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GENETICS

High prevalence of genetic abnormalities in Middle Eastern patients with idiopathic non-obstructive azoospermia

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Abstract
Introduction Our objective is to detect the frequency and types of major genetic abnormalities of idiopathic non-obstructive azoospermia (NOA) to give appropriate genetic counseling before assisted reproductive techniques (ART) in Middle East and to compare the frequencies with other regions of the world.
Material and methods A total of 880 Middle Eastern patients with NOA were recruited in this multicenter study for genetic evaluation prior to use of ART. Karyotyping was performed on peripheral blood lymphocytes according to standard G-banding methods, polymerase chain reaction (PCR) was performed to screen the microdeletions in the AZF region of the Y chromosome.
Results The present study shows that the total prevalence of genetic abnormalities is 28.41 %, including 184 patients (20.91 %) with chromosome disorder and 66 patients (7.5 %) with Y chromosome microdeletions. The most prevalent chromosome abnormality is Klinefelter's syndrome, which includes 161 patients (18.3 %), 7 patients had XX reversal male sex (0.8 %), 2 patients had 47XYY (0.23 %) and 2 patients had 45XO/46XY (0.23 %). Structural abnormalities occurred in 12 patients (1.36 %).
Conclusions The high prevalence of genetic abnormalities (28.41 %) in our study strongly suggests the need for routine genetic testing and counseling prior to assisted reproduction in such population with idiopathic infertility, as a result may help determine the prognosis, as well as the choice of ART. Moreover it allows specific pre-implantation genetic testing to minimize the risk of transmitting genetic defects to offspring.

Capsule Men with Non-obstructive azoospermia need genetic testing and counseling prior to assisted reproduction.

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Introduction

Infertility is a very common health problem, affecting approximately 15–20 % of couples who attempt pregnancy [1]. Male factor is assumed to be responsible in about 40–50 % of the infertile couples [2–5]. About 15 % of the infertile men may carry a genetic abnormality, including numerical and structural chromosomal abnormalities [6, 7]. The frequency of chromosomal abnormalities in subfertile males is estimated to be 2–10 % [8, 9], and may reach 10–19 % in azoospermia cases [10].

Azoospermia is defined as complete absence of sperm from ejaculate and approximately occurs in 10–15 % of infertile

men with abnormal semen analysis [11]. Azoospermia is classified as obstructive and non-obstructive, although in some clinical protocols aetiologies for azoospermia fall into pre-testicular, testicular and post-testicular categories [12, 13].

Among the genetic abnormalities found in azoospermia men, the most frequent one being the 47, XXY karyotype that characterizes the klinefelter syndrome [2, 14]. Microdeletions of the Y chromosome removing the azoospermia factor (AZF) region are the most frequent genetic cause of spermatogenic failure after the klinefelter's syndrome [4, 15–17]. The AZF region is further subdivided into three non-overlapping regions defined as AZFa, AZFb and AZFc [4, 18, 19]. Furthermore, recently a fourth region, AZFd, has been proposed between AZFb and AZFc. So far at least 12 genes have been isolated from these regions [20]. Several genes located in the AZF region are expressed in the testes and could therefore be viewed as "AZF candidate genes". Figure 1 depicts the three different AZF regions of the Y chromosome.

Patients with microdeletions restricted to AZFd may present mild oligozoospermia or abnormal sperm morphology. But the patients with microdeletions restricted to AZFa usually result in patients with sertoli cell only syndrome (SCOS), whereas microdeletions restricted to AZFb or AZFc can result in patients with phenotypes, which range from SCOS to moderate oligozoospermia [22].

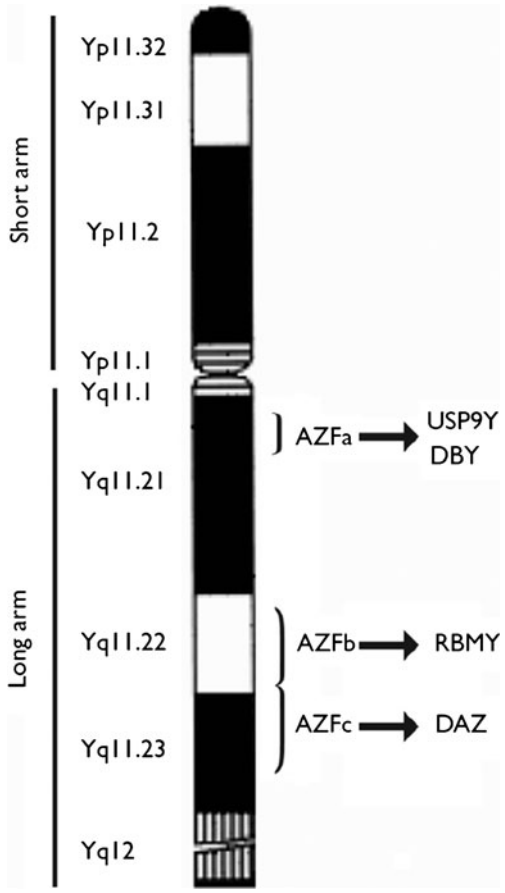

Fig. 1 Diagram shows the AZF region of Y chromosome [21]

Structural chromosomal abnormalities can also cause spermatogenic failure. Structural abnormalities include inversions, balanced translocations and additional chromosomal material. Reciprocal and Robertsonian translocations are also found in azoospermic patients [23].

The aim of this multicenter study is to detect the frequency and types of major genetic abnormalities of idiopathic non-obstructive azoospermia (NOA) to give appropriate genetic counseling before assisted reproductive techniques (ART) in Middle East and to compare the frequencies with other regions of the world.

Material and methods

A total of 880 Middle Eastern patients with NOA were prospectively recruited in this multicenter study for genetic evaluation from January 2008 to January 2011 at faculty of medicine in Damascus university (253 patients), Orient hospital (385 patients), Al-Assad university hospital (87 patients) and department of radiation medicine in atomic energy commission (155 patients). The patients originate from Syria, Iraq, Palestine, Lebanon, Jordan, Saudi Arabia, Qatar and United Arab Emirates.

The mean age of the patients (±SD) was 32.6±6.8. All Participants gave informed consent, according to the protocol approved by local Ethics Committee at Damascus University and health ministry. Routine Clinical and laboratory tests were performed on all patients, Including Semen Analysis, Hormones, Karyotype and investigation of Y chromosome microdeletions.

Semen analysis was performed according to world health organization criteria (WHO laboratory manual for the examination of human semen and semen-cervical interaction, 1999). Clinical examination was performed in all of the patients for anatomical integrity of genital system, and we excluded all the patients who had any history of childhood disease, environmental or radiation exposure, prescription drug usage that could account for their infertility, and other pathologies as varicocele or cryptorchidism. The diagnosis of non-obstructive azoospermia was based on clinical finding and several parameters as FSH, testicular volume and histopathology of a previous testicular biopsy (if available) although no standard method was used. Hormone analysis including follicle stimulating hormone (FSH), luteinizing hormone (LH), Prolactin (PRL) and testosterone were also done by using Enzyme-Linked Immuno-Sorbent Assay (ELISA).

Karyotype analysis was performed using G-Banding methods, and at least 30 peripheral blood metaphases were analyzed for each patient. The number of analyzed metaphases was increased to 100 whenever necessary. All

chromosomal abnormalities have been reported in accordance with the current international standard nomenclature.

Screening for microdeletions in AZF region of Y chromosome was done by polymerase chain reaction (PCR). Genomic DNA was extracted from peripheral blood leukocytes using QIAamp DNA blood mini kit (Qiagen, Germany) according to manufacturer's instruction. Extracted DNA concentration and purity were measured using BioPhotometer Plus (Eppendorf, Germany). The following 20 sequence tagged sites (STS markers) were analyzed using Y chromosome deletion detection system, version 2 kit (Promega, USA):

SY81, SY86, SY84, SY182 (AZF a)
SY121, SYPR3, SY124, SY127, SY128, SY130, SY133, SY134 (AZF b).
SY242, SY208, SY254, SY254, SY255, SY157 (AZF c).
SY145, SY152 (Proximal AZFc (AZFd)).
SY14 (SRY). And SMCX locus, ZFX/ZFY were used as internal controls.

5 Multiplex PCRs was performed for each sample using mastercycler® thermal cycler machine (Eppendorf, Germany). With the following program: 94 °C for 2 min, then 94 °C for 1 min, 57 °C for 30 s, 72 °C for 1 min (35 cycles) and a final extension 72 °C for 5 min. PCR products were electrophoresed on 3 % LMP agarose gel (Promega, USA) in 1X TBE buffer containing 0.5 μg\ml ethidium bromide, and a running buffer 1X TBE buffer containing 0.5 μg\ml ethidium bromide . 5 V\cm for 35 min.

Gel was visualized using UV transilluminator (320 nm) and photographed using gel documentation system. Normal male genomic DNA control and No DNA control were performed with each set of samples. DNA extraction and Mix preparation were performed by female technician. Unamplified STS was confirmed by additional two experiments.

Results

Genetic abnormalities were found in 28.41 % of all non-obstructive azoospermic men studied, Including 184 patients (20.91 %) with karyotype abnormalities as shown in Table 1, and 66 patients (7.5 %) with Y-chromosome microdeletions as shown in Table 2.

Variable translocations and additional chromosomal material occurred in 12 patients (1.36 %) as shown in Table 3.

Discussion

The most prevalent chromosomal abnormality in our study was klinefelter's syndrome (KS), which including 161 patients (18.3 %), 143 patients (16.25 %) were non-mosaic and 18 patients (2.05 %) were mosaic.

Table 1 The Karyotype of the patients with idiopathic NOA

Karyotype	Number	Rate
46, XY	696	79.09
47, XXY	143	16.25
47, XXY/46, XY	18	2.05
46, XX Male	7	0.8
47,XYY	2	0.23
45, XO/46, XY	2	0.23
Structural abnormalities	12	1.36

Klinefelter's Syndrome (KS) is the most common abnormality of sexual differentiation, and occurs in approximately 1 in 500 live births. KS is a form of primary testicular failure with testicular hypotrophy and elevated gonadotropin plasma levels, and it represents the most common form of male hypogonadism [24]. Other studies showed that the prevalence of KS among NOA men is very high, up to 10 % [25]. It has always been assumed that more than 90 % of non-mosaic 47,XXY males are azoospermic. In their series, 74.4 % of mosaic 47,XXY/46,XY patients were azoospermic, whereas the remaining had severe oligospermia [2]. The result of the other international studies in the literature revealed an average frequency of 15.1 % (ranged from 4.2 % to 33 %) chromosomal abnormalities in men with NOA as shown in Table 4. Moreover, the result of the other regional studies reported an average frequency of 14.9 % (ranged from 4.2 % to 33 % also) chromosomal abnormalities in NOA patients [4, 5, 16, 28, 34, 36–38, 40, 41, 43].

Y chromosome microdeletions have been increasing interest to clinician since assisted reproductive techniques were introduced to the main treatment option for severe male factor infertility. The frequency of microdeletions in the literature was internationally reported to be in the range of 1.9 % to 55.6 % with an average frequency of 10.8 % as shown in Table 5. Nevertheless this frequency of microdeletions in the regional studies was reported to be in the range of 1.9 % to 51.2 % with an average frequency of 10.5 % [4, 5, 16, 36, 37, 40, 51, 54].

Table 2 Distribution of microdeletions in AZF region

AZF region	Number	Rate
AZFa	6	9.09
AZFb	22	33.33
AZFc	18	27.27
AZFb+c	11	16.67
AZFb+d	6	9.09
AZFd+c	2	3.03
AZFb+d+c	1	1.52

AZF Azoospermia factor

Table 3 Structural abnormalities of the patients with idiopathic NOA

Karyotype	Number
45, XY t(14; 15)	2
46, XY t(7; 14)	2
46, XY t(14;21)	1
46, XY t(9; X)	1
46, XY t(15; 11)	1
46, XY t(14; 14)	1
46, XY t(13; 14)	1
46, XY t(5; 11)	1
46, XY add(15)	1
46, XY add(9)	1

t chromosomal translocation; *add* additional chromosome material

In the present study, the frequency of AZF microdeletions was 7.5 % in non-obstructive azoospermic men, which is little lower than the average value. And microdeletions in the AZFb region were the most prevalent (60.61 %), followed by the AZFc (48.48 %), AZFd (13.64 %) and AZFa (9.09 %) respectively. All cases of microdeletions in AZFa region were isolated, but other microdeletions were combined or isolated. None of patients showed individual deletion in AZFd region, however deletion in this region was observed in combination with deletions in other AZF regions.

Our finding is in agreement with the study of Mirfakhraie et al. [54] who reported that 12 % of men with NOA had Y microdeletions and the deletion in AZFb region was the most frequent (66.7 %) followed by AZFc (41.7 %), AZFd (33.3 %) and AZFa (8.3 %), respectively. These are in accordance with the finding in Shanghai [46] where majority of microdeletions in men with NOA were in AZFb region. Another study [56] showed that microdeletions of AZF region occur with different frequency, it has been suggested that AZFc is the most frequently deleted region (60 %), followed by deletions of the AZFb and combined deletions involving different AZF region (35 %). AZFa deletions are extremely rare (5 %) and isolated deletions have been reported.

It is suggested that complete deletion of AZFa region may result in complete sertoli cell only syndrome (SCOS) and azoospermia. Identification of deletions in this region is very important since it is impossible to retrieve testicular sperm for Intracytoplasmic sperm injection [57–60].

Most microdeletions of AZF region are de novo events, due to deletions occurring in germ cells or in early cleavage

Table 4 Review of the literature of the chromosomal abnormalities in non-obstructive azoospermia

Author	Year	Region	Cases	Chromosome abnormality		
				Gonosomal (*n*/%)	Autosomal (*n*/%)	Total (*n*/%)
Rucker et al. [26]	1998	USA	101	13 (12.9)	8 (7.9)	21 (20.8)
Tuerlings et al. [27]	1998	Netherlands	62			4 (6.5)
Gunduz et al. [28]	1998	Turkey	41			14 (34.1)
Kleiman et al. [4]	1999	Israel	105	5 (4.8)	5 (4.8)	10 (9.5)
Dohle et al. [7]	2002	Netherlands	37	3 (8.1)	1 (2.7)	4 (10.8)
Vicdan et al. [16]	2004	Turkey	119			5 (4.2)
Rao et al. [29]	2004	India	99			7 (7.1)
Nagvenkar et al. [30]	2005	India	42	5 (11.9)	1 (2.4)	6 (14.3)
Zhou-Cun et al. [31]	2006	China	256			31 (12.1)
Pina-Neto et al. [32]	2006	Brazil	60	12 (20)	–	12 (20)
Meza-Espinoza et al. [33]	2006	Mexico	227	41 (18.1)	2 (0.9)	43 (18.9)
Samli et al. [34]	2006	Turkey	383	38 (10)	9 (2.3)	47 (12.3)
Vutyavanich et al. [5]	2007	Turkey	50	4 (8)	1 (2)	5 (10)
Martinez-garza et al. [35]	2008	Mexico	50	7 (14)	1 (2)	8 (16)
Balkan et al. [36]	2008	Turkey	52	7 (13.5)	–	7 (13.5)
Ceylan et al. [37]	2009	Turkey	30	10 (33.3)	–	10 (33.3)
Akgul et al. [38]	2009	Turkey	86	15 (17.4)	–	15 (17.4)
Ng et al. [39]	2009	Hong Kong	71	13 (18.3)	2 (2.8)	15 (21.1)
Kumtepe et al. [40]	2009	Turkey	1,214			199 (16.4)
Kosar et al. [41]	2010	Turkey	92	5 (5.4)	–	5 (5.4)
Mafra et al. [42]	2011	Brazil	43	5 (11.6)	–	5 (11.6)
Akbari et al. [43]	2012	Iran	132	27 (20.4)	–	27 (20.4)
Total			3,309			498 (15.1)
Our Study	2013	Middle East	880	172 (19.5)	12 (1.4)	184 (20.9)

Table 5 Review of the literature of Y-chromosome microdeletions in non-obstructive azoospermia

Author	Year	Region	Cases	Y-microdeletions (*n*/%)	Most prevalent pattern
Kremer et al. [44]	1997	Netherlands	19	9 (47.4)	AZFc
Foresta et al. [45]	1998	Italy	18	10 (55.6)	AZFc
Rucker et al. [26]	1998	USA	183	17 (9)	AZFc
Kleiman et al. [4]	1999	Israel	105	7 (6.7)	AZFc
Dohle et al. [7]	2002	Netherlands	37	3 (8.1)	AZFc
Tse et al. [46]	2002	Hong Kong	59	5 (8.5)	AZFc
	2002	Shanghai	135	9 (6.7)	AZFb
SaoPedro et al. [47]	2003	Brazil	29	2 (6.9)	AZFc
Vicdan et al. [16]	2004	Turkey	119	17 (14.3)	AZFc
Roa et al. [29]	2004	India	99	11 (12)	AZFc
Zhou-Cun et al. [31]	2006	China	256	38 (14.8)	AZFc
Pina-Neto et al. [32]	2006	Brazil	60	4 (6.7)	AZFc
Omrani et al. [48]	2006	Azerbaijan	60	18 (30)	AZFc
Vutyavanich et al. [5]	2007	Turkey	50	5 (10)	AZFc
Arruda et al. [49]	2007	Brazil	23	10 (43.5)	AZFa
Imken et al. [50]	2007	Morocco	48	4 (8.3)	AZFc
Malekasgar et al. [51]	2008	Iran	31	16 (51.2)	AZFc
Martinez-garza et al. [35]	2008	Mexico	50	6 (12)	
Balkan et al. [36]	2008	Turkey	52	1 (1.9)	AZFc
Ceylan et al. [37]	2009	Turkey	30	5 (16.7)	AZFc
Ng et al. [39]	2009	Hong Kong	71	6 (8.5)	AZFc
Kumtepe et al. [40]	2009	Turkey	1,214	115 (9.5)	AZFc
Stahl et al. [52]	2010	USA	1,153	120 (10.4)	AZFc
Wang et al. [53]	2010	Northeastern China	219	20 (9.2)	AZFc
Mirfakhraie et al. [54]	2010	Iran	100	12 (12)	AZFb
Behulova et al. [55]	2011	Slovakia	239	8 (3.6)	AZFc
Total			4,680	506 (10.8)	AZFc
Our Study	2013	Middle East	880	66 (7.5)	AZFb

stages. We believe that the deletions in our study were de novo too, whereas all deleted patients were born after natural conceptions, unfortunately, we could not perform the genetic testing for all fathers, only 17 fathers did the Y-Chromosome microdeletions investigation and all of them were normal.

It is clear that the frequency and pattern of microdeletions in NOA are variable (possible ethnic or geographic factors), but the most frequent place of deletions was AZFb region in our study. We may conclude that genes located in the AZFb region were more involved in fertility process in Middle Eastern patients with NOA [54], as a result may help determine the prognosis of sperm retrieval. This is contrary to previous reports in which AZFc deletion was reported to be the most frequent deletion in NOA [4, 5, 16, 36, 37, 40, 51].

Y chromosome microdeletion analysis should routinely be offered to all men with NOA. There are several considerations that support a routine assessment of Y deletion. Firstly, a positive test would provide a firm diagnosis of the man's problem. Secondly, knowledge of the type of Y deletion may assist the clinician in determining the best type of assisted reproduction. Thirdly, couples should be offered this information, as they must understand that their male offspring will almost certainly be subfertile and require reproductive monitoring from the time of sexual maturation [61].

Accordingly, men with Y microdeletions are often infertile, but many can still father children through ICSI using the few sperms present in semen or isolated directly from the testis. Modern sperm recovery techniques have made it possible to help men with NOA to achieve fatherhood [62]. Assisted reproductive techniques have given the chance of having a child to infertile males with NOA.

However, using the intracytoplasmic sperm injection (ICSI) in this group with high genetic abnormalities ratio may increase the inheritance of paternal genetic disorders to offspring [41]. It occurs because the presence of the structural chromosomal abnormality predisposes to abnormal segregation in meiosis. Alternate segregation in male

gametes has been reported and may result in fertilization failure or poor embryonic development [63, 64]. In these cases, the couple should be offered pre-implantation genetic diagnosis (PGD) whenever possible [47].

A relationship between structural chromosomal abnormalities and infertility has been reported among azoospermic men [30]. In our study reciprocal translocations were seen in 10 cases (1.14 %) and additional chromosomal materials were seen in two cases (0.23 %). It is hypothesized that balanced translocations interfere with normal chromosome pairing and segregation at meiosis, thus providing a potential for formation of unbalanced gametes and subsequent unbalanced abnormal offspring [31].

Couples experiencing NOA who are interested in pursuing assisted reproduction are encouraged to discuss genetic testing with their physicians or healthcare providers and to consider undergoing genetic counseling [65].

Although our results reflect a regional pattern of those referrals, comparison of our results with the review of the literature shows a relatively higher incidence of chromosomal abnormalities in idiopathic NOA in the Middle East, which may be due to high incidence of consanguinity, geographic and ethnic origins of the studied population, since our samples were collected from eight countries of Middle East region. These variations may reflect selection bias due to our referral pattern and study design including the composition of the study population and sample size.

In conclusion, our results support that the high prevalence of genetic abnormalities 28.41 % in non-obstructive azoospermic men, strongly suggests the need for routine genetic testing and counseling prior to assisted reproduction in such population with idiopathic infertility, as a result may help determine the prognosis, as well as the choice of ART. Moreover, it allows specific pre-implantation genetic testing to minimize the risk of transmitting genetic defects to offspring.

Implications for patients

This prospective cohort study investigates the prevalence of genetic abnormalities (Karyotype and Y-Chromosome microdeletions) in 880 infertile Middle Eastern men with non-obstructive azoospermia (NOA) referred to several academic centers within Syria.

Its implications include having a high frequency of genetic abnormalities (28.41 %) among this population (NOA men) in this region, suggesting strongly the need for routine genetic testing and counseling prior to assisted reproduction in such patients, and gaining further insight into the etiology of NOA by comparing results with internationally published data.

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