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Original Research Article

Cytogenetic and Y Chromosome Microdeletions Screening in Tunisian Infertile Men

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Aim: **Chromosome abnormalities evaluation and Y chromosome microdeletions screening prior to assisted reproduction have an important interest in preserving future generations of genetic risks. The primary aim of this study was to establish the prevalence of chromosomal abnormalities and microdeletions on Yq chromosome in Tunisian infertile men with severe oligozoospermia or non obstructive azoospermia. Secondary to correlate Yq microdeletions with testicular sperm extraction results and cytological evaluation.** ***Methods*****: In this prospective study, Eighty-four infertile patients and 52 controls were screened for karyotypic abnormalities and Yq chromosome microdeletions.** ***Results:*** **No chromosome abnormalities and no microdeletions were detected in controls. Seven infertile males (8.3%) carried chromosomal abnormalities and 8 (9.5%) presented Y chromosome microdeletions. The frequency of chromosome abnormalities in azoospermic patients was 11.1% vs 3.3% in severe oligozzospermic group. The frequency of microdeletions was 11.1% in the azoospermic group and 6.7% in the severe oligozoospermic group. Six out of 84 (71.4%) of the infertile patients had microdeletions in the AZFc region (4 azoospermic and 2 severe oligozoospermic males), 1 azoospermic male (10%) in the AZFb,c regions and one in AZFb, no deletions in the AZFa region were detected. Among the 6 azoospermic patients with microdeletions: 4 had Sertoly cell only syndrome (SCOS) and 2 had maturation arrest (MA).** ***Conclusion:*** **Genetic abnormalities and Yq microdeletions in infertile Tunisian patients are frequent and similar to those reported in other countries. The knowledge of the existence of genetic abnormalities and microdeletions is useful to provide a correct diagnosis of male infertility, it allows the physician to refer the patient to adequate assisted reproduction technique and examine the value of testicular biopsy pertinence.**

Keywords: Infertility, Y chromosome microdeletions, AZF, Azoospermia, Oligozoospermia.

INTRODUCTION

Infertility is defined as the inability to conceive after one year of unprotected intercourse. (Hopps and al., 2003). It affects 10 to 15% of couples attempting pregnancy. Nearly 50% of these are accountable to the male partner. (Foresta and al., 2001) Different factors have been identified for male infertility; genetic factors were implicated in 10 % of cases. These genetic disorders affect semen parameters by causing alteration of chromosome materials. Recently, many researchers had reported a close relation between Y chromosome microdeletions and male infertility. Moreover, great association with birth defects has been reported. (O'Flynn and al., 2010) The investigation of male infertility should include the identification and the analysis of chromosomal anomalies, the most frequent genetic causes of this condition. (Tiepolo and al., 1976). The incidence of chromosomal abnormalities is about ten times higher in infertile men than in the general population (Egozcue et al., 2003). Klinefelter syndrome, 47, XXY and its variants are the most common chromosomal aberrations among men, with an estimated frequency of 1:500 among newborns (Ceylan c and al., 2010). After the Klinefelter

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syndrome, Y chromosome microdeletions are the leading genetic cause of male infertility. (Vicdan and al., 2004) Different studies have shown that the three microdeletions of the Azoospermia factor; AZFa, AZFb and AZFc was seen in 10% of azoospermic men.

These deletions remove many genes likely involved in male germ cell development and maintenance. (Kuroda-Kawaguchi and al., 2001). The genes in the AZF region are considered critical for spermatogenesis, and the frequency of microdeletions in the Yq region has been associated with the severity of the spermatogenic defects (Krausz and al., 2001). This predisposition for infertility can also include gradual alterations in spermatozoa production such that men with oligozoospermia may later develop azoospermia (Kihaile and al., 2005). Chromosomal abnormalities and Y chromosome microdeletions may be transmitted to future generations by ART techniques, clinical diagnosis of the cause of infertility is mandatory.

The primary aim of this study was to establish the prevalence of chromosomal abnormalities and microdeletions on Yq chromosome in Tunisian infertile men with severe oligo-zoospermia or non obstructive azoospermia. Secondary to correlate Yq microdeletions with testicular sperm extraction results and cytological evaluation.

MATERIALS AND METHODS

Patients

In this prospective study, patients were recruited consecutively from our AMP center. The population consisted of 84 infertile Tunisian males with non obstructive azoospermia or severe oligozoospermia according to World Health Organization guidelines 2010 (sperm count $<5 \times 10^6$/ml) (WHO; 2010) who were planning to undergo IVF/ICSI due to male factor infertility. Patients with sperm count higher than 5 millions/mL or with obstructive azoospermia or monomorphe teratospermia were excluded.

The control group consisted of 52 individuals with normal semen analysis who were attending any assisted reproductive technique due to exclusively primary or secondary (same partner) female factor infertility. Semen samples were produced by masturbation and collected in wide mouthed collection vials. Only patients and controls born in Tunisia and with Tunisian parents were included in this study. All participants gave informed consent according to the protocol approved by the ethics review board.

Variables of interest

The following parameters were collected in all patients: age; family history of infertility; tobacco use, medical history (criptorquidia, genital trauma, mumps infection, prostatitis, varicocele, or sexually transmitted disease) previous pelvic surgery; bilateral hypotrophic testicular (estimated clinically), Plasma hormone levels (FSH, LH, Testosterone), cytogenetic analysis with karyotyp, Y chromosome microdeletions and histological examination on testicular tissue (only in azoospermic men when testicular biopsy showed no spermatozoa)

Methods

a) Plasma hormone levels

The plasma concentrations of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were measured by immunoassay using an IM$^{®}_{X}$ ^{system} kit (Abbott Laboratories, Abbott Park, IL, USA), while plasma testosterone levels were determined by radioimmunoassay using a Coat-A-Count® Testosterone Total kit (Diagnostic Products Co., Los Angeles, CA, USA) according to manufacturer's instructions. Normal reference ranges for men are FSH 1-12 mIU/ml, LH 2-12 mIU/ml and testosterone 262-1593 ng/dl.

b) Cytogenetic Analysis

Chromosomal analysis was performed using phytohemagglutinin-stimulated peripheral lymphocyte cultures as standard cytogenetic technique. The number of metaphases analyzed followed the criteria recommended by Hook for detecting 8% mosaicism in 40 metaphases, with a confidence interval of 95%. A resolution of 550-band stage was considered satisfactory. The routine analysis was based on G-bands by trypsin using Giemsa GTG-banded staining (Moorehed and al., 1960; Hook and al., 1977)

c) Molecular analysis

Peripheral blood was collected from each patient using vacutainer EDTA-containing tube (Becton Dickinson UK Ltd, Plymouth, England). Genomic DNA was extracted from peripheral blood, according to a standard protocol (42 Godoy and al). Yq microdeletions Were performed according to the EMQN (European Molecular Genetics Quality Network) (Simoni and al., 2004). Multiplex-PCR was used to detect sequence tagged sites (STS) of AZF microdeletions, a set of 6 STS was chosen for molecular screening in two steps. In the first step, the multiplex A containing: SY86 (AZFa), SY127 (AZFb) and SY254 (AZFc) and in the second multiplex B: SY84 (AZFa), SY134 (AZFb) and SY255 (AZFc).

Each PCR multiplex was carried out in 25µl reaction volumes containing: 50ng genomic DNA, 12.5µl of master mix QIAGEN, 2.5µl of multiplex A or B. Multiplex PCR was performed under the same PCR conditions as follows: initial denaturation at 95°C for 15min followed by 35 cycles of denaturation at 94°C for 30s, annealing at 57°C for 1min 30s, extension at 72°C for 1min and a final extension at 72°C for 10min. PCR products were separated on 2% agarose gel electrophoresis, stained with ethidium bromide, and visualized using the Gel Doc system. Microdeleted sample was performed at least three times. In each multiplex PCR assay, healthy female and fertile male were used as negative and positive controls.

d) *Testicular* sperm extraction

The testicular biopsies (TESE) were performed superficially only in azoospermic patient. The tunica albuginea was incised transversely for about 5 mm in each testis proximal to the sites of the needle sampling. The testis was then gently squeezed and the protruding tissues were excised. Smears of each testicular biopsy were taken immediately to be used as an additional means of sperm identification and for cytological evaluation if no spermatozoa detected. The biopsy material was inserted into 2 separate tubes containing SPERMRINSE™ medium (vitrolife®, sweeden) and transferred to the laboratory

for sperm search and isolation. The tunica albuginea was closed using 6/0 nylon monofiber, and the layers of scrotum were sutured separately. Procedures were performed in the day-surgery clinic. After recovery from anesthesia, the men were discharged and advised to rest for 2 hours. They all received prophylactic antibiotic treatment and were re-examined 1 week later. In the laboratory, the testicular tissue samples taken from each testis were treated and examined separately. Each sample was minced using 25-gauge sterile needles. The shredded tissue was collected, centrifuged at 300 × g for 5 minutes and, after removing the supernatant, the pellet was suspended in G-IVF medium (vitrolife®, sweeden). The isolated spermatozoa were cryopreserved to perform ICSI later.

e) Statistical Analysis

*R*esults are reported as % or mean ± SD. Statistical differences between 2 means were obtained using the Student's 2-tailed unpaired t test. For multiple comparisons, 1-way analysis of variance was used. Data were considered statistically significant when $P < 0.05$. All analyses were performed using the SPSS software for Windows (SPSS, version 20, SPSS Inc., Chicago, IL, USA)

RESULTS

One hundred thirty six males were analyzed, 52 fertile males with normal semen parameters (mean age, 38.2 ± 5.2) and 84 infertile (54 azoospermic and 30 severe oligzoospermic) males (mean age, 39, 3 ± 5,6). Table 1 describes their demographic characteristics.

Cytogenetic Evaluation — Chromosome analysis was performed analyzing 40 metaphases for each patient and control. No chromosome abnormalities were detected in controls and 7 [8.3%; CI 3.9–16.4] abnormalities were identified in infertile males. Six out of 7 aberrations were detected among azoospermic men: Five were 47, XXY; and one was 46, 46, X del Y/45, X. The frequency of chromosome abnormalities in azoospermic patients was 11.1% (6/54). One severe oligozoospermic man out of 30 (3.3%) presented an abnormal karyotype [47, XXY/46, XY] Table 2).

Y Chromosome Microdeletion Screening — All 137 males were screened for the presence of microdeletions in the Y chromosome. No microdeletions were identified in any of the control males. Microdeletions were found in 8 of 84 [9.5 %; CI 4.1–17.2] infertile males. The frequency of microdeletions was 11.1% (6/54) in the azoospermic group and 6.7% (2/30) [CI 1.1 – 22.3] in the severe oligozoospermic group. Six out of 84 (71.4%) of the infertile patients had microdeletions in the AZFc region (4 azoospermic and 2 severe oligozoospermic males), 1 azoospermic male (10%) in the AZFb,c regions and one in AZFb, no deletions in the AZFa region were detected (Table2).

Testicular sperm extraction (TESE) was performed in 48/54 azoospermic patients (6 patients with no Yq microdeletion refused to continue the protocol). Spermatozoa was found in only 13 patients who had benefit of sperm cryopreservation (TESE +). For all other patients histological examination of testicular tissue was performed (Table 3). Among the 6 azoospermic patients with microdeletions: 4 had Sertoly cell only syndrome (SCOS) and 2 had maturation arrest (MA) (Table 4). The correlation between chromosomal abnormalities, cytological finding and Yq microdeletions is summarized in table 5.

DISCUSSION

Several factors, including genetic abnormalities play a crucial role in the diagnosis of male infertility. In our population there was a significant association between infertile men and duration of infertility, family infertility history, varicocele, bilateral hypotrophic testicular and higher FSH level. In general, patient's age and duration of infertility are considered important factors in making decisions regarding how to investigate, treat, and establish a reproductive prognosis. A significant association Yq chromosome micodeletions and chromosomal disorders were strongly implicated in spermatogenesis.

The frequency of Yq chromosome microdeletions and its type were associated with ethnic and regional differences (Krausz and al., 2003). Many studies had reported varying frequencies ranging from 1.3 to 55% (Osterlund and al., 2000; foresta and al., 2001; chiang and al., 2004). Many factors could explain this large variation such as ethnic and regional differences, sample selection criteria and the STS selection for the research of Yq chromosome microdeletions. In this work, we found that 9.5% of patients with azoospermia or severe oligozoospermia had chromosomal anomalies, which ranges with the reported frequency. However, our finding was higher than the previously reported value in our country; Rejeb and al., 2008, has reported 6.85%. The small and unbalanced sample, patient selection criteria, methodological aspects and other factors explain the difference between the present and the previous study.

The most frequent chromosomal alteration was 47, XXY, which was present in 5 patients (9.2% of the azoospermic patients). Autosomal chromosome abnormalities wasn't seen in our cohort. These results support others reports where sex chromosome anomalies and 47, XXY karyotype are the most frequent alterations in azoospermic males (Gardner and al., 2004; Collodel and al., 2006). Only one (3%) olgiozoospermic patient had abnormal karyotype [46, X del Y/45, X]. The association between Yq microdeletions and Primary spermatogenic failure (PSF) has been reported since the 1990s. Even though there are considerable variations in the frequencies reported, it appears that the mean is 7.6% in patients with PSF (Krausz and al., 2003.

In our population, microdeletions were found in 9.5% of infertile males. The frequency of microdeletions in the azoospermic group is 11.1%, which is higher than the 6.7% in the severe oligozoospermic group. However, these findings help us to realize that the frequency of Yq microdeletions in Tunisian infertile males is similar to that in other populations studied (Najmabadi and al., 1996; Silber and al., 1998; Kim and al., 1999; Foresta and al., 2001; Simoni and al., 2004). In this study, AZFa was not deleted, and the AZFc region was the most frequently deleted (75%) followed by the AZFb region (12.5%) and AZFb,c (12.5%). data are in agreement with the literature, in which AZFc was often described as the most frequent deletion of the Y chromosome overlapping the DAZ gene family; encoding for proteins in testicular tissue containing RNA-binding motive which regulates RNA metabolism (Hopps and al., 2003; Simoni and al., 2004).

In this study AZFc was dectected in patients with heterogeneous phenotype because 4 patients were azoospermic and 2 were severely oligozoospermic. AZFb deletions was found in one azoospermic patient and AZFb,c deletion in one other. It has been established that AZFa deletion leads to the absence of germ cells, AZFb deletion is responsible for maturation arrest and AZFc deletion leads to variable attemps of spermatogenesis.

Table 1. Demographic characteristics of patients and controls

Variables	Controls N=52	Infertile patients N=84	p
Mean age; year±SD	38.2 ± 5.2	39,3 ± 5,6	NS
Duration of Infertility; year±SD	3.5±1.4	9.2±4.1	<0.05
Tobacco, n (%)	18 (34.6)	30 (35.7)	NS
Family infertility history, n (%)	2 (3.8)	15 (17.8)	<0.05
Criptorquidia, n (%)	0	1	NS
Prostatitis, n (%)	0	2	NS
Mumps infection, n (%)	0	0	-
Sexual transmitted disease	3 (5.7)	4 (4.7)	NS
Varicocele, n (%)	1 (1.9)	10 (11.9)	<0.05
Varicocelectomy, n (%)	0	8 (9.5)	<0.05
Genital trauma, n (%)	0	1	NS
Hypotrophic testicular, n (%)	0	16 (19%)	<0.05
FSH level, mIU/ml±SD	7.22±3.14	14,52 ±11,05	<0.05
LH level, mIU/ml±SD	4.82±1.12	5,06 ±2,68	NS
Testosterone level, ng/dl±SD	531.32±112	422 ± 195	NS
Ethnicity	All Tunisian	All Tunisian	NS

Table 2. Genetic chromosomal abnormalities and Yq microdeletions

	Controls N=52	Infertile patients N=84	
		AZ (n=54)	SO (N=30)
Klinefilter syndrome homogenous (47,XXY)	0	5 (9.2%)	0
Klinefilter syndrome mosaic (47,XXY/46,XY)	0	0	1 (3.3)
46, X del Y/45,X	0	1 (1.8%)	0
AZFa	0	0	0
AZFb	0	1 (1.8%)	0
AZFc	0	4 (7.4%)	2 (6.7)
AZFb+c	0	1 (1.8%)	0

AZF: azoospermia factor, AZ: azoospermia, SO: severe oligozoospermia

Table 3. TESE findings according to Yq microdeletions in azoospermic patients

	N=48	Microdeletion (-)	Microdeletion (+)	Histological examination
TESE (+)	13	13 (100%)	0	No
TESE (-)	35	29 (82.8%)	6 (17.2%)	yes

TESE: testicular sperm extraction

Table 4. Cytological findings according to Yq microdeletions in azoospermic patients

Cytological findings	TESE (-) N=35	Microdeletion (-) N=29	Microdeletion (+) N=6
SCOS	24	20	4
MA	11	9	2

SCOS: Sertoly cell only syndrome, MA: maturation arrest, TESE: testicular sperm extraction

Table 5. Correlation between chromosomal abnormalities, histological findings and Yq microdeletions

Patient No	Age, Y	Semen analysis	Karyotype	AZF region	Histological findings
P3	47	AZ	46 XY	AZFc	SCOS
P6	38	OS	normal	AZFc	-
P13	45	AZ	46 XY	AZFc	SCOS
P22	31	AZ	46, X del Y/45,X	AZFb,c	MA
P45	29	OS	normal	AZFc	-
P62	41	AZ	normal	AZFc	SCOS
P71	46	AZ	normal	AZFc	SCOS
P81	44	AZ	normal	AZFb	MA

AZ: azoospermia, SO: severe oligozoospermia, SCOS: Sertoly cell only syndrome, MA: maturation arrest

The type of Yq chromosome microdeletion (AZFa, b, c) has been proposed as a potential prognostic factor for sperm retrieval in men undergoing multiple TESE. Same of this finding were supported by our cytological evaluation: all azoospermic and AZFc microdeleted patients showed a Sertoli Cell Only Syndrome. However, AZFb and AZFb,c showed a maturation arrest

CONCLUSION

Our study shows that genetic abnormalities and Yq microdeletions in infertile Tunisian patients are frequent and similar to those reported in other countries. However, the authors recognize that the studied population described here is not representative of the total Tunisian population of infertile men. More studies including men of different ethnicities or from different geographic areas are needed to determine whether the frequencies of genetic abnormalities are much variable in different infertile populations.

Moreover, the knowledge of the existence of genetic abnormalities and microdeletions is useful to provide a correct diagnosis of male infertility, it allows the physician to refer the patient to adequate assisted reproduction technique and examine the value of testicular biopsy pertinence. Before using IVF/ICSI genetic counseling should be provide, it must be considered that the son of a man with microdeletion may inherit this abnormality.

DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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