12$). **Conclusion:** Microdeletions of the Y chromosome in the study were 8% among severe oligozoospermic males. There was no statistically significant relation between testicular size, semen volume, testosterone and LH with the Y chromosome microdeletions. The detection of a deletion in an infertile man provides a proper diagnosis of the disease, allow the clinician to avoid empirical, unnecessary or often expensive treatments to improve fertility. A molecular diagnostic test of Y chromosome microdeletions should be at least performed in all men with a sperm concentration of $<5 \times 10^6$ mL^{-1}, regardless of the presence of other apparent concomitant cause of testicular damage.

Key words: Infertility, Y chromosome, microdeletions, oligozoospermia

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Corresponding Author: Zakaria Mahran, Department of Dermatology, Al-Azhar University, New Damietta, Egypt

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Infertility is defined as the inability of couples to conceive after 12 months or more of unprotected intercourse[1]. Approximately 15% of couples are infertile and among these couples, male factor infertility accounts for approximately 50% of causes. In more than half of infertile men, the cause of their infertility is unknown and could be congenital or acquired[2].

Spermatogenesis is an essential stage in human male gamete development, which is regulated by many Y chromosome specific genes. Most of these genes are present in a specific region located on the long arm of the human Y chromosome (Yq11) known as the azoospermia factor region[3]. The Y chromosomal microdeletions considered as the most frequent structural chromosome anomaly associated with the quantitative reduction of sperm[4], as it associated with about 15-20% of idiopathic azoospermic men and 7-10% of idiopathic oligozoospermic men[5].

The Y chromosome is not essential for life and most regions of it assumed to be functionally inert. Sex determination (controlled by SRY gene) has been long viewed as the sole function related to it. This theory was changed when another important function (spermatogenesis control) was discovered and many genes were mapped to Y chromosome[6].

The Y chromosome plays a fundamental role not only for sex determination but also in the control of spermatogenesis. Microdeletions of the Y chromosome removing AZF region are found in men suffering from azoospermia or oligozoospermia and are the second most frequent genetic cause of spermatogenic failure after Klinefelter's syndrome[7-9].

Patients with AZFd only may present with mild oligozoospermia or even normal sperm count associated with abnormal sperm morphology[10].

As molecular techniques used to identify these microdeletions had much greater resolution than cytogenetics, more than 0.5% of azoospermic men were now shown to have genetic defects of their Y chromosome[11].

Since infertility is largely due to impairment of gametogenesis, in which a number of genes participate, it is logical that mutations in spermatogenic genes would result in impaired spermatogenesis, leading to infertility[12]. Thus, the aim of this study was to detect AZF region microdeletions and their locations on Y chromosome in infertile men having severe oligozoospermia and to correlate these microdeletions with patient's semen analysis, hormonal profile and testicular volume.

MATERIALS AND METHODS

In this study, 50 infertile severe oligozoospermic patients (<5 million) were included, their ages ranged from 20-50 years. The patients included in this study were selected from Damietta Outpatient Clinic of Dermatology and Andrology Department, Faculty of Medicine Al-Azhar University from period of March, 2014-2016 and our patients were subjected to detailed history taking including personal, infertility history and any conditions might affect fertility (medical diseases, trauma, surgery). Full general and genital examination was performed. At least, two semen analyses were performed for every patient by Computer Aided Sperm Analysis (CASA) and further confirmed with repeated microscopic examination. Patients were instructed to bring semen samples by masturbation after sexual abstinence period of at least 2-5 days, 4 weeks apart. Severe oligozoospermia was diagnosed as very low sperm count ($\leq$5 million sperm mL^{-1}). Semen analysis was done according to WHO recommendation[13]. Serum FSH, LH, free testosterone levels were estimated using the Radio Immuno Assay (RIA) technique.

The study was approved by the local ethical committee. Written informed consent was obtained from patients.

DNA studies

DNA extraction from the whole blood: Genomic DNA was extracted by using Genomic Prep™ Blood DNA isolation kit (Amersham Pharmacia Biotech).

RBCs lysis: Three milliliters of whole blood was added to 9 mL RBCs lysis solution. Incubation for 10 min at RT was done. Centrifugation for 10 min at 2000×g was done. The supernatant was removed, the visible white cell (WBCs) pellet was left behind with aresidual liquid of 100-200 µL. The WBCs in the residual supernatant were vortexed vigorously. Three milliliters of cell lysis solution was added, pipetting up and down was done to lyse the WBCs.

RNase trearment: Fifteen microliters of RNase A soluation was added to the cell lysate. The tubes were inverted 25 times and incubated at 37°C for 15 min to mix the sample. Centrifugation at 2000×g for 1 min was done, the ethanol was carefully poured off. The tube was drained on clean absorbent, the sample was allowed to air dry for 15 min.

DNA hydration: The DNA hydration solution (250 µL) was added to the DNA pellet. The DNA was allowed to rehydrate overnight at RT. The DNA was stored at -20°C till used in PCR.

Primer sequences: Two sets of Y specific Sequence Tagged Sites (STS) spanning the euchromatic region of Yq were tested in each patient. We used the SY254 and SY255 for amplification of the AZFc region (DAZ).

PCR amplification: Multiplex PCR was carried out in 50 µL of reaction volume containing 200 ng of genomic DNA extracted from peripheral blood cells. The PCR mixture was added to the DNA:

10x buffer	5 µL
$MgCl_2$ (3 mM)	6 µL
Primer 1_F (30 pmol)	1.5 µL
Primer 1_r (30 pmol)	1.5 µL
Primer 2_F (30 pmol)	1.5 µL
Primer 2_r (30 pmol)	1.5 µL
dNTPs (10 mM)	2.5 µL
Taq (2 U)	1 µL

Nuclease free water was added to final volume of 50 µL

Sequence of SY254	Forward	5'GGG TGT TAC CAG AAG GCA AA 3'
	Reverse	5'GAA CCG TAT CTA CCA AAG CAG C 3'
Sequence of SY255	Forward	5'GTT ACA GGA TTC GGC GTG AT 3'
	Reverse	5'CTC GTC ATG TGC AGC CAC 3'

PCR cycling condition:

94°C	For 5 min	One cycle
94°C	For 40 sec	35 cycles
56°C	For 40 sec	35 cycles
72°C	For 30 sec	35 cycles
72°C	For 10 min	One cycle

Agarose gel electrophoresis: About 3.5% agarose gel was prepared in TBE1x, stained with ethidium bromide. The PCR products were electrophoresed in tris-borade EDTA (TBE) buffer at RT using a voltage gradient of 8 V cm^{-1} for 30-60 min. Positive cases gave bands at 320, 123 bp for SY254 and SY255, respectively under UV transillumination.

Statistical analysis: Descriptive statistics were presented as Mean±Standard Deviation, as well as frequencies and percentages. Chi-square test and Fisher exact test for contingency table analysis.

RESULTS

The mean age of studied cases was 34.26 years, with mean duration of infertility 8.16 years. Normal FSH, LH and testosterone was found in 74, 96 and 88%, respectively. The AZF deletion was reported in 4 cases as shown in Table 1. Regarding relation to studied hormones and testicular size, there was significant association between AZF deletion with both FSH (p = 0.02) and LH (p = 0.025), while there was no significant association with testosterone (p = 0.44) or testicular size (p = 0.12) as shown in Table 2.

Table 1: Data of the studied cases

Parameters	Values
Age (years) (Mean±SD)	34.26±8.77
Duration of infertility (years) (Mean±SD)	8.16±6.64
Testicular size	
Normal	32 (64%)
Small	15 (30%)
Moderate	3 (6%)
Semen volume (mL) (Mean±SD)	2.914±1.0
Serum FSH (No., %)	
Normal	37 (74%)
High	13 (26%)
Serum LH (No., %)	
Normal	48 (96%)
High	2 (4%)
Serum testosterone (No., %)	
Normal	44 (88%)
Decreased	6 (12%)
AZF gene (No., %)	
Normal	46 (92%)
Deleted	4 (8%)
Site of DAZ deletions (No., %)	
SY254	3 (6%)
SY254 and SY255	1 (2%)
Normal	46 (92%)

Table 2: Distribution of vitamin D gene polymorphism in studied groups

	Normal (n = 46)		Deleted (n = 4)		
	No.	%	No.	%	p-value
FSH					
Normal	36	78.3	1	25	0.02*
High	10	21.7	3	75	
LH					
Normal	45	97.8	3	75	0.025*
High	1	2.2	1	25	
Testosterone					
Normal	40	87.0	4	100	0.44
Low	6	13.0	0	0	
Testicular size					
Normal	31	67.4	1	25	0.12
Small	12	26.1	3	75	
Moderate	3	6.5	0	0	

*Significant

DISCUSSION

Genetic factors contribute about 10-15% of male infertility. The Y chromosome microdeletions cannot be predicted cytogenetically or on the basis of clinical findings or on semen analysis. Thus, PCR-based Y chromosome screening for microdeletions is becoming necessary both for providing the correct diagnosis as well as for proper management/ counseling of these cases[11].

In the present study, AZF deletions (8%) were nearly equal to the result of Kleiman *et al.*[14], which was 6.7% and that of Girardi *et al.*[15], which was 7%.

Our results were slightly lower to another study done by Foresta *et al.*[16] who reported that 10-15% of subjects affected by azoospermia or severe oligozoospermia carry a deletion in one or more AZF regions, 60% of which involves AZFc. In contrast, the deletion involving the interval of Yq11 including DAZ was approximately 1.9%, in the study of Vog *et al.*[17], which was lower than the frequency of the present study.

The deletion frequency of 3% reported by Simoni *et al.*[18] of azoospermic and severely oligozoospermic men selected with similar criteria and analyzed using four oligonucleotide primer pairs is in good agreement with the data obtained by Vog *et al.*[17]. This relatively low incidence may reflect ethnic differences.

Foresta *et al.*[19] reported higher percent as AZFc associated with azoospermia was 54% and severe oligozoospermia was (46%). In contrast, Fu *et al.*[20] found that the rate of deletion was higher in severe oligozoospermia (14.3%) than in azoospermia (11%) possibly due to the fact that screening was to strict and the number of cases was too small to represent the true deletion rate.

Among the various studies, remarkable differences in the prevalence of microdeletions exist, ranging[21,22] from 1-35%, reflecting different patients selection criteria, ethnic differences, different number of cases and methodologies. Therefore, the actual incidence of clinically relevant microdeletions in infertile men is still unclear. In fact, male infertility is a heterogeneous diagnostic category that may be classified only on clinical and historical data or on seminological data (normo, oligo, azoospermia) and/or on testicular structure.

Also, Peterlin *et al.*[23] showed that the reported frequency of Y chromosome microdeletions varies from 1-55% which is largely related to different inclusion criteria. Writzl *et al.*[24] concluded that partial deletions of the DAZ genes are associated with oligozoospermia but not with azoospermia, however, an increased number of DAZ genes does not seem to be a statistically significant risk factor for spermatogenic failure.

On the other hand, most problems with the PCR technique in screening for microdeletions derived from the intrinsic nature of the Y chromosome, which largely consists of repetitive elements and gene families widely dispersed along the chromosome. Also, the number of STSs to be used for a first screening is not well established and it varies among the researchers. However, as a general rule, at least two or three STSs for each region should be used and if a deletion is found and confirmed, the number of STSs should be increased[19].

We would like to emphasize that these differences in the frequency of deletions and localization between different studies may be due to the inclusion of varying populations in a study or to the use of different STS primers or they may reflect genuine population variances such as particular Y chromosome haplotypes, genetic background or environmental influences. In the present study, only men with severe oligozoospermia were considered and the STS primers used were those recommended by the European Academy of Andrology.

In agreement with these results, a significantly higher value of plasma FSH was found in azoospermic than those in oligozoospermic males reported by El Awady *et al.*[25].

In contrast to our results, Song *et al.*[26] showed that the mean FSH level among patients with deletions (12.3 ± 4.3 IU L^{-1}) which did not differ significantly from patients without deletions (12.1 ± 9.1 IU L^{-1}).

In this study, FSH elevation was more marked in groups with gene deletion than the normal. Chiang *et al.*[27] matched with those results. Similarly, Frydelund-Larsen *et al.*[28] found elevated serum FSH levels in the majority of 16 patients with AZFc microdeletions compared with fertile subjects. The researchers inferred that a specific alteration of germ cells only partially influences sertoli cell function. However, Foresta *et al.*[16] suggested that sertoli cell function is not damaged in patients with AZFc-DAZ deletions and that the strong reduction of germ cells does not affect the FSH-inhibin B feedback loop and so patients with AZFc-DAZ deletions had only slightly elevated FSH and normal inhibin B plasma concentrations. Inhibin B responded normally during FSH treatment, supporting the hypothesis that Sertoli cells are not altered.

Present study showed 1 case of DAZ deletion with normal FSH level. These results coincided with Najmabadi *et al.*[29], Stuppia *et al.*[30] and Simoni *et al.*[18] who showed one patient with DAZ deletion and normal serum FSH level. Therefore, patients with normal FSH should not be excluded.

SaoPedro *et al.*[31] reported that all their 4 patients carrying Y chromosome deletions (DAZ) had elevated serum FSH. The LH and testosterone levels were within the normal range except one patient who presented with elevated LH and reduced testosterone level. This result was also in agreement with a study by Song *et al.*[26] reporting that testosterone levels were within the normal range in the 21 patients with deletions.

In the study the percent of normal testicular size in the deleted patients was (one patient) 25% Our results are in agreement with Chiang *et al.*[27] who reported that, the testicular size reduction was more marked in groups with gene deletion involving both AZFb and AZFc.

CONCLUSION

In conclusion, microdeletions of the Y chromosome in this study were 8% in severe oligozoospermia. This study confirmed that it is not possible to detect which men with testicular failure will have these deletions based on clinical parameters such as age, infertility duration, testicular volume, semen volume, testosterone and LH, as there was no statistically significant relation between any of these factors and the Y chromosome microdeletions. The detection of a deletion in an infertile man provides a proper diagnosis of the disease, allow the clinician to avoid empirical, unnecessary or often expensive treatments to improve fertility (e.g., hormonal treatments) and has important ethical consequences if the patient is a candidate for assisted reproduction techniques. A molecular diagnostic test of Y chromosome microdeletions should be at least performed in all men with a sperm concentration of $<5\times10^{6}$ mL^{-1} regardless of the presence of other apparent concomitant cause of testicular damage.

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