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Research Article

Genetic Diagnosis in Non-Obstructive Azoospermic Tunisian Men

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Abstract

Purpose: Male infertility is the cause in half of all childless partnerships. Numerous factors contribute to male infertility, including chromosomal aberration and Yq microdeletions. We therefore aimed to evaluate the prevalence of genetic abnormalities among non-obstructive azoospermic (NOA) Tunisian men referred for routine cytogenetic analysis to the department of cytogenetics of the Pasteur institute of Tunis.

Methods: Karyotype analyses were performed on peripheral blood lymphocytes using R-banding for 401 NOA. Molecular diagnosis of classic Yq microdeletions was performed in 90 NOA with normal karyotypes by two multiplex PCRs using six STS markers (Sequence-Tagged Site) recommended by the EAA/EMQN (European Academy of Andrology / the European Molecular Genetics Quality Network).

Results: The overall incidence of chromosomal abnormalities was 12.22% (49/401). Out of the 49 patients with abnormal cytogenetic findings, sex chromosome abnormalities were observed in 42 (85.71%) including Klinefelter syndrome in 37 (75.5%). Structure chromosome abnormalities involving autosomes (14.28%) and sex chromosomes (2.04%) were detected in 8 infertile men. Furthermore, the Yq microdeletions were seen in two patients (2.22%). Both had complete deletion of the AZFc region.

Conclusion: The occurrence of chromosome anomalies and Yq microdeletions among NOA men strongly suggests genetic testing and counseling prior to employment of assisted reproduction techniques in Tunisia.

Keywords: Male infertility; Non-obstructive azoospermia; Chromosomal abnormalities; Y-chromosome microdeletion

Introduction

Infertility is a major health problem affecting up to 15% of couples of reproductive age [1]. For many years, it was assumed that most reproductive problems could be attributed the female partner, but research in recent years has demonstrated that 30-50% of infertility is caused by male factor [2].

Until recently, there was no treatment available for men who have a complete absence of sperm in the ejaculate (azoospermia). This one accounts 10-15% of male infertility patients [3-5], among them 50-60% of which are non-obstructive azoospermia (NOA), which is characterized by the absence of sperm in the ejaculate without the obstruction of the reproductive tract pathway [6]. NOA occurs in ~1% of the male population and approximately 20-25% of NOA patients are caused by known genetic abnormalities [3, 7, 8]. Those involving chromosome anomalies to about 15%-16% [9]. The most frequent one being the 47, XXY karyotype that characterizes the Klinefelter Syndrome (KS) with a frequency 14% [9], followed by the microdeletions of the long arm of the Y chromosome (Yq) removing the azoospermia factor (AZF) region or parts with a frequency 8% [10]. However, there is still a significant proportion of NOA patients that have unknown etiology.

The establishment of in vitro fertilization using intracytoplasmic sperm injection (ICSI) as a standard treatment modality has resulted in a number of these men successfully fathering a child through surgically retrieved sperm from the testis. However, a genetic risk exists for these offspring, implying the necessity for future parents to be appropriately informed on potential consequences [11-13].

The aim of this study was to determine the prevalence of various chromosomal aberrations and the prevalence of Y-chromosome microdeletions among non-obstructive azoospermia Tunisian men attending the Pasteur Institute of Tunis.

Patients and Methods

Patients

The cytogenetic analysis has been focused on 401 Tunisian infertile patients with idiopathic non-obstructive azoospermia. These infertile men with sperm disorders were referred for karyotyping to the department of Histology and Cytogenetics at the Pasteur Institute of Tunis between January 2006 and May 2014. Among these patients, 90 non-obstructive azoospermia with normal karyotype were benefited from molecular analysis.

Patients were checked for the history of relevant medical disorders, e.g., diabetes, renal, liver disease, radiation, endocrine abnormalities (e.g., hypogonadotropic hypogonadism), exposure

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to toxins and/or medical affecting spermatogenesis, acquired and congenital structural defect of urogenital system, history of surgical intervention of genital tract.

Informed consent was obtained for karyotype and a molecular investigation from individual participants included in the study, and approval was given by the local ethics committee of Pasteur Institute of Tunis.

Karyotyping

Cytogenetic analysis was performed from phytohemagglutinin-stimulated lymphocyte cultures by routine laboratory protocol. For microscopic analysis, R-banded metaphase spreads were analyzed and abnormalities recorded according to the current International System for Human Cytogenetic Nomenclature [14]. A resolution of 550 to 700 bands per haploid karyotype was used for the routine analysis. For each patient, at least 20 well-spread metaphases were analyzed and two to five metaphases were karyotyped. When at least one of the 20 showed a loss or gain of a chromosome, especially X or Y chromosome, the number of analyzed metaphases was increased to 30. If a second abnormal cell was observed, the analysis was considered complete; otherwise, the number of metaphases was increased to 50. Sex chromosome mosaics occurring at a level of less 5% were not considered as well as pericentric inversions of chromosome 9 or other structural chromosome variants and polymorphisms that were considered as normal cytogenetic events.

Detection of AZF microdeletions by multiplex PCR

Genomic DNA was extracted from blood lymphocytes using a commercially available kit (FlexiGene Kit; Qiagen), according to the manufacturer's instructions. Each patient was examined for six AZF loci. The STS primers used were: for AZFa sY84 and sY86, for AZFb sY127 and sY134 and for AZFc sY254 and sY255. This primer set was suggested by Simoni et al. [9] and is prescribed by the European Academy of Andrology (EAA) and European Molecular Genetics Quality Network (EMQN) [15, 16]. In addition, sY14 (STS within the SRY gene located in Yp) was tested as an internal positive control. Two multiplex PCRs were carried out in 50 µl reaction volumes containing: 200 ng of each DNA sample, 1.5mM MgCl2, 0.4 mM of each dNTP, 1.6 µM of each oligonucleotide primer: sY86, sY127, sY254 (Mix A) for the first multiplex PCR, sY84, sY134, sY255 (Mix II) for the second one, 5% dimethyl sulfoxide (DMSO) were added to 1 µl of Taq DNA polymerase. A positive control (sample from a normal fertile male), and two negative controls [(i) normal female sample, (ii) every constituent except DNA], were included in every PCR assay. The reaction mixture included thermo cycling consisted of an initial denaturation of 5 minutes to 94°C followed by 35 cycles of 30 seconds at 94°C, 30 seconds annealing at 59°C, 30 seconds extension at 72°C, finally, 7 minutes extension step at 72°C. PCR products were analyzed by electrophoresis on 3% agarose gels containing ethidium bromide and visualized by exposure to ultraviolet light. In the event of detecting deletion with a primer, the PCR assay was repeated thrice for confirmation. A STS was considered absent only after 3 amplification failures in the presence of successful amplification of internal control (SRY).

Results

Cytogenetic analysis

The average age was 38.07 ± 5.96 and the average duration of

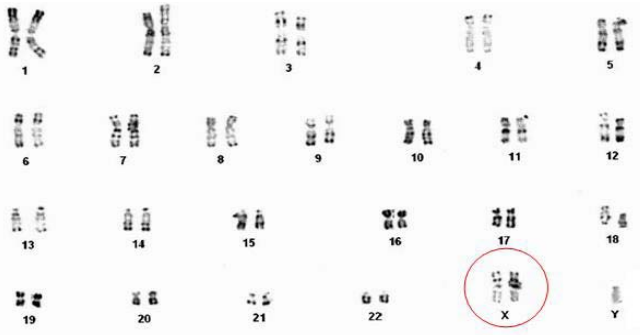

Figure 1: Karyotype of a patient with Klinefelter syndrome: 47, XXY.

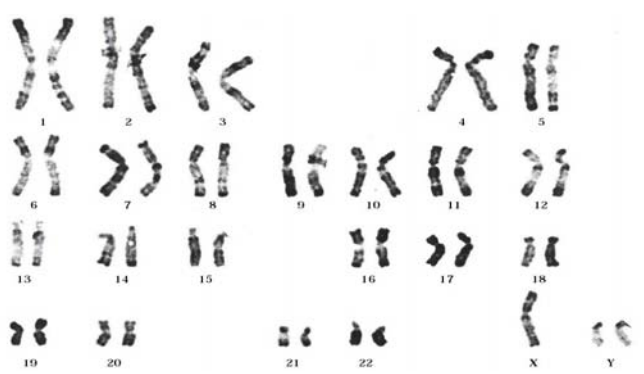

Figure 2: Karyotype of a patient with 47, XYY.

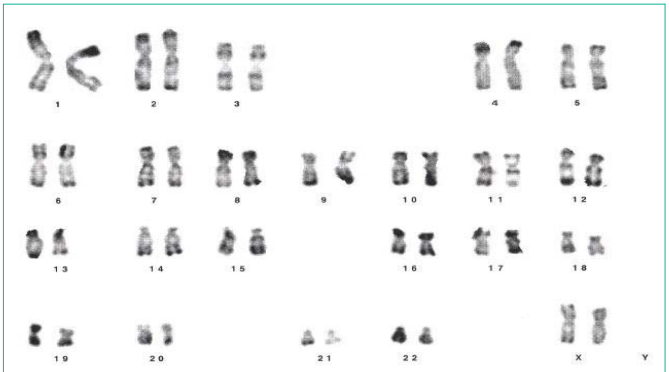

Figure 3: Karyotype of a patient with male syndrome 46, XX.

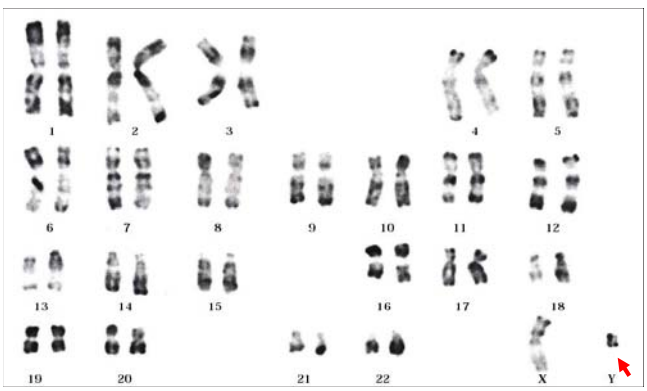

Figure 4: Karyotype of a patient with 46,X,del (Y)(q11.2 qter).

Table 1: Type and frequency of chromosomal anomalies in 401 non-obstructive azoospermia Tunisian men.

	% (No. of men with chromosomal aberration/total No.of men karyotyped)			
	Sex chromosome aberrations		**Autosomal chromosome aberrations**	
	Karyotypes	**%**	**Karyotypes**	**%**
Non-obstructive azoospermia men 12.22 % (49/401)	47, XXY Mosaic 47, XXY: *46, XY/47, XXY *47, XXY (85%)/48, XXXY (15%) 47, XYY 45, X (15%)/46, XY (85%) 46, X, del (Y) (q11.2 qter) XX males (46, XX)	8.23 (33/401) 1 (4/401) (3) (1) 0.25 (1/401) 0.25 (1/401) 0.25 (1/401) 0.5 (2/401)	**Reciprocal translocation** : *46, XY, t (9 ; 22) (q11;p11) *46, XY, t (7;16) (p11;p13) *46, XY, t (4;6) (p12;p22) *46, XY, t (4;17) (q11;p11) **Robertsonian translocation :** *45, XY, der (13;14) (q10;q10) **Inversion:** *46, XY, inv (7) (q22;q35) **Supernumerary marker chromosomes**: * 46, XY/47, XY, +mar	1 (4/401) (1) (1) (1) (1) 0.25 (1/401) (1) 0.25 (1/401) (1) 0.25 (1/401) (1)
	Subtotal	**10.47 (42/401)**	**Subtotal**	**1.75 (7/401)**

infertility was 5.26 ± 4.47 for the azoospermic patients.

Among these 401 patients, 49 cases showed abnormal karyotypes to a prevalence of 12.22%, in which 42 (10.47%) were sex chromosomal abnormalities and 7 (1.75%) were autosomal abnormalities.

The most common abnormality observed was the 47, XXY karyotype or their variant (mosaic 47, XXY/46, XY, 47, XXY/48, XXXY) consistent with KS (Figure 1), which were found in thirty seven cases (9.23%). The other chromosomal abnormalities was represented by: (6) balanced autosomal rearrangements, (2) 46, XX males (Figure 2), (2) unbalanced rearrangements, (1) 47, XYY (Figure 3), and (1) Yq deletion (Figure 4), (Table 1).

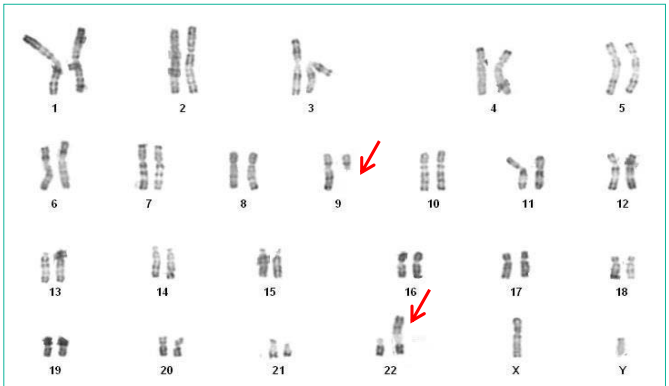

Figure 5: Karyotype of a patient with a balanced reciprocal translocation: 46,XY,t(9;22) (q11; p11).

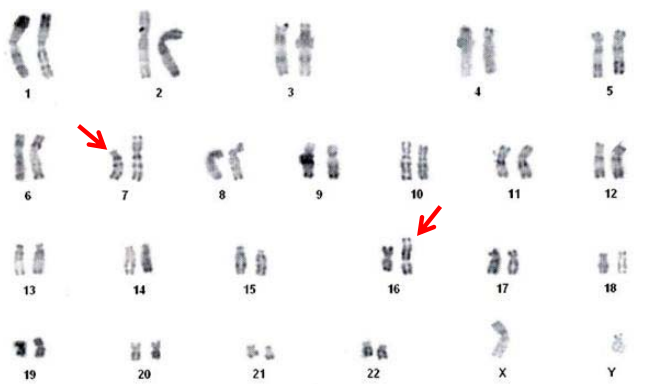

Figure 6: Karyotype of a patient with a balanced reciprocal translocation: 46, XY, t(7;16) (p11;p13).

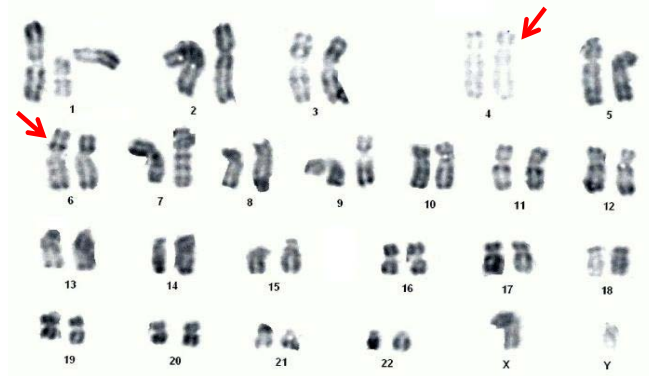

Figure 7: Karyotype of a patient with a balanced reciprocal translocation: 46, XY, t(4;6)(p12;p22).

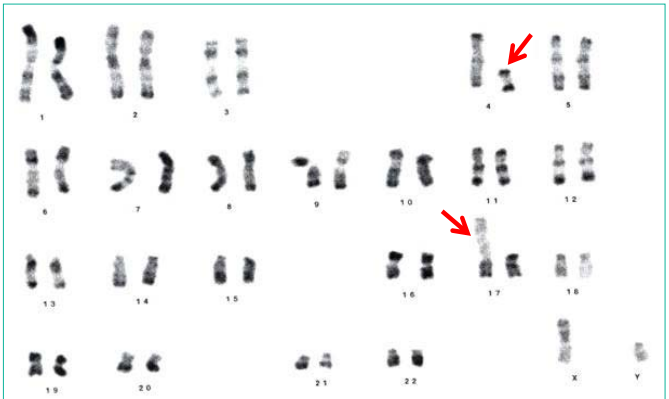

Figure 8: Karyotype of a patient with a balanced reciprocal translocation: 46, XY, t(4;17)(q11;p11).

Of balanced rearrangements are identified in six patients including: four with balanced reciprocal translocations, one with balanced Robertsonian translocations between chromosome 13 and chromosome 14 (Figure 9), and one paracentric inversion in chromosome 7 (Figure 10). An unbalanced rearrangement was identified in the two patients: one patient with supernumerary marker chromosome in a low level mosaic [3 out 20 metaphases] (Figure 11), and the last one had three cells with a 45, X constitution.

Yq chromosome microdeletion screening

Screening for AZF microdeletions was carried out in the 90 NOA

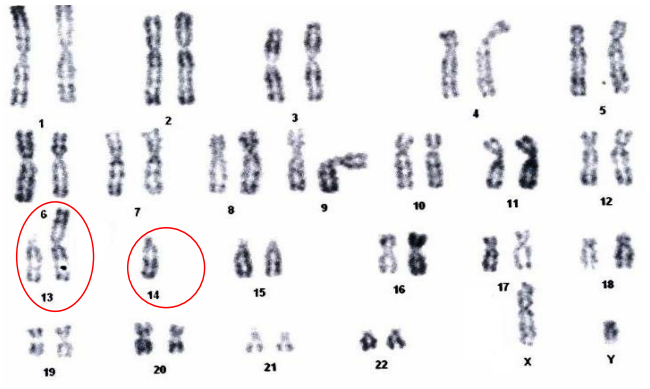

Figure 9: Karyotype of a patient with Robertsonian translocation: 45, XY, der(13;14)(q10;q10).

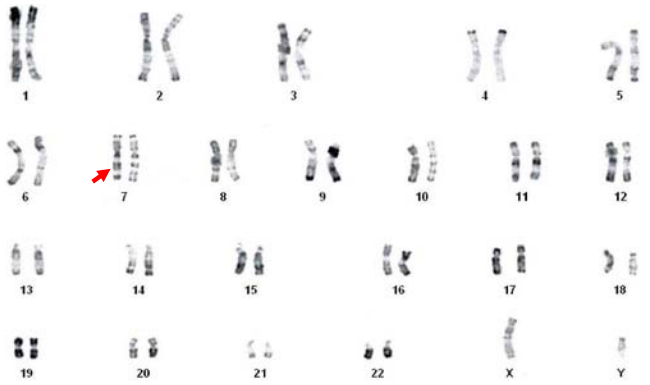

Figure 10: Karyotype of a patient with inversion paracentric of chromosome 7: 46, XY, inv(7)(q22;q35).

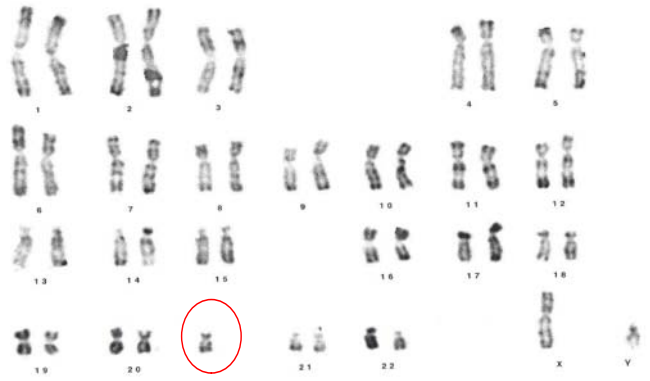

Figure 11: Karyotype of a patient with small supernumerary chromosome: 47, XY, +mar.

men with normal karyotype. Using the EAA/EMQN criteria, AZF region microdeletions on the Y chromosome were found in patients with a frequency of 2.22%. The failed amplification of sY254 and sY255 markers indicates a complete deletion in the AZF sub-region (Figure 12).

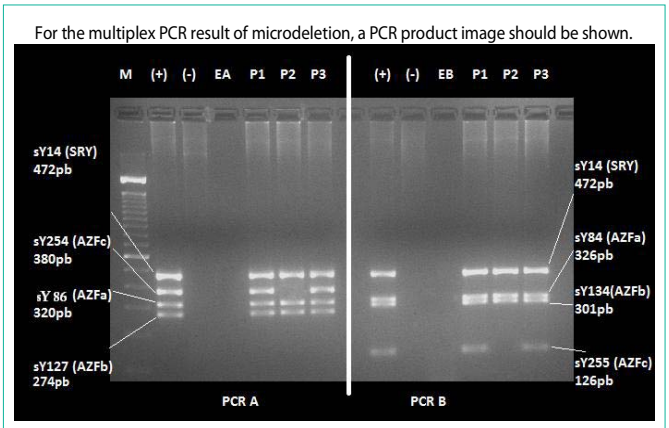

Figure 12: Gel electrophoresis analysis of two multiplex PCRs (PCR A and B) showing deletion in the AZF region of the Y chromosome.
(P1, P2 & P3) three NOA patient with normal karyotype. (M) size marker (100bp ladder), (+) fertile man sample, (-) female sample, (EA and EB) water. Patient (P2) have an absence of the sY254 and sY255 markers, indicating a complete deletion in the AZFc subregion.

Discussion

Cytogenetic analysis

Chromosomal abnormalities have emerged as one of the major genetic factors contributing to male infertility. In this study, the prevalence of major chromosomal anomalies was 12.22% in NOA with primary infertility. This was lower than in previous studies of Tunisian population 14.10 to 23.62%, but the incidence in other populations has been found to be between 5.43% and 19.44% (Table 2).

- Sex chromosome abnormalities

Sex chromosome abnormalities are the most frequent chromosome related cause of infertility. In our study, we have found thirty three men (8.23%) with 47, XXY karyotype. A mosaic 46, XY/47, XXY/ 48, XXXY karyotype was found in four azoospermic male (1%). Clinically, these abnormalities are associated with severe spermatogenic failure causing a marked reduction in testicular size which can be associated with gynecomastia, and/or a reduction of

Table 2: Comparison of chromosomal anomalies between this study and other similar studies.

Authors	Region	Years	Prevalence of chromosomal aberration (No.of cases /Total No;)
Tuerlings *et al.* [39]	Netherlands	1998	6.45% (4/62)
Nagvenkar *et al.* [34]	India	2005	14.29% (6/42)
Elghazel *et al.*[58]	Tunisia	2006	23.62% (81/343)
Mohammed *et al.* [59]	Kuwait	2007	19.44% (21/108)
Ng *et al.* [60]	Hong Kong	2009	21.1% (5/71)
Kosar *et al.* [61]	South of Turkey	2010	5.43% (5/92)
Mafra *et al.* [62]	Brazil	2011	11.62% (5/43)
Ghorbel *et al.* [48]	Tunisia	2012	22.22% (12/54)
Zhang *et al.* [63]	Northeast China	2012	17.28% (14/81)
Cavkaytar *et al.* [64]	Turkey	2012	11.22% (22/196)
Al-Achkar *et al.* [65]	Syria	2013	17.55% (17/97)
Amouri *et al.* [66]	Tunisia	2014	14.10% (46/328)
This study	Tunisia	2015	12.22% (49/401)

Table 3: Frequency of Yq microdeletions in non-obstructive azoospermic men with normal karyotype screened with the EAA/EMQN STSs markers from different region.

Authors	Region	Years	Prevalence of Yq microdeletions (No. of cases / Total)	Karyotype
Arruda *et al*. [55]	Brasilia	2007	43.48% (10/23)	46, XY
Elhawary *et al*.[67]	Egypt	2010	39.28% (11/28)	46, XY
Behulova *et al*.[53]	Slovakia	2011	3.35% (8/226)	46, XY
Sun *et al*. [54]	China (Shanghai)	2012	9.32% (33/354)	46, XY
Wettasinghe *et al*. [52]	Sri Lanka	2012	1.96% (3/153)	46, XY
Saliminejad *et al*.[50]	Iran	2012	2.13% (2/94)	46, XY
Chellat *et al*. [51]	Alegria	2013	2.04% (2/49)	NA
Hammami *et al*. [68]	Tunisia	2014	2.70% (2/74)	46, XY
This study	Tunisia	2015	2.22% (2/90)	46, XY

NA= not available;

pubic and facial and/or hair. And biologically, it is associated with a form of primary hypogonadism. Fluorescence in situ hybridization (FISH) analysis has demonstrated that the frequency of aneuploidy for the sex chromosomes varies from 2% [17] to 45% [18] in the sperm of men who appear to have a non-mosaic KS, and 1.5% [19] to 7% [20] in sperm from mosaics KS. The majority of babies born to men with KS have been normal although chromosomally abnormal fetuses have been reported [21-23] studied embryos by preimplantation genetic diagnosis (PGD) and reported a significant fall in the rate of normal embryos (54%) from KS patients when compared with the controls (72%). Even that there appears to be a small increased risk for these men, it is advised that PGD or prenatal diagnosis be performed before ICSI to ensure that the offspring is not aneuploid [24].

Besides, the second most predominant constituent is represented by the 46, XX, which it has been identified in 2 cases (0.5%). This rare condition was initially named "XX male syndrome". However, this was revised in October 2005 to its current nomenclature of "46, XX testicular disorder of sex development" (DSD) [25, 26]. This disorder has been reported with an incidence of 0.9% in azoospermic males [27]. Phenotypically the adults are similar to patients with KS. However, 46, XX DSD are shorter, and in some cases they have genital abnormalities [28]. The treatment of fertility of these patients can only be handled using the artificial insemination with donation sperm. Moreover, the donor sperm is banned in Tunisia and in the rest of the Sunni Islamic world [29] and the only possibility paternity for these patients remain the adoption.

The 47, XYY karyotype was seen in one patient. Men with the extra Y chromosome are mostly fertile, but azoospermia may be seen in some cases [30-32].

We also observed in our samples, two others gonosomes aberrations related with the Y chromosome aberration: one case with long arm deletions in all cell, and the second one with the 45X/XY mosaicism constituent. Effectively, loss of genes on the Yq, which plays a primary role in the regulation of different stages of spermatogenesis, is particularly dramatic in spermatozoa production [33].

- Autosomal chromosome abnormalities

The prevalence of autosomal abnormalities in our cohort of NOA was 1.75% (7/401), represented by; six cases with balanced rearrangements [(4) reciprocal translocation, (1) Robertsonian translocation and (1) inversion] and one case with unbalanced rearrangement [(1) small supernumerary marker chromosome (sSMC)].

An association between balanced autosomal translocation and infertility has been reported among NOA men [34-37]. In our samples, the reciprocal translocation were seen four cases involving these translocation [t(9;22); t(4;6); t(7;16); t(4;17)] (Figure 5-8). Otherwise, one case had Robertsonian translocation involving chromosome 13 and 14. Most translocations have no effect on other tissues but can severely impair spermatogenesis [38]. Using the ICSI in this group may increase the inheritance of paternal genetic disorder to offspring due to disrupted meiotic pairing and segregation [38, 39]. Indeed, according to the translocations carried the percentage of unbalanced gametes varies between 2.7% to 26.5% [40]. Both the chromosomes involved in translocation and the location of the breakpoints are likely to be determining factors for the fertility status of the patient. In addition, Robertsonian translocation can result in offspring with Down syndrome or Patau's syndrome or in gestational loss of concepts with monosomy of chromosome 13, 14 or 21, or trisomy of chromosome 14, which are lethal [32]. Preimplantation genetic diagnosis (PDG) by FISH (Fluorescent in Situ Hybridation) is recommended for the autosomal chromosome aberrations, in order to have an estimation of the risk of abnormal offspring and to adopt genetic counselling which accordingly may be useful for couples who opt for ART.

A relationship between autosomal inversion and infertility in the male has been reported [41]. The inversions may have complex outcomes, depending on the chromosome and the site and extent of the inversion. Due to the formation of abnormal loops during chromosomal pairing and disruption of meiosis, duplication and deletion events can occur, resulting in a germ cell arrest or the production of sperm with high rates of aneuploidy and consequent adverse birth outcomes [42]. In our study, we have seen only one case with paracentric inversion of chromosome 7. This autosomal abnormality with breakpoint in 7q22 has been reported in other infertile men, from paternal or maternal origin [39, 41]. Furthermore, it is important to document whether structural chromosomal aberration in infertile males are 'de novo' or inherited. In case a structural chromosomal aberration is familial and co-segregates with male infertility, this might pinpoint a chromosomal region harboring

one or more genes involved in spermatogenesis. Attempts were made to obtain blood samples to karyotype other family members, but in most cases the patients do not concur. Except for the Robertsonian translocation, which was inherited from his normally fertile mother, no further information was obtained for the other cases.

Besides these balanced rearrangements, one case of the 401 NOA men was shown to have small marker supernumerary chromosomes (sSMC). Although the relationship between sSMC and infertile males is not clear, the frequency of sSMC detected in infertile patients is higher than that in general population (0.125% versus 0.043%), and it is also different between male (0.165%) and female infertility (0.022%) [43]. Also, the sSMC with the regions of chromosome 15 or 20 have been reported with male infertility [44, 45], which indicates that male infertility may have no direct relation with genetic abnormality of specific genes. However, sSMC are preferentially maternally transmitted [46], suggesting either a reduced fertility in male carriers or that the marker is excluded in spermatogenesis.

Molecular analysis

The diagnosis of Yq microdeletions is considered to be clinically relevant for appropriate counselling and management of male infertility and is highly recommended for cases undertaking ICSI [16]. The Yq chromosome microdeletions have been investigated in many countries with an overall frequency of 1 to 58%, specifically 8% in NOA men and 3-5% in severely oligozoospermic men [10, 47]. According to the studies carried out by various Tunisian researches, the frequency of AZF microdeletions in NOA varies from 0–54.71% [48, 49]. The large variation in these frequencies could be due to the selection criteria of the patients and partly to molecular testing methodology in relation to the choice and number of STSs used (their position and their reliability).

Using the EAA/EMQN criteria, microdeletions of the Yq were found in two out of the 90 NOA men with normal karyotype (2.22%). The low frequency of the AZF microdeletions in our samples is in accordance with some previous studies reported from Iran (2.13%; 2/94) [50], Algeria (2.04%; 1/49) [51] and Sri Lanka (1.96%; 3/153) [52]. However, this rate is lower than the frequencies reported from Slovenia (3.35%; 8/226) [53], China (9.32%; 33/354) [54], Egypt (39.28%; 11/28) and Brasilia (43.48; 10/23) [55] (Table 3). All these studies were performed by the EAA/EMQN STS markers. The frequency variation among these studies is mainly due to the different factors such as environmental influences and ethnic variation.

The two NOA with the Yq microdeletions had a complete microdeletion of the AZFc region (failed amplification of sY254 and sY255 markers). None of them in the present study, showed deletion neither in AZFa nor in AZFb regions showed Yq microdeletions. Microdeletions in the AZFa and AZFb regions are extremely rare while AZFc microdeletion is the most frequently detected in the most published reviews [15]. Histologically, the AZFc microdeletion is associated with various spermatogenetic alterations. Furthermore, AZFc deletions phenotype is less severe because, these deletions are compatible with residual spermatogenesis and, in rare cases, can even be transmitted naturally to the male offspring [56, 57].

Conclusion

Although there is still a significant proportion of NOA patients that have unknown etiology, the cytogenetic and the molecular exploration still has an important role in the workup of male infertility of viewpoint diagnostic, prognostic and preventive in couples who wants to undergo ICSI treatment. Once a genetic cause is observed in an infertile man it helps the clinicians to avoid empirical and often expensive treatments to improve fertility.

Acknowledgment

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