Detection of Y-Chromosome microdeletions in Egyptian infertile males

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Abstract

The major genetic causes in male infertility are chromosomal abnormalities and Y chromosomal microdeletions (YCMs). YCMs occur in approximately 15% of azoospermic patients and 10% of severe oligospermic patients. These microdeletions lead to spermatogenic failure.

This study aims to report the incidence of Azoospermia factor (AZF) microdeletions in Egyptian infertile males with severe oligoospermia & non obstructive azoospermia (NOA) using multiplex PCR.

One hundred-fifty infertile males were included. Semen analysis, hormonal assay, karyotyping, testicular sperm extraction and testicular biopsy were performed.Y chromosome microdeletions were detected by using multiplex polymerase chain reaction (PCR).

Among 150 infertile males; Considering Y chromosome; in severe oligospermic infertile males 3/36 (8.3%) had Y chromosome microdeletions in AZF subregions where; 1/3(33.3%) showed deletions in AZF-c and 2/3(66.7%) showed deletions in both AZF-b+c. However; no deletions were detected in AZF-a region in this group. In NOA group, 21/114(18.4%) had Y chromsome microdeletions in AZF subregions where; 1/21 (4.8%) showed deletions in AZF-b region, 2/21 (9.5%) showed deletion in both of AZF-a+b+c regions, 8/21 (38%) showed deletions in AZF-c region only and 10/21 (47.6%) showed deletions in both AZF-b+c regions.

Conclusion: The frequency of Y chromosome microdeletions in our studied patients was similar to many ethnic reports. Detection of AZF microdeletions is necessary for proper genetic diagnosis in infertile males. AZFc can help informed decisions regarding positive testicular sperm extraction outcome.

Keywords: Male infertility, Y chromosome microdeletions, AZF, NOA, TESE

1. Introduction:

Infertility is a major problem affects approximately 15% of couples around the world [1]. The failure of conception after 12 months of unprotected intercourse is known as infertility [2**].** Male infertility divided into primary infertility and secondary infertility [3]. Major causes of infertility are idiopathic infertility, testicular failure, obstruction, cryptorchidism, low semen volume, sperm agglutination, varicocele, erectile or ejaculatory dysfunction, abnormal viscosity, endocrine disorder, high density of sperm, environmental causes and genetic abnormalities [4]. Genetic factors play well-recognized roles in male infertility, and genetic alterations of the Y chromosome are especially important [5].

Chromosomal anomalies and Y chromosomal microdeletions (YCMs) are the main genetic

factors in male infertility. YCMs occur in approximately 15% of azoospermic patients and 10% of severe oligospermic patients, and are frequently found at the azoospermia factor (AZF) locus in the q11.23 band**.** The AZF locus was subdivided by molecular analyses into three (probably Four) sub regions: AZFa, AZFb and AZFc, with a fourth probable AZFd region [6]. AZFc region is haboring Candidate genes that include a cluster of four genes termed the Deleted in Azoospermia (DAZ) cluster and expressed only in germ cells [7].

As for the AZFb region, it includes a family of genes known as the RNA-binding motif (RBMY) family whose expression is also restricted to the testis [8, 9, 10, 11]. Microdeletions in these regions lead to different degrees of spermatogenetic failure; although, the exact genotype phenotype relationship of microdeletions and AZF gene function and infertility in the AZF locus have not been fully discovered [12].

Genes that control sex differentiation are carried on Sex-determining region Y (SRY) present on Y chromosome short arm (Yp). Other genes affecting spermatogenesis have been found on another region on (Yq), known as AZF which has been correlated to male infertility. AZF deletion were first observed by in azoospermic infertile males [11]. AZF microdeletions reveals the possibility of spermatogenesis related genes spontenous loss [12, 13]

The purpose of this work was to report the incidence of AZF microdeletions in Egyptian infertile males with severe oligoospermia and non obstructive azoospermia (NOA) using multiplex PCR.

2. Patients and Methods

Between May 2016 and October 2018, 150 infertile males who presented to the Andrology Department, Kasr El-Einy, Cairo University, were included. Their median age was 36.0 years (19-54). The required documents are submitted according to the committee guidelines & have been received &showed by Central Directorate of Research & Health Development and the committee decision is Approval Com. No/Dec. No: 5-2019/4

All-purpose clinical records and blood samples were obtained in addition to complete semen analysis according to WHO 2010 criteria [14]. Based on semen analysis, participants were classified into two groups: Non obstructive azoospermia (zero sperm count) and severe Oligozoospermia (<5 million sperm count$/\mu l$). Testicular biopsies were collected and divided into four different groups; Mixed sertoli pattern , Primary spermatocyte arrest, Sertoli cell only syndrome and Hypospermatogenesis [15]. Hormonal profile for each patient was evaluated including: follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone levels and performed by enzyme immunoassay sandwich method with a final fluorescent detection (ELFA)[11]. Karyotyping was performed by G-banding techniques according to standard methods as described by [16]. Patients with normal (46, XY) and abnormal Klienfilter's syndrome (47, XXY) karyotypes were included. Testicular sperm extraction (TESE) was performed for some patients with results recorded as positive or negative trial.

2.1 Specimen:

A- Blood

a. 3ml whole blood on ethylene diamine tetra acetic acid (EDTA) tube for DNA extraction and multiplex PCR screening of Y-chromosome microdeletions;

b. 6ml whole blood divided into two lithium heparin tubes 3ml per each one (duplicate) for karyotyping to detect any chromosomal aberration.

c. 3ml whole blood on plain tube was left for 15 minutes at water bath then centrifuged and serum was withdrawn for hormonal assay (FSH, LH, and T. TT).

B-Semen:

For all 150 Seminal analyses were done to classify the infertility case into two main groups; whether Non-obstructive Azoospermia (NOA) or severe oligospermia.

Semen samples were collected by masturbation in sterile cups following 3 days with no sexual intercourse. Semen samples were let to liquefy for 20 minutes, and then volume, concentration, total spermatic count, motility, and abnormalities were evaluated.

C-Testicular biopsy

Some patient underwent testicular pathology, was done for NOA infertile males to reflect spermatogenesis state and determine to which category it belongs whether sertoli cell only syndrome (SCOS), Maturation arrest, mixed sertoli or hypospermatogeneis Patients' samples were sent to histopathology lab and results were recorded.

2.2 DNA extraction:

Genomic DNA was extracted using Gentra Puregene Blood Kit (Qiagen, Germany). 900 μl RBCs lysis solution was added to 300 μl of whole blood followed by mixing by inversion for 10 times.

2.3 Multiplex PCR for sequence tagged sites (STSs):

Polymerase chain reaction (PCR) amplification using 25 sequences tagged sites (STSs) within the AZF region of Yq11 were used in five multiplex PCR sets for AZF microdeletions detection of the Y chromosome. Each multiplex set contained five pairs (forward and reverse) of different designed primer (Bio Basic Inc., Canada).in order to cover euchromatic region of Yq11 (AZFa, AZFb and AZFc regions) where microdeletions might take place. Multiplex polymerase chain reactions were prepared in five different mixes; A, B, C, D and E

Briefly, each PCR mixes contained 500 ng genomic DNA added to hot start master mix MyTaq™ HS Red Mix (2x, Bioline, London, UK)containing (10x Buffer, dNTPs& hot start enzyme) in addition to 0.8 μM of forward and reverse primers and adjusted with distilled water into a total reaction volume of 25 μl.

Amplification was performed in a T-Personal thermal cycler (Biometra Göettingen, Germany)**.**The amplification protocol was as follows: initial denaturation at 95°C for 10 min, a consequent series of 45 cycles 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. A STS for SRY (sex-determining region on the Y-chromosome SRY gene) sY14 was used as a control primer to distinguish a negative result from a technical failure. Female genomic DNA which controls for specificity and contamination was used as a negative control. No template control (NTC) was used to check for contamination in every PCR reaction.

Table 1: Multiplex sets containing 25 sequences tagged sites (STSs) used for detecting Y microdeletions

Multiplex set	STSs	Left primer	Right primer	Fragment size in (bp)
Multiplex PCR I	sY272 sY152 sY132 sY84 sY14	GGTGAGTCAAATTAGTCAATGTCC AAGACAGTCTGCCATGTTTAGAGAGTCATAATGCCGACGT AGAAGGGTCTGAAAGCAGGT GAATATTCCCGCTCTCCGGA	CCTTACCACAGGACAGAGGG ACAGGAGGGTACTTAGCAGT TGGTCTCAGGAAGTTTTTGC GCCTACTACCTGGAGGCTTC GCTGGTGCTCCATTCTTGAG	93 125 143 326 472
Multiplex PCR II	sY269 sY139 sY153 sY138 sY155	CTCTGGGACAAGTGTTCCTTG TTCAGAGGAATCATGTGGGT GCATCCTCATTTTATGTCCACACATGAAGCACTGGAACTG ATTTTGCCTTGCATTGCTAG	CATTGGCATGAATGTGTATTCA AATGTTTCATCACCATTATCCC CAACCCAAAAGCACTGAGTA AGGGCCTGAGTCTCCAGG TTTTTAAGCCTGTGACCTGG	94 120 139 170 349
Multiplex PCR III	sY255 sY144 sY160 sY143 sY254	GTTACAGGATTCGGCGTGAT TCATCTGCCACCATCAACAT TACGGGTCTCGAATGGAATA GCAGGATGAGAAGCAGGTAG GGGTGTTACCAGAAGGCAAA	CTCGTCATGTGCAGCCAC ACGTGTTTCTACACCTGCCC TCATTGCATTCCTTTCCATT CCGTGTGCTGGAGACTAATC GAACCGTATCTACCAAAGCAGC	126 143 236 311 350
Multipl	sY273 sY24	GGTCTTTAAAAGGTGAGTCAAATT GTTTCTTCATAAGCAACCAA	AGACAGAGGGAACTTCAAGACC CAGATTATGCCACTGCCCT	93 118 400

ex PCR IV	**3 SPGY sY164 RBM1**	**ATTG TTTCACATACAGCCATTAAG TTTAGC AATGTGCCCACACAGAGTTC ATGCACTTCAGAGATACGG**	**T CAATTTTGATAGTCTGAAC ACAAGC TGGAAGACCAGGATTTCAT G CCTCTCTCCACAAAACCAA CA**	**590 800**
Multiplex PCR V	**sY166 sY150 sY158 sY117 sY277**	**GAACTCCAATCATTCCCTGA GGGAGAGTCACATCACTTG G CTCAGAAGTCCTCCTAATAG TTCC GTTGGTTCCATGCTCCATAC GGGTTTTGCCTGCATACGTA ATTA**	**TTGGCTCTACTTTTCCCCTT TTGAATTATCTGCCTGAGT GC ACAGTGGTTTGTAGCGGGT A CAGGGAGAGAGCCTTTTAC C CCTAAAAGCAATTCTAAAC CTCCAG**	**115 158 231 262 310**

2.4 Detection of amplified product using gel electrophoresis:

A 2% agarose gel (GenAgarose L.E. Genaxxon bioscience GmbH Söflinger, Germany**)** was prepared in 1x TAE buffer (Bio Basic Inc., Canada) mixed with 4 µl ethidium bromide. 12.5 µl of the final PCR product were electrophoresed with Gene DireX® 50 bp ladder RTU (ready to use) by GeneDirex, Co. on a MultiSUB Horizontal Gel Systems andnanoPAC-300 (Cleaver Scientific LTD, United Kingdom) connected to supply voltage power which was adjusted at 160V for 25 min at room temperature. The gel was visualized under ultraviolet light of UV trans-illuminator (Spectroline, New York).

3. Results:

One hundred fifty infertile males were included in this study with a median age of 36.0 years (19-56). Based on semen analysis, patients were divided into two groups; non-obstructive azoospermia 114/150 (76%) and severe oligozoospermia 36/150 (24%). TESE was performed for 102/150(68%) patients, 25/150 had a successful TESE (16.7%). Karyotyping was performed for 26/36(72.2%) of oligospermic and 74/114 (65%) of NOA patients, results showed both normal karyotype (46, XY) and abnormal karyotype (47, XXY) where Klinefelter's syndrome.

Table 2: Patients characteristics

Parameters	Percentages
Age (years) (Mean ± SD)	
NOA	35.17± 7.85
Severe oligospermia	35.72± 6.48
Semen analysis	
NOA	114 (76%)
Severe Oligozoospermia	36(24%)
FSH	
NOA	14.70± 9.45
Severe oligospermia	14.32± 7.93
LH	
NOA	9± 5.27
Severe oligospermia	7.75± 3.85
Testosterone	
NOA	3.47± 2.45
Severe oligospermia	3.16± 2.23
Testicular sperm extraction	
Positive	25 (16.7%)
Negative	77 (75.3%)
Karyotype	
Normal	83 (83%)
Abnormal	17 (17%)

Y chromosome microdeletions:

Y chromosome microdeletions were detected in twenty-four patients 24/150 (16%) had microdeletions; 3/150(2%) had severe oligospermia while 21/150(14%) had NOA. A total of 15 deleted STS were detected; we used 25 STSs for AZF microdeletions detection, 7/25(28%) deleted STSs of oligospermic group were detected. In NOA group, a total of 15/25 (60%) deleted STS were detected as shown in details in table (3) & (4)

Table 3: distribution of different STS microdeletions in 21/114 (18.4%) of NOA.

No. of cases 21/114	AZF-a	AZF-b	AZF-c
1			Sy277, Sy158
2		Sy143	Sy254, Sy158, Sy166
3			Sy243, Sy158
4			Sy152, Sy243, Sy273
5		Sy117	

6			**Sy166**
7	**Sy84**	**Sy117**	**Sy277, Sy243**
8		**Sy117, Sy132**	**Sy277, Sy243, Sy158, Sy272, Sy273, Sy152**
9		**Sy117**	**Sy277, Sy243, Sy144, Sy254**
10		**Sy117**	**Sy277**
11			**Sy273**
12			**Sy273**
13			**Sy273**
14		**Sy117**	**Sy254, Sy158, Sy273, Sy160**
15	**Sy84**	**Sy117, Sy132**	**Sy254, Sy277, Sy243, Sy158, Sy272**
16		**Sy117, Sy132**	**Sy277, Sy243, Sy272**
17		**Sy117, Sy132**	**Sy255, Sy277**
18		**Sy117**	**Sy277, Sy243, Sy158, Sy272**
19		**Sy117**	**Sy277**
20			**Sy277**
21		**Sy117**	**Sy277, Sy243, Sy273**

Table 4: The distribution of AZF microdeletions in subregions a, b and c in both groups.

Groups / **AZF**	**Azoospermia factor-AZF**			
	AZF-b	**AZF-c**	**AZF-b+c**	**AZF-a+b+c**
Severe Oligoospermia		**1/3 (33.3%)**	**2/3 (66.7%)**	
NOA	**1/21 (4.8%)**	**8/21 (38%)**	**10/21 (47.6%)**	**2/21 (9.5%)**

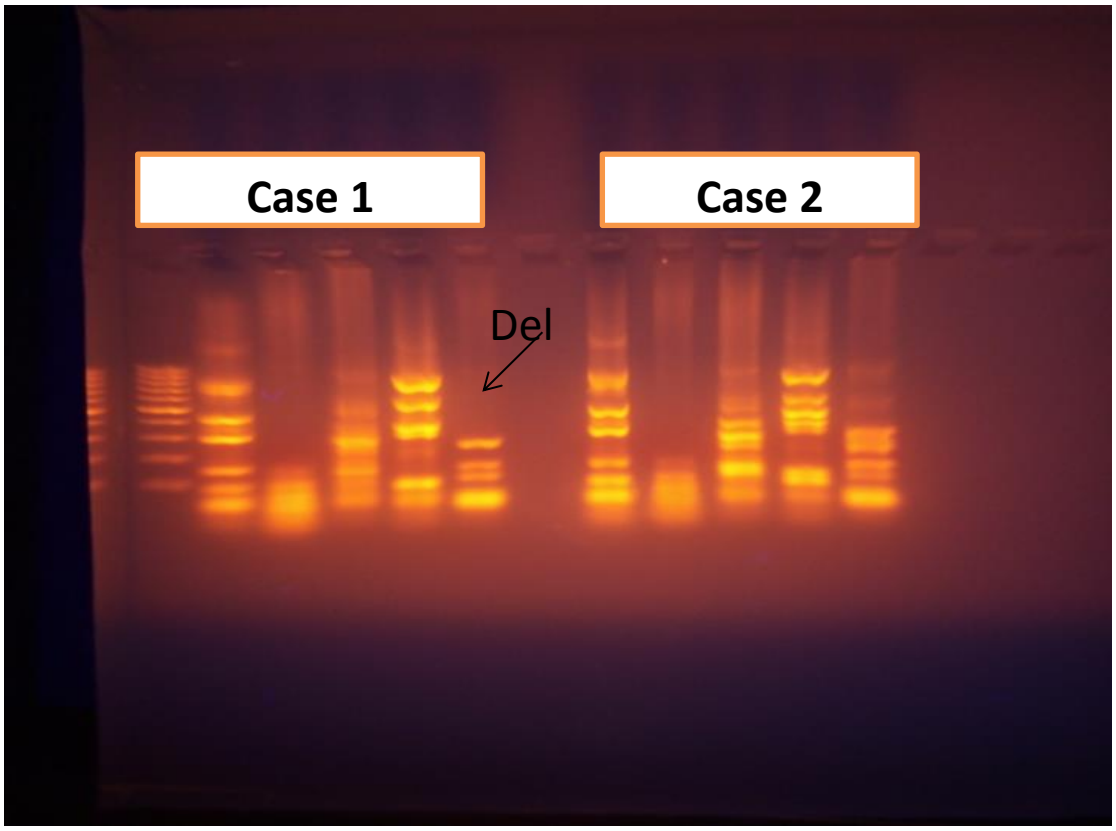

Figure 1: Gel electrophoresis for included infertile male with Y-Chromsome microdeletions in Case 1 showed deletion in Sy277 and sY117 while Case 2; showed normal infertile male.

Table 5: The distribution of Y microdeletions correlated to Karyotyping and TESE.

Groups	Karyotyping		TESE		Y chromosome microdeletions	
	Normal (46, XY)	Abnormal Klienfilter's syndrome (47, XXY)	Negative	Positive	No deletion	Microdeletion
Severe Oligoospermia	20/26 (77%)	6/26 (23%)	5/8 (62.5%)	3/8 (37.5%)	33/36 (91.7%)	3/36 (8.3%)
NOA	63/74 (85%)	11/74 (15%)	72/94 (76.6%)	22/94 (23.4%)	93/114 (81.6%)	21/114 (18.4%)
N (%) within Y micro deletion	9/12 (75%)	3/12 (25%)	17/19 (89.5%)	2/19(10.5 %)		

Table 6: Distribution of Y microdeletions correlated to histopathological patterns

		Histological patterns of testicular biopsies			
		Sertoli cell only syndrome	**Primary spermatocyte arrest**	**Mixed sertoli pattern**	**Hypospermatogenesis**
Deletions in Y-chromosome	**Number**	**7**	**7**	**4**	**3**
	N (%) within Y micro deletion	**1/6 (16.7%)**	**2/6 (33.3%)**	**1/6 (16.7%)**	**2/6 (33.3%)**

Correlation of Y chromosome microdeletions with Hormonal assesment:

In oligoospermic group, FSH with 10.60± 0.95 mIu/ml, LH with 6.40± 1.98) mIu/ml and total testosterone with 6.05± 3.39mIu/ml while in NOA group FSH with 11.85±6.78mIu/ml, LH with 7.187±3.75mIu/ml and total testosterone with4.29±2.76mIu/ml.

Statistical analysis showed there was no significant difference in hormonal level between both groups.

4. Discussion

Nowadays Y-chromosome microdeletions are considered a potential genetic cause of male infertility [3]. In our study, 24/150 (16%) patients had microdeletions. Previous studies reported different frequencies in Y microdeletions; 24.2% [17], 50% [18], 12% [19], 5% [20], 6% [21],5.2% [22],[5.4%[23] , 5% [24]. 16.87% [25].

Variation in the reported incidences of Yq microdeletion could be due to the difference in sample size variability and ethnicity. In our study, we used a multiplex PCR technique detecting 25 different STS in 5 multiplex PCR reactions for each patient. Using a higher sensitivity technique leads to increase in detection limit of Y microdeletions which explains the difference between our results and previous studies that reported a lower incidence of microdeletions due to using PCR technique detecting 6 STS [22], 10 STS[26]

Fourty seven percent of Y microdeletions in our study were detected in the AZFb+c, Thirty eight percent of microdeletions were identified in AZF-c patients, 9.5% in AZF-a+b+c and only 4.8%in AZF-b. Our data are showed that the highest incidence in Y microdeletions was in AZF-b+c; most of international reports revealed that, deletions of AZFc were at highest frequency Deletions in AZFc region was the most frequent 75% [27], (48.1%) [28], (46.6 %) [29].Variation in the reported frequencies could be related to the difference in ethnicity and sample size variability.

In our study, among 114 azoospermic patients, 21/114 (18.4%) had detectable Y chromosome microdeletions vs. only 3/36 patients (8.3%) in the oligospermic group. Supporting our results, previous reports revealed a comparable incidence of 16% 13.5% , 12.8% ,10% and 6.4% in azoospermic patients and1.5%, 8%,8.8% ,2% and 5.8% in oligospermic patients).[23**,** 31, 30,21, 29] respectively.

According to Asadi et al., 2017 [22] Concerning histopathological patterns; 3/21 patients (14.2%) showed a hypospermatogenic pathology, 7/21 (33.3%) showed C1 arrest while 7/21 (33.3%) patient was sertoli cell only and 4/21 (19%) 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. Within patients having Y microdeletion; 5/6 (83%), 4/6 (67%) and 2/6 (33%) showed normal FSH, LH and testosterone, respectively. A previous report revealed a normal FSH, LH and testosterone levels in 36% of patients with Y microdeletions.

TESE extraction showed two successful trials while seventeen trails were negative. Both patients were NOA and had AZFc microdeletions which indicates that AZFc is a god prognosis for successful TESE outcomes.

5. Conclusion:

We can conclude that the frequency of Y chromosome microdeletions in our studied patients was similar to many ethnic reports. Detection of AZF microdeletions is necessary for proper genetic diagnosis in infertile males. AZFc can help informed decisions regarding positive TESE outcome.

6. Conflict of interest:

The authors declare no conflict of interest.

7. References:

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الملخص باللغة العربية

دراسة مسحية لتعيين الحُذف الجينية الدقيقة للكروموسوم الصبغي الجنسي في الرجال المصريين العقماء بأستخدام تفاعل البلمرة المتسلسل المتعدد

الخلفية: من الأسباب الرئيسية الوراثية في العقم عند الرجال هي الخلل الكروموسومي والحُذف الجينية الدقيقة للكروموسوم الصبغي الجنسي في الرجال.هذه الحُذف الجينية تحدث في ما يقرب من ١٥ ٪ من المرضى الذين يعانون من عدم وجود حيوانات منوية أو وجودها بنسبه قليله جدا في السائل المنوى و ١٠ ٪ من مرضى قلة سكر الدم الحاد. هذه الحُذف الجينية الدقيقة تؤدي إلى فشل في الخلايا المسئولة عن أنتاج الحيوانات المنوية.

طرق البحث: أجري البحث علي ١٥٠ من الرجال المصابين بالعقم وتقسيمهم ألي مجموعتين أساسيتين :المجموعه الأولي تمثل قله شديدة في عدد الحيوانات المنوية (<٥ ملايين عدد الحيوانات المنوية / مل) والمجموعه الثانية تمثل عدم وجود حيوانات منوية ، تم عمل تحليل السائل المنوي، فحص هرموني،تحليل كروموسومات (ل ١٠٠ رجل) وعملية أستخراج للحيوانات المنوية من الخصية (ل ١٠٢ رجل) .

النتائج: من بين ١٥٠ من الذكور المصابين بالعقم. كان ١٥٠/٣٦ (٢٤ ٪) من المرضى الذين يعانون من قله شديدة في عدد الحيوانات المنوية و ١٥٠/١١٤ (٧٦ ٪) من المرضى الذين يعانون من عدم وجود حيوانات منوية. بالنسبة للفحص الهرموني: لم تكن هناك فروق ذات دلالة إحصائية في المستويات الهرمونية بين المجموعتين ، أظهر ٢٦/٢٠ (٧٧٪) من المجموعه الأولي في التحليل الكروموسومي (٤٦ ، XX) بينما أظهر ٢٦/٦ (٢٣٪) خلل كروموسومي غير طبيعي (٤٧ ، XXY). في المجموعة الثانية 63/74 (٨٥٪) كان
(٤٦، XY) بينما ٧٤/١١ (١٥ ٪) كان (٤٧، XXY). لم تنجح نتيجة أستخراج الحيوانات المنوية إلا في ٨/٣ (٣٧,٥٪) من لديهم حذف حيني في الكروموسوم الصبغي الجنسي. بينما في مجموعة الثانية ، ٩٤\٢٢ ٢٣،٤%). أعتبارا للكروموسوم الصبغي الجنسي ؛ يوجد في المجموعه الأولي ٣٦/٣ (٨,٣ ٪) لديهم حُذف جينية دقيقة للكروموسوم الصبغي الجنسي في مناطق AZF حيث ؛ أظهر ٣/١ (٣٣,٣٪) عمليات حذف في AZF-c و ٣/٢ (٦٦,٧٪) أظهر عمليات حذف في كل من AZF-b + c. ومع ذلك؛ لم يتم اكتشاف أي عمليات حذف في AZF-a. في مجموعة الثانية ، كان ١١٤/٢١ (١٨,٤ ٪) لديهم حُذف جينية دقيقة للكروموسوم الصبغي الجنسي في المناطق AZF حيث ؛ أظهر ٢١/١ (٤,٨٪) عمليات حذف في منطقة AZF-b ، وأظهر حذف ٢١/٢ (٩,٥٪) في كل من منطقتي AZF-a + b + c ، وأظهر ٢١/٨ (٣٨٪) عمليات حذف في منطقة AZF-c أظهر فقط و ٢١/١٠ (٤٧,٦٪) عمليات حذف في كل من مناطق AZF-b + c.

الخلاصه: كان معدل حدوث الحُذف الجينية الدقيقة للكروموسوم الصبغي الجنسي في الرجال في المرضي الذين شملتهم الدراسة مشابهًا لبعض الأبحاث المهتمة بفحص الحُذف الجينية الدقيقة للكروموسوم الصبغي الجنسي في الرجال.[19] [25]

الكشف عن الحُذف الجينية الدقيقة للكروموسوم الصبغي الجنسي ضروري للتشخيص الوراثي السليم في الذكور المصابين بالعقم. وجود حُذف جينية دقيقة في منطقة AZFc يمكن أن تساعد في اتخاذ قرارات مستنيرة بشأن نتائج إيجابية في عملية أستخراج الحيوانات المنوية من الخصية.