Y-Chromosome Microdeletions and their association with male factor infertility in Egyptian Patients

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Abstract: Background: Y chromosome microdeletions of the azoospermia factor regions (AZFa, AZFb, AZFc) are considered among the most important causes of male infertility. These microdeletions lead to spermatogenic failure. **Aim:** This study aims to assess the incidence of Y chromosome microdeletions in azoospermic and oligospermic Egyptian infertile males and to correlate Y microdeletions with histopathological patterns and testicular sperm extraction (TESE). **Patient and Methods:** Fifty infertile males were included. Semen analysis was performed according to WHO criteria. Y chromosome microdeletions were detected after genomic DNA extraction by a multiplex Polymerase Chain Reaction (PCR) covering 25 different sequence tagged sites (STSs) for AZFa, AZFb and AZFc in 5 different reactions mix to each patient. **Results:** Among 50 infertile males; 34/50 (68%) patients were azoospermic and 16/50 (32%) were oligospermic. Six/50 patients (12%) had detectable Y microdeletions with a total of 13 deleted STSs; 11/13 (85%) in AZFc versus 2/13 (15%) in AZFb. STSs deletions detected were SY158 (3/13, 23%), SY243 (2/13, 15%), SY166 (2/13, 15%) followed by SY143, SY117, SY277, SY273, SY254 and SY152; each detected in 1/13 (7%). Five/6 patients (83%) with Y microdeletions were azoospermic vs. 1/6 (17%) oligospermic patient. TESE result was only successful in 2/6 patients (33%) having AZFc deletions. **Conclusion:** The incidence of Y chromosome microdeletions in our studied population is similar to different ethnic reports. Screening of Y microdeletions is essential for appropriate genetic diagnosis in infertile males. AZFc microdeletions can help informed decisions regarding positive TESE outcome.

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1. Introduction

Infertility is defined as the inability of a couple to conceive after one year of unprotected intercourse [1]. Several causes can lead to male infertility including non-motile spermatozoa, varicocele, obstruction of spermatic ducts, hormonal imbalance and diabetes [2]. Fifteen percent of male infertility cases are idiopathic. Some of these patients were found to have detectable Y chromosome microdeletions in the Azoospermia factor (AZF) region which lead to spermatogenic failure [3]. Successful spermatogenesis is controlled by sex as well as autosomal genes. Therefore, deletions or mutations in spermatogenesis-related genes result in male infertility[4].

Sex-determining region Y (SRY) on the short arm of Y chromosome (Yp) carries genes that control sex differentiation[5]. Another region on the long arm (Yq) has been found to carry candidate genes implicated in spermatogenesis [6]. This region is known as the AZF region and has been associated with spermatogenesis and male infertility. Deletions of AZF region were first observed by Tiepolo et al in infertile males with azoospermia [7, 8].

By mapping deletions on Yq11, three subregions carrying candidate genes were identified within the AZF locus; AZFa, AZFb and AZFc [9]. Candidate genes located on the AZFc region include a cluster of four genes termed the Deleted in Azoospermia (DAZ) cluster and expressed solely in germ cells[10]. As for the AZFb region, it comprises a family of genes known as the RNA-binding motif (RBMY) family, the expression of which is also limited to the testis[11, 12].

Molecularly detected Y-chromosome microdeletions described in AZF locus indicate the susceptibility to spontaneous loss of spermatogenesis-related genes [13, 14].

This study aims to assess the incidence of Y-chromosome microdeletions in a population of idiopathic azoospermic and oligospermic infertile Egyptian males and to correlate these microdeletions with their hormonal profile, histopathology, and testicular sperm extraction (TESE) outcome.

2. Patients and Methods

Patients

Between May 2016 and January 2017, 50 infertile males who presented to the Andrology Department, Kasr El-Einy, Cairo University, were included. Their median age was 36.0 years (19-54). All-purpose clinical records and blood samples were obtained in addition to complete semen analysis according to WHO 2010 criteria [15]. All patients gave written informed consents. The study was approved by the Institutional Review Board.

Based on mean sperm concentrations, participants were categorized into 2 groups: Azoospermia (zero sperm count) and Oligozoospermia (<15 million sperm count/ul). Histological patterns of testicular biopsies were collected and divided into four different groups; Sertoli cell only syndrome, Primary spermatocyte arrest, Mixed sertoli pattern and Hypospermatogenesis [16]. Testicular sperm extraction (TESE) was performed to available patients with results recorded as positive or negative trial. Hormonal profile for each patient was assessed including: follicle-stimulating hormone (FSH), luteinizing hormone (LH) and testosterone levels and performed by enzyme immunoassay sandwich method with a final fluorescent detection (ELFA) [17]. Chromosomal banding analysis (Karyotype) was performed by G-banding techniques according to standard methods as previously described [18]. Patients with normal (46, XY) and abnormal (47, XXY) karyotypes were included.

B) Methods

Sample Collection: Two mL EDTA blood were withdrawn from each patient for DNA extraction.

DNA extraction: Genomic DNA was extracted using GentraPuregene Blood Kit *(Qiagen, Germany).* Nine hundred ul RBCs lysis solution was added to 300 ul of whole blood followed by mixing by inversion for 10 times; incubation for 1 min at room temperature (RT) 15–25°Cthen for 7-10 min at 2-8°C to ensure maximum RBCs lysis. Centrifugation for 2 min at 2000×g was performed. The supernatant was discarded by pipetting leaving approximately 10 ul of the residual liquid with visible white cell (WBCs) pellet. The residual supernatant containing WBCs was vortexed vigorously to resuspend the pellet.

Three hundred ul cell lysis solution was added to lyse WBCs and ensure that the solution is homogenous. 100 ul of protein precipitation solution were added after cell lysis to precipitate protein. The solution was vortexed vigorously for 20sec at maximum speed. Precipitated protein formed a tight dark brown pellet. The supernatant was transferred in a new tube containing 300 ul isopropanol and mixed 50 times by inverting until the DNA was visible as threads followed by centrifugation for 1 min at maximum speed. The supernatant was carefully discarded leaving the pellet in the tube.

300 ul 70% ethanol was added to the pellet followed by mixing several times in order to wash the DNA pellet followed by centrifugation for 1 min at maximum speed. Residual liquid was discarded and the tube was left opened for 5 min to ensure evaporation of ethanol. 50 ul of DNA hydration solution were added and tube was vortexed at medium speed. Tube was left for 5 min incubation at 37 °C to dissolve DNA. The DNA was stored at (-20 °C) till used for polymerase chain reaction (PCR) analysis.

Multiplex PCR for sequence tagged sites (STSs):

25 sequence tagged sites (STSs) were used in five multiplex PCR raections. Each multiplex set contained 5 pairs of different primer pairs *(Bio Basic Inc., Canada)* in order to cover euchromatic region of Yq11 (AZFa, AZFb and AZFc regions) where microdeletions might take place. The STSs primers sequence and appropriate amplicon size on ethidium bromide gel are summarized in table 1 [19]. Multiplex PCR reactions were prepared in five different mixes; I, II, III, IV and V.

Briefly, each PCR mix contained 500 ng of genomic DNA added to Hot Start Master Mix; My Taq™ HS Red Mix *(2x, Bioline, London, UK)* containing (10x Buffer, dNTPs&Hot Start enzyme) in addition to 0.8 uM of forward and reverse primers and adjusted with distilled water into a total reaction volume of 25ul.

Amplification was performed in a T-Personal thermal cycler *(BiometraGöettingen, Germany)***.**The amplification protocol was as follows: initial denaturation at 95°C for 10 min, consequent series of 45 cycles of amplification at 94°C for 45 sec (denaturation), 60°C for 1 min (annealing) and 72°C for 2 min (extension). A final extension was carried out at 72°C for 7 min. STS for SRY (sex-determining region on the Y-chromosome); SY14 was used as internal control primer to distinguish a negative result from a technical failure. Female genomic DNA for control of specificity and contamination was used as a negative control. No template control (NTC) was used to check for contamination in every PCR reaction.

Detection of amplified product usingethidium bromide gel electrophoresis:

2% agarose gel *(Gen Agarose L.E. Genaxxon bioscience GmbH Söflinger, Germany)*waspreparedin 1x TAE buffer *(Bio Basic Inc., Canada)*with 4 µl ethidium bromide. 12.5 µl of the final PCR product were electrophoresed with Gene DireX® 50 bp ladder*(RTU, GeneDirex Co)* on a Multi SUB

Horizontal Gel Systems andnano PAC-300 *(Cleaver Scientific LTD, United Kingdom)*connected to supply voltage power adjusted at 160V for 25 min at RT. The gel was visualized under ultraviolet light (UV)transilluminator *(Spectroline, New York).*
Figure (1) shows ethidium bromide gel electrophoresis of a normal patient. Figure (2) shows a patient with SY158 and SY277 deletions

Statistical analysis:

Data were analyzed using Statistical Package of Social Science win statistical package version 23(SPSS Inc., Chicago, IL). Numerical data were expressed as mean and standard deviation (SD) or median and range as appropriate. Qualitative data were expressed as frequency and percentage.Chi-square test was used to examine the relation between qualitative variables.

Table 1: Multiplex primer sets covering 25 STSs used for detecting Y microdeletions

Multiplex set	STSs	Forward primer	Reverse primer	Amplicon size (bp)
Multiplex PCR I	sY272	GGTGAGTCAAATTAGTCAATGTCC	CCTTACCACAGGACAGAGGG	93
	sY152	AAGACAGTCTGCCATGTTTA	ACAGGAGGGTACTTAGCAGT	125
	sY132	GAGAGTCATAATGCCGACGT	TGGTCTCAGGAAGTTTTTGC	143
	sY84	AGAAGGGTCTGAAAGCAGGT	GCCTACTACCTGGAGGCTTC	326
	sY14	GAATATTCCCGCTCTCCGGA	GCTGGTGCTCCATTCTTGAG	472
Multiplex PCR II	sY269	CTCTGGGACAAGTGTTCCTTG	CATTGGCATGAATGTGTATTCA	94
	sY139	TTCAGAGGAATCATGTGGGT	AATGTTTCATCACCATTATCCC	120
	sY153	GCATCCTCATTTTATGTCCA	CAACCCAAAAGCACTGAGTA	139
	sY138	CACATGAAGCACTGGAACTG	AGGGCCTGAGTCTCCAGG	170
	sY155	ATTTTGCCTTGCATTGCTAG	TTTTTAAGCCTGTGACCTGG	349
Multiplex PCR III	sY255	GTTACAGGATTCGGCGTGAT	CTCGTCATGTGCAGCCAC	126
	sY144	TCATCTGCCACCATCAACAT	ACGTGTTTCTACACCTGCCC	143
	sY160	TACGGGTCTCGAATGGAATA	TCATTGCATTCCTTTCCATT	236
	sY143	GCAGGATGAGAAGCAGGTAG	CCGTGTGCTGGAGACTAATC	311
	sY254	GGGTGTTACCAGAAGGCAAA	GAACCGTATCTACCAAAGCAGC	350
Multiplex PCR IV	sY273	GGTCTTTAAAAGGTGAGTCAAATT	AGACAGAGGGAACTTCAAGACC	93
	sY243	GTTTCTTCATAAGCAACCAAATTG	CAGATTATGCCACTGCCCTT	118
	SPGY	TTTCACATACAGCCATTAAGTTTAGC	CAATTTTGATAGTCTGAACACAAGC	400
	sY164	AATGTGCCCACACAGAGTTC	TGGAAGACCAGGATTTCATG	590
	RBM1	ATGCACTTCAGAGATACGG	CCTCTCTCCACAAAACCAACA	800
Multiplex PCR V	sY166	GAACTCCAATCATTCCCTGA	TTGGCTCTACTTTTCCCCTT	115
	sY150	GGGAGAGTCACATCACTTGG	TTGAATTATCTGCCTGAGTGC	158
	sY158	CTCAGAAGTCCTCCTAATAGTTCC	ACAGTGGTTTGTAGCGGGTA	231
	sY117	GTTGGTTCCATGCTCCATAC	CAGGGAGAGAGCCTTTTACC	262
	sY277	GGGTTTTGCCTGCATACGTAATTA	CCTAAAAGCAATTCTAAACCTCCAG	310

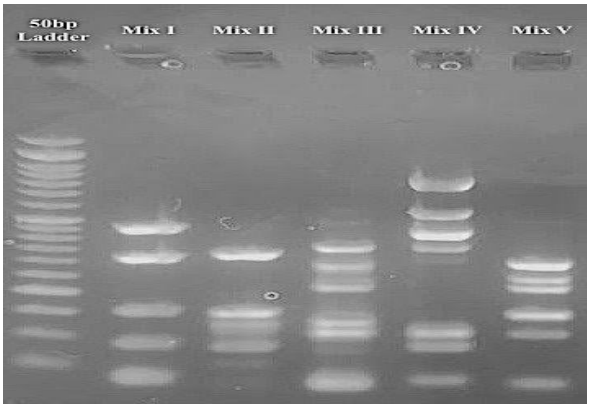

Figure (1): Ethidium bromide gel electrophoresis of a normal patient showing 25 STSs.

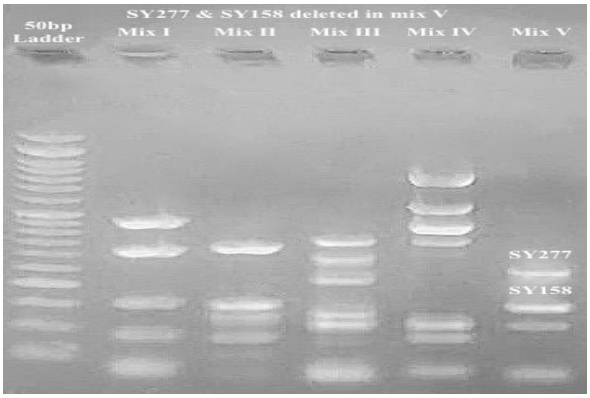

Figure (2): Ethidium bromide gel electrophoresis of a patient with SY158 and SY277 deletions

3. Results:

Fifty infertile males with a median age of 36.0 years (19-54) were included in this study. Based on semen analysis, patients were divided into two groups; non-obstructive azoospermia 34/50 (68%) and oligozoospermia 16/50 (32%). Twenty nine/50 patients (58.3%) showed normal FSH level while 21/50 patients (41.7%) showedhigh FSH level. Twenty-five/50 (50%) of patients had high LH while the other 50% had normal LH level. Twenty-six/50 (52%) of examined patients had low testosterone level while 24/50(48%) had elevated testosterone level. TESE was performed for 35 candidate patients and gave a positive result in 11 (31%) patients. Table 2 shows patients characteristics.

Table 2: Patients characteristics

Parameters	
Age (years) (Mean ± SD)	35.64 ± 6.72
Semen analysis (N,%)	
Azoospermia	34 (68%)
Oligozoospermia	16 (32%)
FSH (N, %)	
Normal	29 (58.3%)
High	21 (41.7%)
LH (N, %)	
Normal	25 (50%)
High	25 (50%)
Testosterone (N, %)	
Low	26 (51.5%)
High	24 (48.5%)
Testicular sperm extraction (N,%)	
Positive	11 (31.4%)
Negative	24 (68.4%)
Karyotype (N,%)	
Normal	36 (90%)
Abnormal	4 (10%)

Y chromosome microdeletions:

Y chromosome microdeletions were detected in six patients; 6/50 (12%). A total of 13 deleted STSs were detected; 2 in AZFb and 11 in AZFc versus no STS deletions detected in AZFa. STS deletions were; SY158 3/13 (23%), SY243 2/13 (15%), SY166 2/13 (15%) followed by SY143, SY117, SY277, SY273, SY254, SY152 each detected in 1/13 (7%). Five/6 (83%) patients with microdeletions were azoospermic versus1/6 (17%) oligospermic patient. Table 3 shows distribution of Y microdeletions in relation to histopathological patterns and table 4 shows distribution of different STS microdeletions in relation to semen analysis, patterns of testicular biopsies and TESE. Among 6 patients with Y microdeletions, 5/6 (83%) presented with normal FSH level vs. 1/6 patient (17%) who presented with high FSH level. Four/6 patients (67%) presented with normal LH level vs. 2/6(33%) with high LH level. Four/6 patients (67%) had low testosterone level vs. 2/6 (33%) who presented with normal testosterone level. Table 5 shows median hormone levels in relation to Y chromosome microdeletions.

Cytogenetic analysis revealed that 5/6 patients (83.3 %) had a normal karyotype (46, XY) vs.1/6 patient (16.7 %) with 47, XXY. Finally, 4/6 patients (66.7%) gave a negative TESE result versus 2/6(33.3%) patients with AZFc deletions revealed positive TESE outcome.

4. Discussion

Y-chromosome microdeletions are considered nowadays a potential genetic cause of male infertility[3]. In our study,6/50 (12%) patients had Ymicrodeletions. Previous studies reported different frequencies in Y microdeletions; 24.2% [20], 50%[21], 12%[22], 5% [23], 5.2%[2], 5%[24] and16.87%[25]. However, most studies revealed an incidence of less than 10% [2, 23, 24, 26].

Table 3: Distribution of Y chromosome microdeletions in relation to histopathological patterns

		Histological patterns of testicular biopsies			
		Sertoli cellonly syndrome (n:17)	**Primary spermatocyte arrest (n:11)**	**Mixed sertoli pattern (n:4)**	**Hypospermato-genesis (n:4)**
Patients with Y-chromosome microdeletions (N=6)	Number	1	2	1	2
	N (%) within Y microdeletions	1/6 (16.7%)	2/6 (33.3%)	1/6 (16.7%)	2/6 (33.3%)
	N (%) Within hisopathology	1/17 (5.9%)	2/11 (18.2%)	1/4 (25.0%)	2/4 (50%)

Table 4: Distribution of different STS deletions in relation to semen analysis, patterns of testicular biopsies and TESE.

	Semen Analysis	Testicular biopsy	TESE	AZFb		AZFc						
				SY143	SY117	SY166	SY243	SY277	SY273	SY158	SY254	SY152
Case 1	Azoospermia	Hypospermatogenesis	-ve					+		+		
Case 2	Azoospermia	Primary spermatocyte arrest	-ve	+		+				+	+	
Case 3	Azoospermia	Mixed sertoli pattern	+ve				+			+		
Case 4	Azoospermia	Sertoli cell only syndrome	-ve				+		+			+
Case 5	Azoospermia	Primary spermatocyte arrest	-ve		+							
Case 6	Oligozoospermia	Hypospermatogenesis	+ve			+						

Table5: Median hormone levels in relation to patients with Y chromosome microdeletions

Group		Testosterone Hormone	Follicle Stimulating Hormone	Luteinizing Hormone
Patients with Y-microdeletions	Mean±SD Median (Range)	3.33±2.43 2.9 (0.9-3.2)	13.41±13.35 9.5 (4-40)	8.361± 6.9 6.9 (2.47-22)
Patients without Y-microdeletions	Mean±SD Median (Range)	2.67±1.150 2.8 (0.7-5)	18.78±13.28 13 (3.7-57)	9.21± 5.9 8 (2.4-25)
p		0.745	0.262	0.531

Variation in the reported frequencies of Yq microdeletions could be related to the difference in ethnicity and sample size variability. In our study, we used a multiplex PCR technique detecting 25 different STSs in 5 multiplex PCR reactions for each patient. Using a higher sensitivity technique leads to increase in the detection limit of Y microdeletions which explains the difference between our results and previous studies that reported a lower incidence of microdeletions when using PCR technique detecting 6 STSs only[2].

Eighty-five percent of Y microdeletions in our study were detected in the AZFc (11/13 STS) and only 15% of microdeletionswere identified in AZFb (2/13). Our data are in concordance with most of international reports who previously stated that the majority of Y microdeletions are located in AZFc.Asadi et al.reported 70.7%[2] and Naasseet al reported 75% [27].

In our study, among 34 azoospermic patients, 5/34 (15%) had detectable Y chromosome microdeletions vs. only 1/16 patients (6%) in the oligospermic group. Supporting our results, previous reports revealed a comparable incidence of 13.5% and 12.8% in azoospermic patients and 8% and 8.8%in oligospermic patients[4, 28].

Regarding histopathological patterns;2/6 patients (33%) showed a hypospermatogenic pathology, 2/6 (33%) showed C1 arrest while 1/6 (16.7%) patient was sertoli cell only and 1/6 (16.7%) showed mixed sertoli testicular pathology. It was previously stated that AZF deletions were associated with altered testicular histological characteristics which ranged from sertoli cell only to hypospermatogenesis[2].

Within patients having Y microdeletions; 5/6(83%), 4/6 (67%) and 2/6 (33%) showed normal FSH, LH and testosterone levels, respectively. A previous report revealed normal FSH, LH and testosterone levels in 36% of patients with Y microdeletions [2].

TESE extraction revealed two successful trials while four trials were negative. One patient was Azoospermic while the other patient was oligospermic. Both patients had Y microdeletions in AZFc which indicates that successful TESE outcome is possible with microdeletions in AZFc. However, none of patients with AZFb deletion gave a positive outcome which was in agreement with Stahl et al.[29].

In conclusion, this study postulates a frequency of Y chromosome microdeletions similar to most studied populations worldwide. Routine screening of Y chromosome microdeletions should be an important practice and a prerequisite for appropriate genetic counseling in infertile males. Accurate detection of Y microdeletions can help informed decisions regarding treatment options.

Conflict of interest

The authors declare no conflict of interest.

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