

Genetic screening (Chromosomal abnormalities, Y chromosome deletions and Androgen receptor CAG repeat length) in infertile Mexican men.

Running head:

Genetic screening in infertile men.

Authors:

Sandra Guadalupe Martínez-Garza
Mayra Celina Gallegos-Rivas
Marcos Vargas-Maciél
Juan Manuel Rubio-Rubio
Mario Espinosa de los Monteros-Rodríguez
Claudia González-Ortega
Patricia Cancino-Villarreal
Luis G. Vazquez de Lara*
Antonio Martín Gutiérrez-Gutiérrez

Institutions:

Instituto de Ciencias en Reproducción Humana (Instituto Vida)
Plaza las américas 115
Col. Jardines del Moral,
León, Guanajuato, México CP 37160

*Facultad de Medicina, Benemérita Universidad Autónoma de Puebla.
Calle 13 Sur 2702, col. Volcanes
Puebla, Puebla, México CP 72410

To whom the correspondence should be addressed

Sandra Guadalupe Martínez-Garza
Instituto de Ciencias en reproducción Humana Vida (Instituto Vida)
Plaza las américas 115
Col. Jardines del Moral,
León, Guanajuato, México
CP 37160
Tel/Fax: (477) 779-0835/36/37
Email: samartin30@yahoo.com

ABSTRACT

We analyzed chromosomal abnormalities, Y-chromosome deletions, androgen receptor CAG repeat length and its association with defective spermatogenesis in infertile Mexican men. Eighty-two infertile patients and 40 controls were screened for karyotypic abnormalities, Y-chromosome microdeletions and CAG repeats. Nine infertile males (11%) carried chromosomal abnormalities and 10 (12.2%) presented Y chromosome microdeletions. Mean CAG repeat length was 21.6 and 20.88 base pairs in idiopathic infertile males and controls, respectively. Chromosomal aberrations and Y-chromosomal microdeletions are related to male infertility in our population. Expansion of the CAG repeat segments of the androgen receptor is not correlated with male idiopathic infertility.

KEY WORDS

Oligospermia, karyotype, microdeletion, trinucleotide repeat.

INTRODUCTION

Around fifty percent of the problems in infertile couples are due to male factor (Wong *et al*, 2000). In these patients, severe oligozoospermia or azoospermia are frequently observed and most of them undergo some kind of assisted reproductive technique (ART). Direct injection of single spermatozoa into the cytoplasm of an oocyte (ICSI) is the current standard technique. ICSI has high success rates even in cases of severely impaired spermatogenesis. Even though this method allows infertile males to have their own child without knowing the cause of their infertility, it also carries the potential risk of transmission of genetic aberrations to the descendants. The cause of alterations in sperm production is unclear, and recent work is focusing on environmental and genetic causes. Among the genetic causes of male infertility that can be passed on to the offspring are cytogenetic abnormalities, Y chromosome microdeletions and CAG repeats in the androgen receptor (AR) gene (Gottlieb *et al*, 2005; Carrell *et al*, 2006). The incidence of chromosomal aberrations in azoospermic males has been found to be as high as 20%, being the sex chromosomes more commonly involved (Wong *et al*, 2000). On the contrary, aberrations have been shown to be less frequent among oligozoospermic males (0 – 8%) and when present, they are found mainly in the autosomes (Elghezal *et al*, 2006; Levron *et al*, 2001).

Azoospermia Factor (*AZF*) is a region in the Y chromosome that encodes genes necessary for normal spermatogenesis. Small deletions in this region can be detected by using molecular biology techniques [for example, analyzing STS (sequence tagged sites) markers]. Extensive physical, functional and genetic analyses of the Y chromosome have now identified three *AZF* regions (*AZF*a, *AZF*b and *AZF*c), which encode spermatogenic genes such as *USP9Y*, *RBM1Y*, *BPY2* and *DAZ* (Deleted in AZoospermia) (Vogt, 1997). However the refinement of Yq mapping and the availability of a complete Y chromosome DNA sequence (Skaletsky et al, 2003) emerges a new classification of Y chromosome deletions: *AZF*a, *AZF*b (P5/proximal P1), *AZF*b_c (P5/distal P1 or P4/distal P1) and *AZF*c (b2/b4), used for clinical diagnostic purposes (Simoni *et al*, 2004). Several groups found Y microdeletions in azoospermic and oligozoospermic patients with an incidence range of 7-21% and 0-14%, respectively (Foresta *et al*, 2001; Najmabadi *et al*, 1996; Silber *et al*, 1998; Kim *et al*, 1999). Although genotype-phenotype correlations have been difficult to establish, multiple studies support the idea that Y microdeletions are a common cause of spermatogenic failure. Complete deletions of *AZF*b and *AZF*b_c (P5/proximal P1, P5/distal P1, P4/distal P1) are characterized by a histological picture of SCO (sertoli cell only) or spermatogenetic arrest resulting in azoospermia (Simoni *et al*, 2004). In oligozoospermic patients, *AZF*c deletions had been associated with a decline in sperm production over time (Simoni *et al*, 1997; Girardi *et al*, 1997). In general, *AZF*c deletions are compatible with residual spermatogenesis. *AZF*c deletions can be found in men with azoospermia or severe oligozoospermia (simoni *et al*; 2004). Detection of Yq microdeletions are very important, not only to define the cause of spermatogenic failure, but also because these patients need genetic counseling since this chromosome defect can be transmitted to 100% of the male offspring.

It is well known that androgens determine male sexual differentiation and also promote the initiation and maintenance of spermatogenesis. Androgens act on target cells through the androgen receptor (AR). The AR gene is located in the X chromosome at Xq11-1. The AR gene has 8 exons that encode 3 protein domains: transactivation domain (exon 1), DNA binding domain (exons 2 and 3) and ligand binding domains (exons 4-8). A polymorphic CAG (glutamine) repeat sequence is in exon 1. The AR-CAG repeat region is unstable and its length may sometimes undergo expansion or contraction during meiotic DNA replication. Mutations in AR gene cause various degrees of androgen resistance, resulting in wide androgen insensitivity syndromes (from 46, XY sex-reversed infertile women to phenotypically normal 46, XY infertile males with severe oligozoospermia or azoospermia) (Mifsud *et al*, 2001; Milatiner *et al*, 2004; Wallerand *et al*, 2001). *In vitro* studies have demonstrated a negative correlation between CAG repeat size and AR function, specially in terms of transcriptional activity (Chamberlain *et al*, 1994). There are some clinical studies showing that longer CAG repeats are associated with defective spermatogenesis (Komori *et al*, 1999; Mengual *et al*, 2003; Mifsud *et al*, 2001;

Wallerand *et al*, 2001; Mengual *et al*; 2003 Milatiner *et al*, 2004; Katagiri Y *et al*; 2006); however, other studies have failed to show a significant correlation (Dadze *et al*, 2000; Thangaraj *et al*, 2002; Asatiani *et al*, 2003; Ruhayel *et al*; 2004; Singh R *et al*; 2006). Since this association may be dependant of the population studied and local environmental conditions, we aimed to study the CAG repeat size in the Mexican population and determine if it correlates with abnormal sperm counts.

In this work, we investigated chromosomal abnormalities, Y chromosome deletions, androgen receptor CAG repeat length and its association with defective spermatogenesis in infertile Mexican men.

MATERIALS AND METHODS

Patients.

Patients were recruited consecutively from the Instituto de Ciencias en Reproducción Humana Vida. The population consisted of 82 infertile Mexican males with abnormal semen analysis according to World Health Organization (WHO) criteria ($\leq 15 \times 10^6/\text{mL}$) who were planning to undergo any assisted reproductive technique due to male factor infertility. All cases of azoospermia/oligozoospermia resulting from endocrine or obstructive causes were excluded from our study. The control group consisted of 40 individuals with normal semen analysis who were used as donors at our semen bank. All of them had proven paternity. Only patients and controls born in Mexico and with mexican parents were included in this study. All were from the center of the country and represent a homogenous group of mixed race people named “mestizos”. All participants gave informed consent according to the protocol approved by the ethics review board. Peripheral blood samples were drawn with heparin and EDTA and stored at 4°C until cell culture and DNA isolation were done.

Cytogenetic analysis.

Chromosome analysis was performed on peripheral lymphocytes cultures. Cultured cells were treated with colchicine to obtain prometaphase chromosomes. The GTG banding technique was applied and 20 metaphases were counted in each patient and control.

Molecular analysis.

DNA isolation was performed using the TSNT lyses buffer (1% Triton, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl pH 8.0, and 1 mM EDTA) followed by phenol-chloroform extraction. DNAs were diluted and stored at 4°C before analysis.

The Y Chromosome Deletion Detection System, version 1.1 (Cat. No. MD1101, Promega, Madison, WI, USA) was used to determine the presence or absence of 18 STS by performing 4 PCR amplifications. This kit was available when these patients were tested. Commercial protocols were followed to perform

PCR and electrophoresis. Multiplex PCRs were repeated whenever a deletion was found to confirm the results. This kit analyzed AZFb (SY121, SYPR3, SY124, SY127, SY128, SY130, SY133), AZFc (SY145, SY153, SY152, SY242, SY259, SY208, SY254, SY255, SY157). We also decided to use SY84 and SY86 to analyze AZFa plus SY134, to analyze the complete AZFb according to Simoni.(Simoni M, *et al*, 2004).

CAG repeats in exon 1 of the AR gene were generated as described by Dadze *et al.*, with some modifications (Dadze *et al*, 2000) and only patients with normal cytogenetic analysis and without microdeletions were analyzed. One hundred ng of DNA were used in a single 15 μ l PCR containing 1X PCR buffer, 0.2 mM of each dNTP, 1.5 mM MgCl₂, 0.5 U Taq DNA polymerase (Promega, Inc.), 0.2 μ M of each AR-1/AR2 primer (Invitrogen, life technologies, Carlsbad, California, USA). PCR conditions were an initial denaturation step at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 45 sec, annealing at 68°C for 1min 30 sec. Again, a final step of extension at 68°C for 5 min was added. PCR products were electrophoresed through 2% agarose gel to confirm amplification. PCR products were mixed with an equal amount of loading buffer, denatured at 95°C for 4 min and put on ice before loading. Denatured reactions were separated on a 6% urea-polyacrylamide gel with 0.5X TBE at 250 V for ~ 10 hrs, followed by silver staining. The size of the PCR bands was determined by comparing the size of this band with the size of sequenced PCR products containing CAG repeats of known length kindly donated by Dr. M. Aevizaki. Quantity One Quantitation Software of Bio-Rad was used to determine the size of PCR products.

Statistical analysis.

Unless otherwise stated, results are reported as mean \pm SD. Statistical differences between two means were obtained using the Student's two-tailed unpaired t-test. For multiple comparisons, one way analysis of variance was used. Data was considered statistically significant when $p < 0.05$.

RESULTS

One-hundred twenty-two males were analyzed, 40 fertile males with normal semen parameters (mean age 21.5 ± 1.9) and 82 infertile males (mean age 32.2 ± 5.2), table 1 describes their demographic characteristics. Infertile males were classified according to concentration of spermatozoa in semen analyses. Four patients had moderate oligospermia (5-15 million cells/mL), 28 patients showed severe oligospermia (< 5 millions cells/mL), and 50 were non obstructive azoospermic males. Table 2, illustrates the proportion of men undergoing each genetic test.

Cytogenetic evaluation

Chromosome analysis was performed analyzing 20 metaphases for each patient and control. No chromosome abnormalities were detected in controls and 9 [11% (CI 4.2-17.8)] abnormalities were identified in infertile males (Table 3). Eight out of 9 aberrations were detected among azoospermic men: five were 47,XXY; one was 47,XYY; one was 46,XY, Yq- and one was 46, XY inv (9). The frequency of chromosome abnormalities in azoospermic patients was 16% (8/50). One severe oligozoospermic man out of 28 (3.6%) presented an abnormal karyotype [46XY t(1;15)Lq(12;q25)].

Y chromosome microdeletion screening

All 122 males were screened for the presence of microdeletions in Y chromosome. No microdeletions were identified in any of the control males. Microdeletions were found in 10 of 82 [12.2% (CI 5.1-19.3)] infertile males (Table 3 and Figure 1). The frequency of microdeletions in the azoospermic group was 12 % (6/50) and in severe oligozoospermic group was 14.3% (4/28) (CI 1.3-27.3). 70 % of the infertile patients had microdeletions in the *AZFc*, region (b2/b4) (3 azoospermic and 4 severe oligozoospermic males), 20% (2 Azoospermic males) in the *AZFb* region, and 1 azoospermic male (10%) in the *AZFb,c* regions. No deletions in *AZFa* region were detected. The larger microdeletion involving 2 complete *AZF* regions (b and c) was detected in an azoospermic male and this finding was observed in his karyotype (46XY, Yq-). Also, one patient presented both a microdeletion and an abnormal karyotype [46 XY inv(9)].

CAG repeat length analysis

Patients with chromosomal abnormalities and microdeletions were excluded from the CAG repeat analysis. This study included 65 DNA from patients with idiopathic infertility. After analyzing gels, we were able to identify 15 and 13 different alleles in the infertile and control group, respectively. The frequency distribution of the alleles in both groups is depicted in the figure 2. The most common allele was 20 (19.7%) followed by 22 (18.1%) in the fertile group while in controls it was 23 (17.5%) followed by 19, 20 and 22 (15%). The mean CAG repeat length was 21.6 ± 3.39 (range 11-35) and 20.88 ± 3.19 (range 10-28) in infertile males and controls, respectively ($p=0.256$). There was no correlation between severity of impairment of spermatogenesis and CAG length (Table 4).

DISCUSSION

Primary spermatogenic failure (PSF) accounts for more than half the cases of infertility due to the male partner, yet, the pathogenesis of this condition is poorly understood. In this work, we found that 11% of patients with PSF had chromosomal anomalies, which is not different from other reports in the

literature (CI95% 4.2-17.8). On the other hand, the prevalence of chromosomal aberrations in the group of azoospermic males in this work is among the highest reported (16%) and sex chromosomes were the only altered chromosomes, assuming that only one patient for this group had a karyotype 46XY inv(9) and this alteration is considered a polymorphism (Gardner RJM and Sutherland GR, 2004), however, Collodel G et al. (Collodel *et al*, 2006) analyzing semen samples of 18 inv(9) carrier males found 5 patients with azoospermia and two of them also carried Y microdeletions, same as our patient. The most frequent alteration was 47XXY and was present in 5 patients. These results support previous reports where sex chromosome anomalies and 47XXY karyotype are the most frequent alterations in azoospermic males. Only one (3.6%) oligozoospermic patient had an abnormal karyotype [46XY t(1;15)Lq(12;q25)] where two autosomes were involved.

Our findings support the previous notion that abnormalities in sex chromosomes are primarily involved in azoospermic patients, while balanced autosomal anomalies are the most frequent in oligozoospermic males (Elghezal *et al*, 2006).

The association between Yq microdeletions and PSF has been reported since 90s. Even though there are considerable variations in the frequencies reported, it appears that is around 7.6% in patients with PSF. Our population has a frequency of microdeletions in severe oligozoospermic group (14.3%) higher than azoospermic group (12.2%), however these frequencies are similar to previously reports. (Simoni *et al*, 2004, Foresta *et al*, 2001; Najmabadi *et al*, 1996; Silber *et al*, 1998; Kim *et al*, 1999).

In this study, AZFa was not deleted and AZFc region was the most frequently deleted (70%) followed for AZFb region (20%) and AZFb,c (10%); this is in agreement with the literature, where the most commonly affected region reported is AZFc (Hopps *et al*, 2003; Simoni *et al*, 2004). We found that deletions of AZFc region were all b2/b4, the phenotype of these patients are heterogeneous because 3 patients were azoospermic and 4 were severe oligozoospermic. These results are similar to those where a variable clinical and histological phenotype have been found (Reijo *et al*, 1996; Oates *et al*, 2002).

AZFb deletions were found in 2 azoospermic patients: patient 2 has missed only sy128, while patient 15 has missed sy121 and sy128. Both patients have amplified sy127 and sy134 indicating that AZFb deletions are partial. However, with the methodology used it is not possible to determine if the deletions correspond to the P5/proximal P1 pattern.

Complete AZFb,c deletion was found also in an azoospermic patient. This deletion was indicated by the lack of amplification of sy127, sy134, sy254 and sy255; however, because of the methodology employed, we were not able to identify P5distalP1 or P4/distal P1 patterns.

These findings help us to realize that frequency of Yq microdeletions in Mexican infertile males are similar to others studied populations (tabla 5).

The role played by androgens in spermatogenesis regulation is well established. Some groups from Spain (Mengual *et al*, 2003), France (Wallerand *et al*, 2001), USA, Singapore (Mifsud *et al*, 2001) and Japan (Komori *et al*, 1999) found an association between longer CAG repeat and low sperm count, while studies from Germany (Asatiani *et al*, 2003; Dadze *et al*, 2000), India (Thangaraj *et al*, 2002) and Israel (Milatiner *et al*, 2004) have not shown a significant correlation. A recently metaanalysis (Davis-Dao *et al*, 2007) using 33 published studies found association of CAG repeat length in AR and male infertility, the mean difference (95% confidence interval) was 0.19 (0.09-0.29) and 0.31 (0.1400.47) for a subset of 13 studies with more stringent case and control criteria. It seems that this association varies according to patient ethnicity: however not statistically significant differences were found in Davis-Dao *et al*, stratified analysis for race/ethnicity.

Our study investigated the association between number of CAG repeats and sperm counts in infertile Mexican males. In this context is necessary to clarify the Mexican ethnicity from a Genomic point of view. The recently created Mexican National Center of Genomic Medicine (<http://www.inmegen.org.mx/>) is performing an analysis of the Mexican population genetic background for the first time, at a big and high throughput scale. This study has revealed that Mexicans are represented by Mestizos resulting from an admixture of Amerindian, Spaniards and, at a lesser extent, African populations, giving a unique genetic component. Around 65% of the Mexican genetic background can be classified as “amerindian”, the result of a mixture of 35 ethnic groups (unpublished observations). This preliminary findings show the importance of our infertility results from an ethnical point of view and warrants further investigation in genetics particularities of male infertility in Latino populations.

Men were first screened for genetic chromosomal aberrations and Yq microdeletions to eliminate genetic factors known to be correlated with infertility. We found no differences in the mean number of CAG repeats between infertile [21.6 ± 3.39 (range 11-35)] and controls [20.88 ± 3.19 (range 10-28)] men. The infertile group was further subdivided according to sperm counts and no differences were found in any subgroup when compared to controls. These results are in agreement with the studies where no association was found.

In conclusion, our study shows that genetic abnormalities and Yq microdeletions in infertile Mexican patients are frequent and similar to those reported in other countries. We found no difference in CAG repeats between patients with PSF and normal sperm donors; accordingly, we did not find a correlation between CAG repeats and sperm count. We suggest that genetic chromosomal abnormalities and Yq microdeletions be analyzed in all patients who undergo any assisted reproductive technique.

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FIGURE LEGENDS

Figure 1. Schematic map of Y chromosome with STS used and deletions detected.

Figure2 . Distribution of the CAG repeat sizes in the androgen receptor gene of infertile men (squares) and fertile controls (triangles).

Table 1. Demographic characteristics of patients and controls

	Controls	Infertile Patients
n	40	82
Sperm Count (million/mL)	52.0±21.1	1.72±3.38
ethnicity	All mestizos	All mestizos

Table 2. Genetic test analyzed

	Karyotype	Microdeletions	CAG repeat
Patients	82	82	65*
Controls	40	40	40

*Patients without cytogenetic abnormalities and microdeletions

Table 3. Genetic chromosomal abnormalities and Yq microdeletions in the infertile group.

Patient	Age	Semen Analysis*	Karyotype	AZF region	Type deletion
2	31	Az	normal	b	NI
4	25	Az	46,XY,inv(9)(p11q13)	c	b2/b4
6	27	Az	47,XXY		
7	35	Az	47,XXY		
12	39	Az	47,XXY		
15	27	Az	normal	b	NI
20	24	SO	normal	c	b2/b4
24	33	Az	normal	c	b2/b4
26	26	SO	normal	c	b2/b4
27	28	SO	normal	c	b2/b4
29	29	Az	47,XXY		
45	34	SO	normal	c	b2/b4
65	39	Az	47,XXY		
67	29	Az	normal	c	b2/b4
72	32	Az	46,XY Yq-	b, c	complete
74	33	Az	47,XXY		
77	34	SO	46,XY t(1;15) Lq(12;q25)		

AZ= Azoospermia; SO= Severe oligozoospermia, NI=Not identified

Table 4. CAG repeat length in controls and in infertile men grouped according to the degree of oligospermia.

Sperm concentration	n	Mean CAG	p
Azoospermia	38	21.05 ± 3.36	NS*
Criptoospermia	7	21.29 ± 2.28	NS
Oligoospermia	20	22.75 ± 3.61	NS
Controls	40	20.88 ± 3.19	

*NS: non significant.

Table 5. Prevalence of Yq microdeletions in infertile males.

Author	%	Country
Ristanovic <i>et al</i> , 2007	15.6	Serbia
Arruda <i>et al</i> , 2007	38.3	Brazil
Mohammed <i>et al</i> , 2007	10.4	Kuwait
Chernykh <i>et al</i> , 2006	7.5	Russia
Fernández-Salgado <i>et al</i> , 2006	3.4	Venezuela
Isidoro-Garcia <i>et al</i> , 2005	13	Spain
Vic dan <i>et al</i> , 2004	9.1	Turkey
El Awady <i>et al</i> , 2004	12	Egypt
Peterlin <i>et al</i> , 2002	4.4	Slovenia
Van Landuyt <i>et al</i> , 2000	3.9	Belgium
Kleiman <i>et al</i> , 1999	6	Israel
Kim <i>et al</i> , 1999	20	Korea
Brandell <i>et al</i> , 1998	7.7	USA
Silber <i>et al</i> , 1998	17.2	USA

Figure 1.

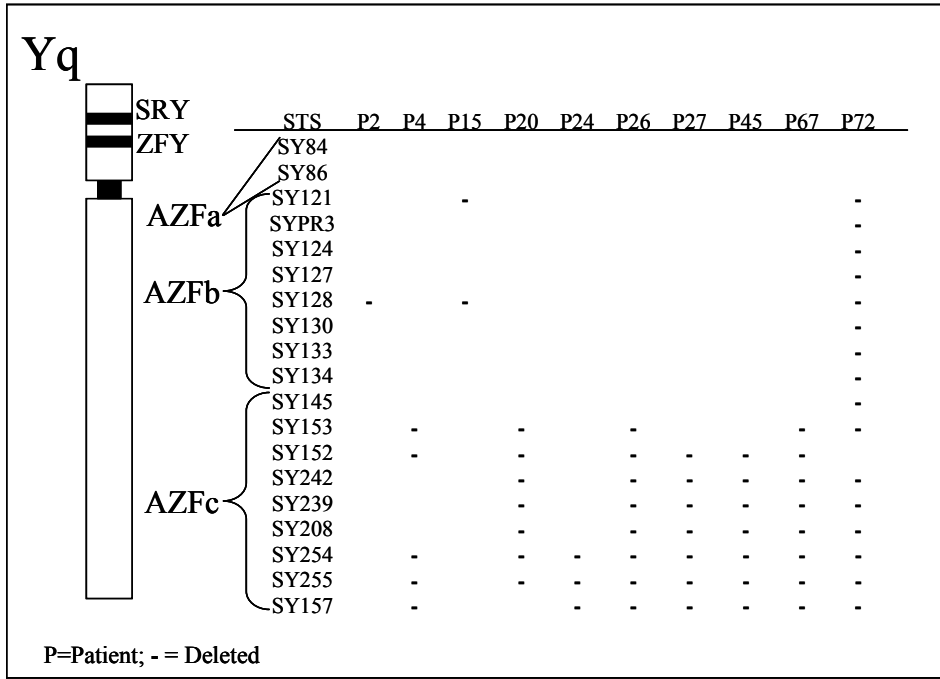


Figure 2.

