Cytogenetic Investigation in a Group of Ten Infertile Men with Non-Obstructive Azoospermia: First Algerian 46, XX Syndrome

*Meriem BAZIZ [1], *Zohra HAMOULI-SAID [1], Ilham RATBI [2], Mohamed HABEL [3], Soukaina GUAOUA [2], Aziza SBITI [4], Abdelaziz SEFIANI [2,4]*

1. L.B.P.O/Section Endocrinology, Faculty of Biological Sciences, University of Sciences and Technology Houari Boumedienne, BP 32, EL ALIA, Bab-Ezzouar, Algiers, Algeria
2. Human Genome Center, Faculty of Medicine and Pharmacy, University Mohammed V, Rabat, Morocco
3. Feconde Clinic El Bordj, Bordj-El Kiffan, Algiers, Algeria
4. Department of Medical Genetics, National Institute of Health, Rabat, Morocco

Corresponding Author: Email: hamoulisaid@gmail.com

Abstract

Background: In Algeria, the data on infertility and its various causes are rare. Recently, the introduction of assisted reproduction has allowed expecting that 300000 couples, which represent 7% of couples of reproductive age, face difficulty conceiving a child. Knowing that most idiopathic cases are likely to be due to chromosomal abnormalities, we aimed to investigate genetic defects by karyotype analysis in Algerian infertile men, using peripheral blood lymphocytes.

Methods: A cytogenetic study was conducted on 10 men from infertile couples by Karyotype analysis of R-banding performed by lymphocyte culture technique. Fluorescence in situ hybridization was performed and molecular abnormalities were investigated by polymerase chain reaction. Follicle stimulating hormone (FSH) and luteinizing hormone (LH) levels were evaluated by immunoradiometric method.

Results: Chromosomal abnormalities were observed in 30% of the patients. We identified a homogenous Klinefelter syndrome patient with 47, XXY karyotype, a mosaic Klinefelter syndrome patient with 47, XXY/46, XY karyotype and a 46, XX male. Fluorescence in situ hybridization showed that the sex-determining region Y was translocated to the short arm of the X chromosome in patient with 46, XX chromosomal constitution and the presence of the SRY gene was confirmed by polymerase chain reaction and electrophoresis.

Conclusion: The occurrence of chromosomal abnormalities in 30% of the infertile men strongly supports the inclusion of routine cytogenetic testing for diagnostic establishment and suitable counseling for couples seeking for assisted reproduction technologies.

Keywords: Male infertility, Cytogenetic, Azoospermia, Severe oligozoospermia

Introduction

According to the World Health Organization (WHO) infertility is defined as the inability of a couple to conceive after 24-months period of regular unprotected intercourse and 8-12% of couples around world experience difficulty conceiving a child (1). In Algeria, the data on infertility and its various causes are rare. Recently, since the intro-

duction of assisted reproduction, the statistics revealed that 300000 couples, 7% of couples of reproductive age, have difficulty conceiving a child (2). Only two studies, reported in the literature, were conducted in East of Algeria (3, 4). Absence of data in the Algerian population is probably related to the fact that the woman was considered, for a

long time, solely responsible for the delay of pregnancy and to the late introduction of assisted reproductive technologies. However, since their apparition twenty years ago, the involvement of man has been clearly demonstrated and began to be accepted. "Indeed, male factor infertility or subfertility is responsible for up to 50% of cases" (5). Investigation of male infertility includes clinical diagnosis, semen analysis, endocrine evaluation (serum total testosterone, luteinizing hormone an dfollicle stimulating hormone levels) and microbiological assessment. However, the underlying cause of male infertility remains undefined in nearly 50% of cases referred to as idiopathic infertility (6). Since most idiopathic cases are likely to be of genetic origin, some tests may be useful for a few patients to identify a male factor contributing to unexplained infertility or to select therapy or assisted reproduction technology (ART). Indeed, karyotyping should be offered to men with i/non-obstructive azoospermia; ii/severe oligozoospermia less than 10^6 sperm/ml over successive samples; iii/ oligozoospermia with a count greater than 10^6 sperm/ml associated with a family history of reproductive disorders (7).

This study explored genetic abnormalities in ten men consulting for infertility in the central region of Algeria.

Materials and Methods

Patients
This cytogenetic study was conducted on 10 men selected from infertile couples consulting in an Algiers's center of ART (Feconde Clinic El Bordj) between Jan and May 2014 after clinical investigation and semen analysis. All patients presented primary infertility of one year or more, non-obstructive azoospermia and female partners without abnormalities. The average age of the selected patients was 45±9.06 yr (range 33-64 yr). This investigation was approved by the Ethics Committee of University of Science and Technology Houari Boumedienne, in accordance with the revised declaration of Helsinki (8). Blood samples were collected and analyzed after the written informed consent was obtained. The clinical characteristics and hormonal levels of each patient are presented in Table 1.

Table 1: Clinical characteristics and endocrine hormone levels

Patients	Age (yr)	Anomalies	FSH (IU/L)	LH (IU/l)	Height (cm)	Weight (Kg)
P1	50	Varicocele at the left, right testicular hypotrophy, diabetes	19,20	7,04	175	95
P2	44	Bilateral testicular atrophy, gynoid appearance	51,01	11,71	165	62
P3	41	Bilateral varicocele, bilateral testicular atrophy	30,04	17,54	173	85
P4	40	Sexual impotence	11,68	3,88	165	93
P5	44	Testicular ectopia	14,09	4,58	180	98
P6	51	Bilateral varicocele	8,05	6,68	175	62
P7	38	Without clinical anomalies	9,0	5,74	173	74
P8	39	Left testicular hypotrophy, ectopia, absence of right testicle	62,12	17,59	163	100
P9	64	Without clinical anomalies	45,59	26,69	176	79
P10	33	Bilateral varicocele	21,31	4,53	183	90

Karyotype analysis of R-banding in lymphocytes
A constitutional karyotype R band according to a specific protocol of National Institute of Health

(Morocco) was performed for each patient. Lymphocytes separated from the peripheral blood were cultured at 37 °C in Karyotyping medium PB-MAX™ (Gibco, Life Technology, USA) for 72

h and colchicine (100μl) was added to incubate for 50 min. After incubation, the centrifuge tubes in which the cultures are set were centrifuged at 1500 rpm/min for 5 min. Mitotic cells were harvested and dispersion of chromosomes is obtained by incubation in hypotonic buffer (KCl). Then, centrifugation is repeated followed by addition of 3:1 methanol-acetic acid, which acts as a fixative. After centrifugation, spread over wet slides was made and stored at 37 °C for minimum 45 min for aging process, heat denatured in a phosphate buffer and stained with 4% Giemsa for the microscopic observation. The classification of chromosomes for at least 11 mitoses has been performed using semi-automatic Cytogen software; chromosomal abnormalities were reported according to the recommendations of the International System for human Cytogenetic Nomenclature (ISCN) 2013.

SRY Fluorescence in Situ Hybridization

FISH analysis on proband metaphases was performed using SRY and X centromeric probes (Vysis SRY Probe LSI SRY Spectrum Orange/CEP X Spectrum Green; Abbot Molecular, USA).

Polymerase chain reaction

Y-chromosome sequence analysis was conducted by PCR. Genomic DNA was extracted from the cells using a Qiagen kit (Germany). To ascertain the presence of sex-determining region Y (SRY), gene SRY was amplified using Bioline® buffer (50X), a MyTaq™ DNA polymerase Bioline®, 5′-GTCG-CACTCTCCTTGTTTTTGAC-3′ as forward primer and 5′-CCGATTGT-CCTACAGCTTTGTC-3′ as reverse primer. After PCR amplification, reaction products (7μl) were submitted to 1% agarose gel electrophoresis prepared with ethidium bromide (40mg/ml). DNA of a man (46, XY), woman (46, XX), a negative control and a size marker (11037421 DNA Molecular Weight Marker XIV, 100 base pair ladder, Roch ® Diagnostics GmbH, Mannheim, Germany) were used.

Hormonal evaluations

Serum concentrations of follicle stimulating hormone (FSH) and luteinizing hormone (LH) were measured by immunoradiometric assay using kits (FSH: IM2125-IM3301, LH: IM1381-IM3302; Beckman Coulter®, Germany). Samples or calibrators are incubated in tubes coated with the first monoclonal antibody in the presence of the second monoclonal antibody labeled with iodine 125. After incubation, the contents of the tubes are rinsed to remove unbound ^{125}I-labeled antibody. The bound radioactivity is then determined in a gamma counter.

Results

Karyotype analysis of R-banding in lymphocytes

As summarized in Table 2, seven of the ten patients (70%) tested to rule out cytogenetic causes of male infertility had a 46, XY karyotype and three had abnormal karyotypes (30%) including an homogenous Klinefelter syndrome patient with 47, XXY karyotype (P3; Fig. 1), a mosaic Klinefelter syndrome patient with 47, XXY/46, XY karyotype (P9; Fig. 2) and a 46, XX male (P2; Fig. 3). Concerning the P9 patient, 11 cells were analyzed: 6 with 47, XXY and 5 with 46, XY were detected, 47, XXY [6]/46, XY [5].

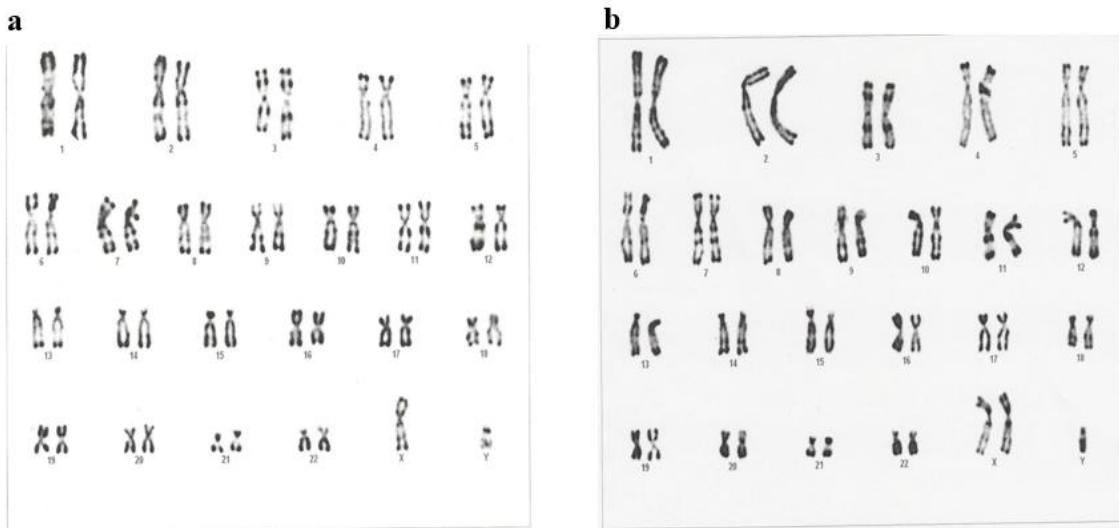**Fig. 1:** Constitutional karyotype of patient P3

a

b

Fig 2: Constitutional karyotype of patient P9 with mitosis in 46, XY (**a**) and mitosis in 46, XXY (**b**)
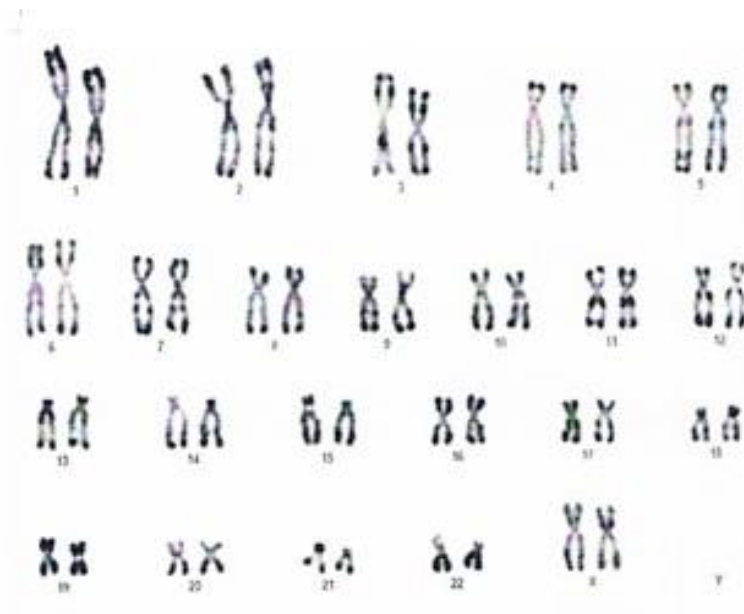
Fig. 3: Constitutional karyotype of patient P2

SRY Fluorescence In Situ Hybridization and Polymerase chain reaction

SRY FISH and molecular analysis have been performed for the phenotypic male P2 with 46, XX chromosomal constitution. FISH analysis has allowed the detection of *SRY* gene on one of the two X chromosome in all metaphases (Fig. 4) and the nuclei observed. Electrophoresis of PCR amplification products showed a 845bp *SRY*-gene-specific fragment (Fig. 5). According to ISCN 2013 (9), the P2's final formula is 46 XX.ish der (X) t (X, Y) (p22.3; p11.3) (SRY +).

Table 2: Karyotypes analysis results

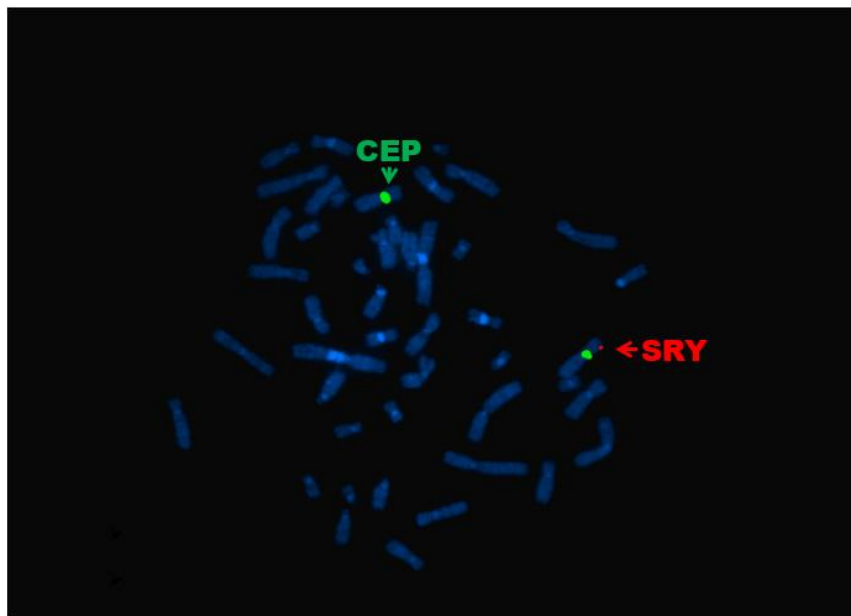| Patients	Karyotype	Diagnostic	Final formula (according to the ISCN nomenclature 2013)
P1	46,XY	-	-
P2	46,XX	46, XX male	*46 XX.ish der (X) t (X, Y) (p22.3; p11.3) (SRY +)*
P3	47,XXY	Homogenous Klinefelter syndrome	*47,XXY*
P4	46,XY	-	-
P5	46,XY	-	-
P6	46,XY	-	-
P7	46,XY	-	-
P8	46,XY	-	-
P9	47,XXY/46,XY	Mosaic Klinefelter syndrome	*47,XXY[6]/46,XY[5]*
P10	46,XY	-	-

Fig. 4: Fluorescence in situ hybridization (FISH) result. FISH analysis was performed on metaphase spreads of P2 and showed a copy of CEPX on each X chromosome and a copy of sex-determining region Y (SRY) on one chromosome X
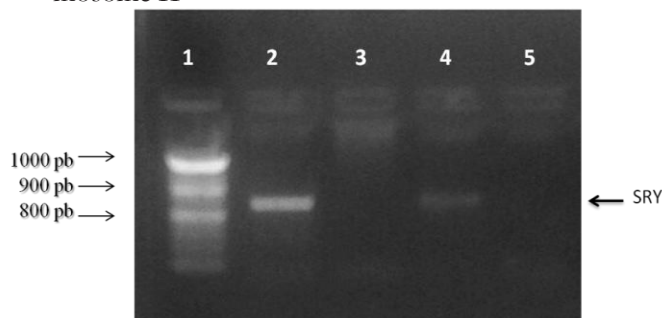

Fig. 5: Electrophoresis of the PCR products. Line **1**: seize markers; line **2**: SRY amplification of P2 (46, XX male); line **3**: female control (46, XX); line **4**: male control (46, XY); line **5**: negative control

Levels of FSH and LH hormones

Seven and four of the ten patients showed high levels of FSH and LH respectively. The mean FSH was 27.21±6.42 IU/L (normal: 1.3-11.5IU/L) and mean LH, 10.60±2.55 IU/L (normal: 0.5-10 IU/L).

Discussion

In our study, constitutional chromosomal abnormalities were identified in 30% of infertile patients. Idiopathic infertility found in most cases of non-obstructive azoospermia or severe oligozoospermia is due to chromosomal abnormalities or mutations of genes involved in sex determination and spermatogenesis (6). The incidence of cytogenetic abnormality has been estimated at 5.8% in infertile men and only 0.5% in the normal population (10).

Our study was designed to explore the implication of chromosomal abnormalities, which play a prime role in male infertility with abnormal semen parameters, in a small population recruited in Feconde clinic El Bordj in Algeria. Klinefelter syndrome, present with a variety of subtle age-related clinical signs, is a group of chromosomal disorders in which there is at least one extra X chromosome to a normal male karyotype, 46, XY. 47,XXY aneuploidy is the most common disorder of sex chromosomes in humans, with a prevalence of 1 in 500 males (11). Our patients with Klinefelter syndrome are 41 and 64 yr old indicating that this syndrome isn't often diagnosed until adulthood when men seek medical advice on small testes or for a difficulty conceiving a child, since most men with Klinefelter syndrome produce little or no sperm. Indeed, genetic abnormalities particularly Klinefelter's (47, XXY) as well as Y chromosome microdeletions have severe adverse influence on normal hormone levels, testicular volume and sperm count (12). The two patients with 47, XXY and 46, XY/47,XXY karyotypes presented an hyper gonadotropic hypogonadism, a negative testicular biopsy and had no spermatozoa in semen. However, in all cases, germinal aplasia must be confirmed histologically since some cases of Klinefelter syndrome present focal spermatogenesis histologically and can benefit from ICSI technique with a pre-implantation genetic diagnosis in order to father a child (13). Only patient 3 with 47, XXY karyotype, presented anatomical anomalies with a bilateral varicocele and bilateral testicular atrophy.

In literature, men with mosaic Klinefelter syndrome appeared to be more androgenized than those with non-mosaic Klinefelter syndrome, with lower baseline LH levels and similar FSH level (14). At the contrary, in this study, the patient with mosaic Klinefelter syndrome presented higher LH and FSH values when compared with non-mosaic Klinefelter syndrome counterpart. Our third patient was a phenotypically normal male with normal external genitalia, pubic hair, penile size and sexual life, gynoid appearance, high levels of FSH and LH hormones, no hypospadias, gynecomastia or cryptorchidism and a SRY-positive 46, XX karyotype reported for the first time in Algerian population. The incidence of Sex Reversal Syndrome is 1: 20000 in newborn males (15) and despite patients with 46, XX karyotype have male external genitalia, they generally have small testes and may also have abnormalities such as cryptorchidism or hypospadias, azoospermia, hypergonadotropic hypogonadism, varying degrees of gynaecomastia, poor facial hair growth, diminished libido and normal intelligence evaluation (16, 17). The first case was described in 1964 by De la Chapelle in France (18) as one of the rarer causes of ambiguous external genitalia or primary infertility in phenotypic males. Since then, several other cases have been reported in different regions worldwide. During the two last decades, cases from China (15, 17, 19, 20), Japan (21, 22), Turkey (23, 24), Romania (25), Spain (26), Pakistan (27), Mexico (28), Middle East (29), Kuwait (30) and Tunisia (31) have been reported. Both Klinefelter and 46, XX patients have small testes, elevated gonadotropins and low to normal testosterone levels. However, overall men 46, XX were reported to be much shorter than Klinefelter patients or healthy men (32). In our study, height of patient with 46, XX syndrome (P2) was 165 cm while those of homogenous Klinefelter syndrome

and mosaic Klinefelter syndrome were 173 and 176 cm respectively.

There are various phenotypic properties of 46 XX male patients, some are accompanied by micro deletion of Y chromosome as shown by Xia et *al* who presented the case of 4 patients of short stature with 46, XX, SRY-positive, testicular atrophy and absence of AZFa, b and c of the Y chromosome (20*).* This suggests a fortuitous association between the microdeletions of Y chromosome and different testicular abnormalities. The 46, XX karyotype with normal phenotype is consequences of sex reversing translocation due to an unequal meiotic recombination of the distal X and Y short arms during male gametogenesis (33). A breakpoint has been characterized within a protein kinase gene, PRKY, previously described as a hotspot of ectopic recombination between homologous regions on X and Y chromosomes during male meiosis (34, 35). Much rarer, the SRY autosomal translocation could have infertility and lead to the XX male syndrome (36). The sex-determining region of the Y or the SRY, was thought to be the master regulator of sex determination knowing that the presence of just this region from the Y chromosome is sufficient to cause male development (37) but a description of normal male phenotype in 46, XX in absence of SRY (38) and ambiguous external genitalia both in 46,XX SRY positive and SRY negative patients (39, 40) led to the conclusion that the XX males may be divided in 3 groups : i/ the 46,XX SRY positive patients with male differentiation (80% of cases), ii/ the 46, XX SRY negative subjects with abnormal development due to other genes than SRY; the presence of testicular tissue may be the result of a mutation in a gene that functions in the cascade triggered by SRY (10%) and iii/ XX/XY chromosomal mosaïcism (10%) (41).

In this study, 4 of the 10 patients (P1, P5, P8, P10) have non-obstructive azoospermia, high levels of FSH, testicular abnormalities (Varicocele, testicular atrophy, testicular ectopy…) and normal karyotypes. Wang et al. have investigated the relationship between FSH and AZF microdeletions on Y chromosome and concluded that this micro deletion was one of the important causes of high level of FSH (42). This is confirmed by a team from East of Algeria who searched for micro deletions of Y chromosome in 80 patients and revealed a deletion of the region AZFc in one patient (1.3%) with non-obstructive azoospermia and high levels of FSH (4).

Conclusion

We reported a case of an infertile 46, XX male in Algeria and showed that the establishment of a karyotype with a testicular biopsy is an important step for the diagnosis, further investigations orientation, early replacement therapy and proper counseling for couples seeking for ART.

Ethical considerations

Ethical issues (including plagiarism, data fabrication or falsification, double publication or submission, informed consent, etc.) have been completely observed by the authors.

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